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Original Article

CD146 inhibits the human umbilical vein endothelial cell expression of transforming growth factor β1 in high-glucose environment in vitro

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Abstract: Background: CD146, a cell adhesion molecule, belongs to the immunoglobulin superfamily. It plays an important role in cell proliferation and activation, and contributes to inflammatory reaction. High glucose leads to abnormally high expression of adhesion molecules in renal cells. Up to now, TGF-β1 has been known as a marker for glomerular sclerosis. Objective: We studied the association between CD146 and TGF-β1 in normal and high glucose ((NG and HG) environment to understand its role in the pathogenesis of diabetic nephropathy. Methods: To mimic the environment of diabetic nephropathy, human umbilical vein endothelial cells (HUVECs) were cultured with glucose in dose- (5 mM NG, 15 mM HG, 30 mM HG or 45 mM HG) dependent manner for 24 hours. Then CD146 monoclonal antibody (McAb) was co-incubation with 5 mM NG (5 mM NG-I) or 30 mM HG (30 mM HG-I) for further 24 hours. CD146 and TGF-β1 mRNA expression were determined by RT-PCR. The soluble form of CD146 and TGF-β1 protein was evaluated by ELISA. Results: The expression of CD146 mRNA and protein on HUVECs was increased after high glucose treatment, 30 mM glucose gave the maximum response. Meanwhile, the expression of TGF-β1 mRNA and protein on HUVECs was also significantly upregulated in a dose-dependent manner in the high glucose group, and the peak level was achieved at 45 mM glucose. However, after CD146 McAb blocked the expression of CD146, the expression of TGF-β1 mRNA and protein on HUVECs were dramatically increased in high glucose group. Conclusions: We speculate that CD146 expressed on HUVEC may have a certain inhibitory effect on TGF-β1 in high glucose environment, through which CD146 plays a vital role on delaying the process of glomerular sclerosis to some extent.

Keywords: CD146, endothelial cell, transforming growth factor-β1, high glucose

Introduction

Diabetic nephropathy (DN) is a serious complication of diabetic mellitus (DM). The manifestations of diabetic nephropathy may be a consequence of the effect of certain cytokines and growth factors. It has been shown that high glucose leads to the abnormally expression of adhesion molecules in renal cells, such as integrins, intercellular cell adhesion molecule-1 (ICAM-1) and cadherins. These lead to cell activation and stimulate the inflammatory cascade [1]. CD146, known as Mel-CAM, MUC18, S-Endo-1 and P1H12, is a cell adhesion molecule of the immunoglobulin (Ig) superfamily. It was initially identified as a marker of tumor progression and metastasis in human melanoma through homotypic or heterotypic cell-cell adhesion [2]. Later further studies explored that CD146 has a wider tissue distribution, including endothelial cells [3], activated T cells [4], cerebellar cortex [5], and keratinocytes. Until now, CD146 is regarded as an endothelial cell marker which locates at the endothelial conjunction and plays a critical role in cell proliferation and activation [6, 7]. However, the role of CD146 in the pathogenesis of DN is unclear.

Previous studies have confirmed that transforming growth factor β (TGF-β) mediated the pathologic changes of diabetic kidney disease. TGF-β promotes renal cell hypertrophy and extracellular matrix accumulation, which are hallmarks of diabetic renal disease. In addition,

TGF-β1 is considered as a key marker of glomerular sclerosis in the development of kidney diseases [8, 9]. However, the exact mechanism of the TGF-β1 expression in DN has not been fully determined. The aim of the study was to investigate the association between CD146 and TGF-β1 on HUVECs in the condition of DN.

Subjects and methods

Cell culture

A human umbilical vein endothelial cell (HUVEC) was obtained from the Institute of Shanghai Biochemistry and Cell Biology. Cells were cultured in RPMI1640 (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Hyclone, USA) in 6-well cell culture plates or flasks. Cells were incubated at 37°C in 5% CO_2 95% air for 3-4 days until a confluent monolayer was attained. Cells were released for passaging or intervention with 0.25% trypsin/1 M methylenediaminetetraacetic acid (EDTA) (Gibco, Grand Island, NY, USA).

Experimental conditions

The passaging HUVECs were deprived of serum (10% FCS) for 24 hours, and cultivated in RPMI 1640 media at different glucose concentrations for 24 hours. Cell were divided into a normal glucose group (NG) with 5 mmol/L D-glucose, high glucose groups (HG) with 15 mmol/L, 30 mmol/L or 45 mmol/L D-glucose (HG 15 mM, HG 30 mM and HG 45 mM). In some experiments, certain amount of CD146 monoclonal antibodies (McAb, sc-53369, Santa Cruz, mouse anti-human MEL-CAM (OJ79c MUC 18) monoclonal antibody) was co-incubated with NG 5 mM or HG 30 mM for 24 hours more (NG 5 mM-I, HG 30 mM-I). The CD146 monoclonal antibody's final concentration in the medium was 30 μg/ml. Meanwhile, control groups were added with isotope murine IgG1 (sc-3877, santa cruz) till 10 μg/ml. Then the supernatants were collected to evaluate the soluble form of CD146 and TGF-β1 by enzyme-linked immunosorbent assay, and the cells were washed three times with PBS and then harvested for mRNA expression by reverse transcriptase polymerase chain reaction analysis.

Reverse transcriptase polymerase chain reaction analysis of CD146 and TGF-β1 mRNA expression

HUVECs total RNA was isolated using Trizol (Invitrogen, San Diego, CA, USA). After adding chloroform and centrifuging, the aqueous phase was transferred and the RNA recovered by precipitation with isopropyl alcohol. RNA purity and quantification were analyzed by spectrophotometry. Two micrograms of total RNA was reverse transcribed using oligo-dT, dNTP, RNase inhibitor and M-MLV reverse transcriptase (Fermentas, USA). The cDNA was amplified in 20 mL of 2 mL cDNA and 1 mL Taq DNA polymerase (Promega, USA) with dNTP and 10 × PCR buffer. CD146 primers were designed as follows: human sense, 5'-AGA ACC GGG TCC ACA TTCAG-3'; antisense, 5'-GTC GGG TAG AAA ACA GGG ACG-3'. And TGF-β1 primers: sense, 5'-CAA GTG GAC ATC AAC GGG TT-3'. antisense, 5'-GCT CCA AAT GTA GGG GCA GG-3'. The β-Actin primers were used as controls: sense, 5'-GGT CAG AAG GAT TCC TAT GT-3'; antisense, 5'-ATT GCC AAT GGT GAT GAC CTG-3'. PCR reactions were done in a Light Cycler using the following profile: 5 minutes at 94°C, followed by 30 cycles of amplification, consisting of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 90 seconds. The PCR products were 193 bp for CD146, 296 bp for TGF-β1 and 615 bp for β-actin. The amplification products were separated by 2% agarose gel electrophoresis. The band densities were analyzed by Tanon imaging software. Each experiment was repeated three times with similar methods.

Enzyme immunoassay for sCD146 and TGF-β1 protein

For soluble CD146 and TGF-β1, concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using ELISA kits (Marseille, France, and DY1679, R & D, USA).

Statistical analysis

Results are expressed as means ± SD. Comparisons between the groups were performed using a paired Student's *t* test and one-way ANOVA, followed by the Dunnett test for multiple comparisons. All Statistical analysis were performed by using SPSS 17.0 (Chicago, IL). Significance was defined as a p value <0.05.

Results

High glucose increases CD146 and TGF-β1 mRNA expression in HUVECs in vitro

HUVECs were exposed to different glucose concentrations (NG, HG 15 mM, HG 30 mM and HG 45 mM) for 24 hours. The CD146 mRNA

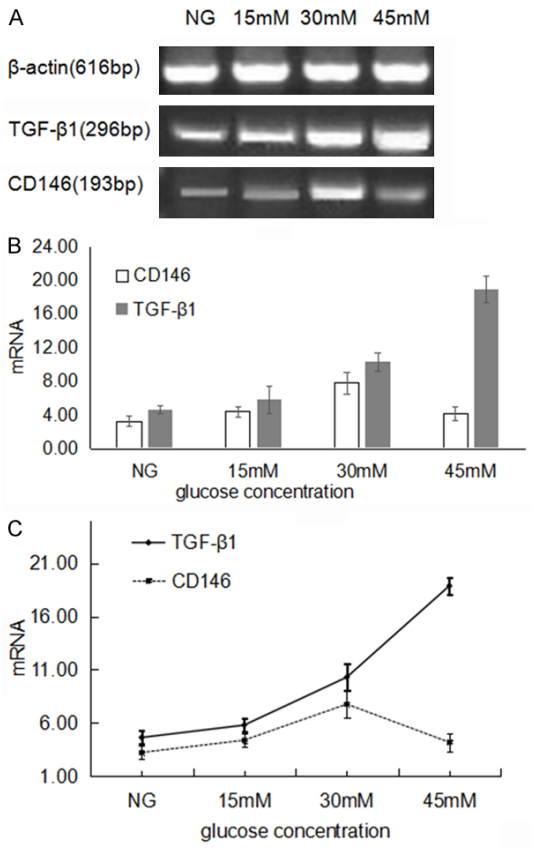

Figure 1. Effect of high glucose on the expression of TGF-β1 and CD146 mRNA in HUVECs (RT-PCR). A: TGF-β1 and CD146 mRNA expression in different glucose concentration media. B: The relative TGF-β1 and CD146 mRNA levels (compared with βactin). C: The mRNA expression trendy contrast between TGF-β1 and CD146. $^{*}P<0.05$, compared with NG 5 mM group. $^{\#}P<0.05$, compared with HG 30 mM group. NG: normal glucose group, HUVECs were cultivated in RPMI 1640 media containing 5 mmol/L D-glucose. 15 mM: HG 15 mM grorp, HUVECs were cultivated in RPMI 1640 media containing 15 mmol/L D-glucos. 30 mM: HG 30 mM group, HUVECs were cultivated in RPMI 1640 media containing 30 mmol/L D-glucose. 45 mM: HG 45 mM group, HUVECs were cultivated in RPMI 1640 media containing 45 mmol/L D-glucose.

expression levels were (/β-actin): 3.23±0.63, 4.38±0.59, 7.77±1.28, 4.19±0.83, respectively; Meanwhile the TGF-β1 mRNA expression were (/β-actin): 4.64±0.52, 5.80±1.69, 10.29±1.12, 18.87±1.56, respectively (**Figure 1A**, **1B**). One-way analysis of variance shows CD-146 mRNA expression were significantly upregulated in the HG 30 mM group compared to NG group (7.77±1.28 vs. 23.23±0.63, $P<0.05$) (**Figure 1B**). There was a slight decline in CD146 mRNA expression in the 45 mM HG group, indicating there is a dose effect of high glucose on

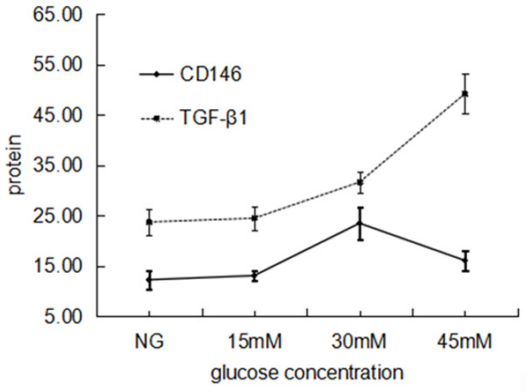

Figure 2. CD146 and TGF-β1 protein expression in different concentration of culture media in HUVECs. $^{*}P<0.05$, compared with NG 5 mM group. $^{\#}P<0.05$, compared with HG 30 mM group. NG: normal glucose group, HUVECs were cultivated in RPMI 1640 media containing 5 mmol/L D-glucose. 15 mM: HG 15 mM grorp, HUVECs were cultivated in RPMI 1640 media containing 15 mmol/L D-glucos. 30 mM: HG 30 mM group, HUVECs were cultivated in RPMI 1640 media containing 30 mmol/L D-glucose. 45 mM: HG 45 mM group, HUVECs were cultivated in RPMI 1640 media containing 45 mmol/L D-glucose.

the expression of CD146 in HUVECs. At the same time, TGF-β1 mRNA expression was significantly upregulated in a dose-dependent manner in the high glucose group, and the peak level was achieved at 45 mM glucose (18.87±1.56 vs. 4.64±0.52, $P<0.05$) (**Figure 1B** and **1C**). All these results indicate that high glucose increases the expression of CD146 and TGF-β1 in HUVECs.

High glucose increases CD146 and TGF-β1 protein expression in HUVECs supernatants

The cell supernatants were collected from each group to detect the expressions of CD146 and TGF-β1 extracellular proteins by ELISA. The CD146 protein expression levels in the NG, HG 15 mM, HG 30 mM and HG 45 mM groups were: 23.73±2.64, 24.47±2.43, 41.54±3.04, 26.18±2.17, respectively. Meanwhile, the TGF-β1 expression levels were 12.26±2.18, 13.09±1.06, 20.28±2.52, and 34.03±2.4, respectively. Similar to the molecular level, CD146 and TGF-β1 extracellular protein expressions were enhanced by the increasing glucose concentration, which were markedly enhanced in the HG 30 mM group, showing a significant difference from the NG group (**Figure 2**). As the glucose concentration further rose to HG 45 mM, the

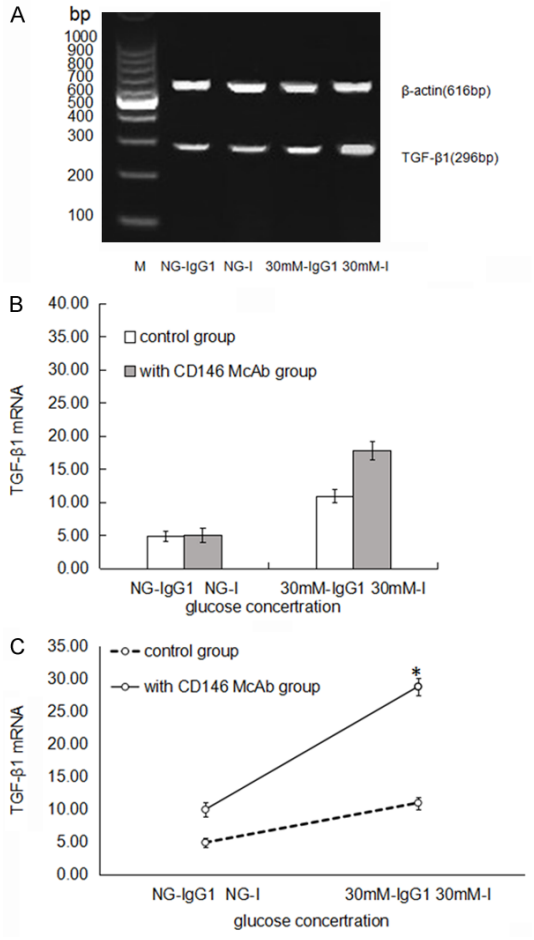

Figure 3. Effect of CD146 monoclonal antibody on expression of TGF-β1 mRNA in high-glucose cultured HUVECs (RT-PCR). NG-I,: NG 5 mM with 30 μg/mL CD146 monoclonal antibody. 30 mM-I: HG 30mM groups with 30 μg/mL CD146 monoclonal antibody. NG-IgG1, NG 5 mM with 10 μg/mL isotope murine IgG1 till. 30 mM-IgG1 (Control group): HG 30 mM with 10 μg/mL isotope murine IgG1. *Compared with control group *P*<0.05

expression of TGF-β1 protein further increased compared to the HG 30 mM group, while the expression of CD146 protein decreased slightly.

Effect of CD146 monoclonal antibody on expression of TGF-β1 mRNA in high-glucose cultured HUVECs

Cells cultured for 24 h were collected from NG group and HG 30 mM groups with or without 30 μg/ml CD146 monoclonal antibody. Meanwhile, control group was added with 10 μg/ml isotope murine IgG1. After culturing for an additional 24 h, TGF-β1 mRNA was amplified by PCR. The

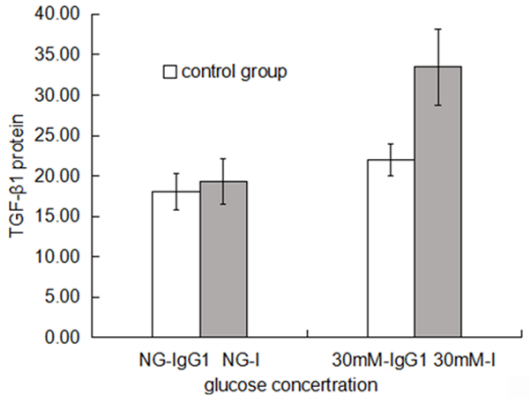

Figure 4. Effect of CD146 monoclonal antibody on expression of TGF-β1 extracellular protein in high-glucose cultured HUVECs. NG-I: NG 5 mM with 30 μg/mL CD146 monoclonal antibody. 30 mM-I: HG 30 mM groups with 30 μg/mL CD146 monoclonal antibody. NG-IgG1: NG 5 mM with 10 μg/mL isotope murine IgG1. 30 mM-IgG1 (Control group): HG 30 mM with 10 μg/mL isotope murine IgG1. *Compared with HG 30 mM-IgG1 group *P*<0.05.

expression levels of TGF-β1 mRNA were 5.54±0.54 vs. 5.66±0.81 (*P*>0.05, **Figure 3A**, **3B**) for NG 5 mM-I vs. control group, whereas were 13.41±1.82 vs. 8.32±1.96 (*P*<0.05, **Figure 3A**, **3B**) for HG 30 mM-I group vs. control group. Paired T test revealed that there was no significant difference of TGF-β1 mRNA between the NG 5 mM-I and control groups. However, the HG 30 mM-I group expressed significantly higher level of TGF-β1 mRNA than the isotope control group, indicating that blocking the expression of CD146 by CD146 monoclonal antibody dramatically increased the expression of TGF-β1 mRNA on HUVECs.

Effect of CD146 monoclonal antibody on expression of TGF-β1 extracellular protein in high-glucose cultured HUVECs

The cell supernatants were collected from each group to detect the expression of TGF-β1 extracellular protein by ELISA. The expression levels of TGF-β1 protein were 18.06±2.26 vs. 19.36±2.81 (*P*>0.05, **Figure 4**) for NG 5 mM-I vs. control group, whereas were 33.46±4.72 vs. 21.95±1.97 (*P*<0.05, **Figure 4**) for HG 30 mM-I vs. control group. There results showed that no significant difference was found between NG 5 mM-I group and control group. However, after inhibiting the expression of CD146 by by CD146 monoclonal antibody, level of TGF-β1 extracel-

lular protein was highly increased in the HG 30 mM-I group compared with the isotope control group, This finding was consistent with the variation of mRNA expression of TGF-β1 under different treatments. Therefore, we demonstrated that the CD146 expressed by HUVECs may have a certain inhibitory effect on TGF-β1 in high-glucose environment.

Discussion

CD146 is a cell adhesion molecule with a transmembrane structure, belongs to the immunoglobulin superfamily. It has been considered as a component of the endothelial junction and played a critical role in cell cohesion, permeability and monocyte transmigration [6, 10, 11]. In multiple human organs, its multifunctionality has been reported, including mediation of lymphocyte transmembrane migration and homing in the thymic microenvironment [12], regulation of neurite extension, and formation of a neural network [13] and participation in heterotypic intercellular adhesion [14, 15]. Sema Ciftci Dogansen found that CD146 levels may be an useful marker for earlier identification of high risk for atherosclerosis in type 1 diabetic patients, which may be more sensitive than CIMT [16]. Tomoko Nakai's study suggested that annulus fibrous cells gradually changed to a contractile phenotype after CD146 expression increased. Furthermore, TGF-β1 enhanced the above cellular commitment [17]. There results indicated that there is an association between CD146 and TGF-β1. However, the role of CD146 and the relationship between CD146 and TGF-β1 in the pathogenesis of DKD remain rarely reported.

It has been described there were two different isoforms of CD146. One short isoform displays angiogenic properties and one long isoform presents at the cellular junction with structural properties [17]. Meanwhile CD146 also exists as a soluble form (sCD146) in endothelial cells [2], which is shed from the membrane CD146. Our study found that CD146 expressed in HUVECs increased significantly at high concentration of glucose. In our experiment, HUVECs were treated with different dose of glucose concentrations including 15 mM, 30 mM and 45 mM. After culturing in the high glucose medium for 24 h, the expressions of both CD146 mRNA and extracellular protein (sCD-146) were higher than the normal glucose group. These expressions were enhanced with the increasing glucose concentration, which showed significant differences between the NG group in HG 30 mM group. When the glucose concentration was further increased to 45 mmol/L, the expression of CD146 did not continue to increase, but decreased slightly compared to the HG 30 mM group, indicating that there was a different dose response of high glucose on the expression of CD146 in the HUVECs. Meanwhile, TGF-β1 expression in HUVECs presented the same trend as CD146 in high glucose group, and significantly upregulated at 45 mM glucose. The increased expression of CD146 along with TGF-β1 in HUVECs after high glucose treatment lead us to speculate the association of the CD146 and TGF-β1 under the condition of high glucose.

To confirm this hypothesis, cells were cultured for 24 h in the NG and HG 30 mM groups with or without CD146 monoclonal antibody to block the binding of the endothelial-expressed CD146. According to the CD146 antibody instruction, we used 30 μg/ml as the final antibody concentration in the culture medium to block CD146 antigen. The control group was added with a certain amount of isotope murine IgG1. The results showed that after CD146 antibody was added, the expressions of TGF-β1 mRNA and extracellular protein did not change significantly in the NG group. However, after CD146 antibody was added, the HG 30 mM group exhibited significantly higher TGF-β1 expression than the control group. Thus, the endothelial cell expression of CD146 in high-glucose environment seems to have a certain inhibitory effect on TGF-β1 expression. The glomeruli of patients with diabetic kidney disease (DKD) are in a long-term high-glucose state, which is similar to our experimental setting of different glucose concentrations. Based on the above results, we presume that under mild high-glucose stimulation, glomerular endothelial cells express an elevated level of CD146 to promote the proliferation and adhesion of endothelial cells, thereby maintaining the endothelial cell integrity. Nevertheless, high glucose concentration also stimulates increased TGF-β1 production to cause glomerulosclerosis, while the increased CD146 can partially inhibit the production of TGF-β1 to delay the progress of glomerulosclerosis. However, if the blood glucose level of patients continues to elevate without efficient control, the response

of the increased CD146 expression after more higher glucose will be weakened as showed that there is a dose effect of high glucose on the expression of CD146 in HUVECs in 45 mM HG. Once it happened, the lowering effect of CD146 on TGF-β1 will be broken. Meanwhile, positive stimulation by the high glucose will persist and enhance. Hence, the TGF-β1 expression show a greater rate of increase than before, thereby accelerating the progress of glomerulosclerosis. Thus, we believe that in the early stage of DKD, the increased expression of CD146 in endothelial cell has a inhibitory effect on TGF-β1, which to some extent can delay the occurrence and progression of DKD. This study firstly demonstrated the association of the CD146 and TGF-β1 in diabetes, and may provided a new direction for the early treatment of DKD.

Finally, it can not be ignored that the up-regulated expression of high glucose-induced adhesion molecules not only has an endothelial protective function, but also is involved in mediating the activation of immune inflammatory responses to cause endothelial injury. High glucose stimulates the proliferation of glomerular endothelial cells with over-expression of a variety of adhesion molecules [1, 18, 19], and mediates leukocyte homing and releasing of numerous inflammatory and growth factors, thereby activating the immune inflammation cascade to cause tissue damage [20-22]. Therefore, looking for a balance between the maximization of adhesion molecules' protective function and the avoidance of their injury-inducing risk may be an important direction in the future research of adhesion molecules in DKD.

It should be pointed out that in this study, we used CD146 antibody to block the CD146 protein expression in HUVECs. There are still some deficiencies need to be improved in the further experiments. We would try siRNA to knockdown CD146 or over-experssion of CD146 to fully verify the above inhibitory effect of CD146 on TGF-β1 in DN. Thus, in order to definite the role CD146 and exact relationship between CD146 and TGF-β1 in the pathogenesis of diabetic nephropathy, we need a long way to go.

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Disclosure of conflict of interest

None.

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