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Original Article

Effect of α1-AT on VEGF and MMP-2 in HUVECs exposed to high glucose and hypoxia: a possible therapeutic approach towards diabetic retinopathy

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Abstract

Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus caused by hyperglycemia. Due to sustained hyperglycemia, the endothelial cells are damaged, leading to its dysfunction, which results in hypoxia. The hypoxic condition upregulates vascular endothelial growth factor (VEGF) activity that induces matrix metalloproteases (MMPs) to cause extracellular matrix degradation resulting in angiogenesis. Alpha-1 antitrypsin (α1-AT), an anti-protease, is known to downregulate MMPs. The objective is to assess the effect of α1-AT on VEGF and MMP-2 levels in an *in vitro* culture setup with various glucose concentrations and hypoxic conditions. Human Umbilical Vein Endothelial Cells (HUVECs) were cultured with different concentrations of glucose and cobalt chloride to induce high glucose and hypoxic conditions, respectively. Later, the cells were treated with α1-AT to assess its effect on VEGF and MMP-2. The VEGF and MMP-2 levels were evaluated in conditioned media by Enzyme-Linked Immunosorbent Assay. VEGF and MMP-2 levels were observed to be increased in the conditioned media of those cultured with high glucose concentrations and $CoCl_2$. In contrast, the levels of VEGF and MMP-2 were observed to be reduce upon treatment with α1-AT. In conclusion, α1-AT reduced the levels of VEGF and MMP-2 in cells treated with high glucose concentration and hypoxia. This suggests the beneficial effect of α1-AT and its approach as a possible therapeutic target towards DR.

Keywords: *in vitro*, diabetes mellitus, endothelial cells, extracellular matrix, neovascularization.

Introduction

Diabetic Retinopathy (DR) is the most widespread vision-threatening microvascular complication of Diabetes Mellitus (DM). Based on the clinical manifestation of DR, it is classically categorized into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [1]. An estimated 22.27% of subjects suffering from Diabetes Mellitus are affected by DR globally [2].

Chronic hyperglycemia is considered as a potential risk for developing DR [3]. Hyperglycemia activates different pathways, such as the formation of advanced glycosylation end products and receptors, pro-inflammatory cytokines and chemokines, proliferator-activated receptor-γ disruption, growth factors, oxidative stress, and microRNA [4]. Activation of these pathways leads to retinal microvasculopathy, inflammation, and retinal neurodegeneration, all of which result in the breakdown of the blood-retinal barrier (BRB). Disruption of BRB leads to endothelium damage resulting in the formation of acellular capillaries and edema in retinal vascular structure [5]. This increases endothelial cell permeability resulting in vascular leakage, thickening of the vessel wall and coagulation, further resulting in hypoxia. Hyperglycemic-induced hypoxia stimulates

angiogenesis in DR by modulating a balance between pro-angiogenic and anti-angiogenic mediators [6, 7]. The hypoxic effects are mediated by hypoxia-inducible factor-1alpha (HIF-1a), an oxygen-sensitive transcription factor [8]. Induction of HIF-1α is known to be responsible for the production of vascular endothelial growth factor (VEGF), which is responsible for retinal neovascularization, a classic hallmark of progressive DR [9, 10].

VEGF is a homodimer glycoprotein and a potent mitogen for endothelial cells and mediates angiogenesis [11]. VEGF, also known as vaso permeable factor, leads to the development of new blood vessels which are fragile in nature [12]. VEGF regulates the proliferation and migration of endothelial cells at the molecular level and enhances vascular permeability by activating its tyrosine kinase receptors. Studies have demonstrated that VEGF knockout during embryogenesis in mice is lethal as it leads to a phenotype that displays delayed endothelial cell differentiation and impaired blood island formation resulting in impaired blood vessel formation [13, 14]. Extracellular matrix (ECM) degradation has been implicated in pathological angiogenesis during the development and progression of DR [15]. VEGF-activated endothelial cells secrete matrix metalloproteases (MMPs); zinc-dependent endopeptidases, leading to ECM degradation [16].

Among the identified MMPs, MMP-2 and MMP-9 are known to play an important role in neovascularization during DR [17]. The activity of MMPs is regulated by a group of endogenous inhibitors such as tissue inhibitors of metalloproteinase, α2-Macroglobulin, and Alpha-1 antitrypsin (α1-AT) [18].

The α1-AT is a 52kDa serine protease inhibitor belonging to the serpin superfamily and is encoded by the SERPINA1 gene. The α1-AT is an acute phase protein produced in hepatocytes and changes its concentration in response to inflammation and tissue injury [19]. Existing evidence suggests that α1-AT not only possesses the ability to inhibit proteases but also possesses anti-inflammatory and anti-apoptotic properties [20, 21]. Owing to these properties, α1-AT has been proposed as a potential therapeutic agent to hinder the progression of DR.

In addition, clinical studies have demonstrated the decreased/deficiency of α1-AT in Type 1 and Type 2 diabetes mellitus (T2DM) patients suggesting the protective role of α1-AT in the pathogenesis of DM [22, 23]. Thus, this study aimed to investigate the effects of α1-AT on VEGF and MMP-2 using human umbilical endothelial cells (HUVECs) exposed to high glucose and oxygen deprivation to mimic DR *in vitro* to corroborate its beneficial effects in DR progression.

Material and methods

Cell culture

HUVECs were procured from ATCC, USA, #CRL-1730. Cells were cultured in Dulbecco's modified eagle media with 10% fetal bovine serum and antibiotics until confluency was obtained. After attaining confluency, cells were seeded in a 24-well cell culture plate at an approximate 0.25×10^6 density. The cells were then cultured in different glucose concentrations (from 15 mM to 33 mM) for 3 days. The hypoxic condition was induced from the fourth day of culture in cells growing in 33 mM glucose by the addition of cobalt chloride ($CoCl_2$) at different concentrations of 50 µM and 100 µM maintained at 37°C and 5% CO_2 (to mimic diabetic retinopathy milieu) [19, 21]. Later, the cells were incubated with and without 1mg/ml α1-AT (normal concentration) for 10 hours as follows: 5 mM glucose (with and without α1-AT treatment), 15 mM glucose (with and without α1-AT treatment), 33 mM glucose (with and without α1-AT treatment), 33 mM glucose + 50 µM $CoCl_2$, (with and without α1-AT treatment) and 33 mM glucose+100 µM $CoCl_2$ (with and without α1-AT treatment). Cell supernatant was collected and an enzyme-linked immunosorbent assay (ELISA) was performed to estimate VEGF and MMP-2.

Statistical analysis

The results were analyzed statistically using Prism GraphPad 5 software. The observations were represented as mean and standard deviation. One-way ANOVA was conducted to compare the means between the groups. P-value<0.05 was considered statistically significant and p<0.001 as highly significant.

Results

Effect of α1-AT on VEGF and MMP-2 in cells cultured with low glucose concentration

The effect of α1-AT on VEGF and MMP-2 was evaluated by estimating the levels of VEGF and MMP-2 in the HUVEC cells treated with and without α1-AT (1 mg/ml) under low glucose concentration (5 mM). Under the

low glucose condition, no significant difference in the VEGF and MMP-2 levels was observed between the untreated and the treated group (Figure 1 and Figure 2). These results suggest that Alpha 1-antitrypsin did not have any significant effect on VEGF and MMP-2 secretion under normal glucose condition.

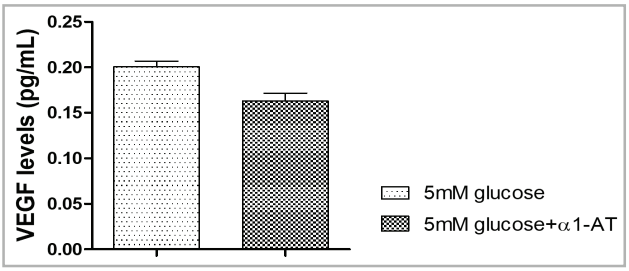

Figure 1: VEGF levels in cells treated with 5 mM glucose without and with α1-AT treatment.

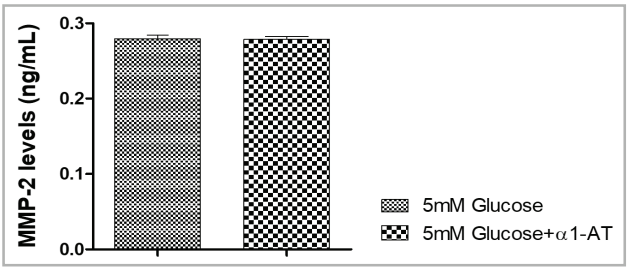

Figure 2: MMP-2 levels in cells treated with 5 mM glucose without and with α1-AT treatment.

Effect of α1-AT on VEGF and MMP-2 in cells cultured with 15 mM and 33 mM glucose concentration

To mimic a diabetic environment, cells were cultured in media with high glucose concentrations, i.e., 15 mM and 33 mM. We observed that high glucose concentrations significantly increased the levels of VEGF and MMP-2 ($p<0.001$). To determine whether a normal concentration of α1-AT would be sufficient to reduce the increased levels of MMP-2 and VEGF at high glucose concentration, the cells were supplemented with 1 mg/ml α1-AT. The results indicated significantly reduced levels of VEGF and MMP-2 (Figure 3, $p<0.001$ and Figure 4, $p<0.001$), indicating that α1-AT at the physiological level may have a protective effect in diabetic patients.

Effect of α1-AT on VEGF and MMP-2 in cells cultured with 33 mM glucose concentration with hypoxia-induced by $CoCl_2$

The cells cultured in glucose concentration of 33 mM were treated with different concentrations of

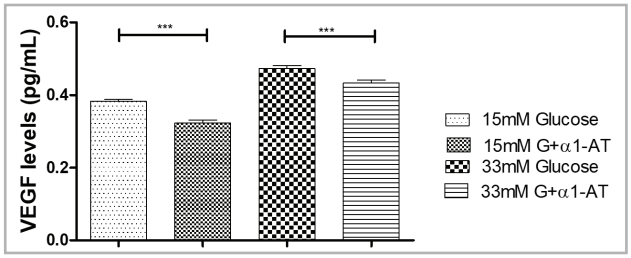

Figure 3: VEGF levels in cells treated with 15 mM glucose and 33 mM glucose without and with α1-AT treatment.

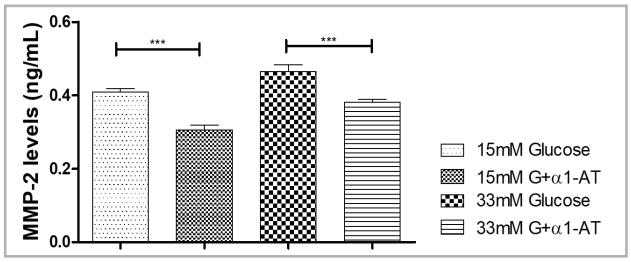

Figure 4: MMP-2 levels in cells treated with 15 mM glucose and 33 mM glucose without and with α1-AT treatment.

$CoCl_2$ (50 µM and 100 µM). This was done to achieve a hypoxic condition to mimic the DR environment. The effect of α1-AT on VEGF and MMP-2 under high glucose and hypoxic conditions was evaluated. The conditioned media showed significantly elevated levels of VEGF and MMP-2 in the untreated group when compared to the treated group (Figure 5, $p<0.001$, and Figure 6, $p<0.001$). Furthermore, the levels of VEGF and MMP-2 increased in cells induced by hypoxia as compared to VEGF and MMP-2 levels without hypoxia. We also observed increased levels of VEGF and MMP-2 in cells treated with 100 µM of $CoCl_2$ as compared to cells treated with 50 µM $CoCl_2$. This shows that an advanced hypoxic condition further induces VEGF activity and this in turn upregulates the activity of MMPs. The α1-AT at 1 mg/ml concentration was observed to significantly decreased levels of VEGF and MMP-2, suggesting its protective effect in DR.

Discussion

Evidence from clinical studies supported the critical role of VEGF in the progression of DR and is considered a reliable biomarker for DR. A meta-analysis involving twenty-nine studies pointed towards the increased VEGF levels in patients with DR as compared to controls [24–26]. Studies conducted by Selim KM *et al.*, Endo M *et al.*, and Catrina SB *et al.*, reported increased levels of VEGF in DR [27–29]. Supporting these reports,

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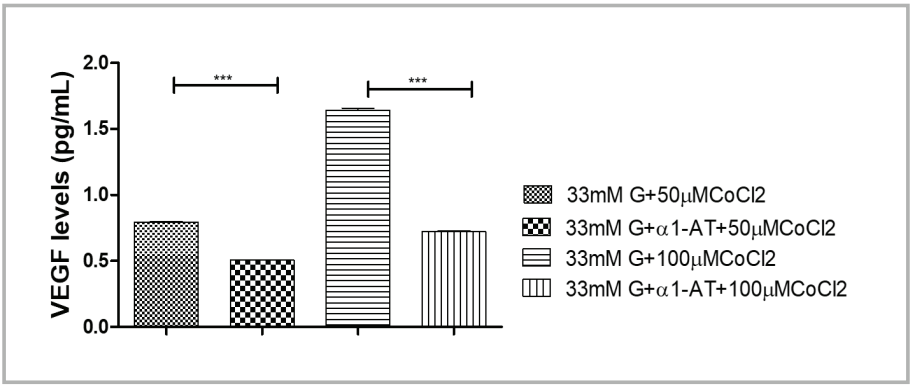

Figure 5: VEGF levels in cells treated with 33 mM glucose and different concentrations of $CoCl_2$ without and with α1-AT treatment.

in this *in vitro* study, we found significantly elevated levels of VEGF in cells treated with 15 mM and 33 mM glucose concentrations as compared to cells treated with 5 mM glucose. Our study also revealed that with an increase in the hypoxic environment in cells treated with 33 mM glucose, the VEGF levels were also observed to be elevated. This suggests hypoxic condition as the primary inducer of VEGF production conditioned with high glucose condition.

VEGF-activated endothelial cells induce MMP-2 activity during DR progression, facilitating the degradation of collagen, one of the major components of ECM [16]. MMPs facilitate the migration of endothelial cells through degraded matrix resulting in neovascularization [30]. The levels of MMP-2 are observed to be increased during DR development [16, 31–34]. Our study showed an increase in the levels of MMP-2 in cells treated with 15 mM glucose and 33 mM glucose compared to 5 mM glucose. We also observed an increase in MMP-2 levels with an increase in the hypoxic condition. This suggests that VEGF accelerates MMP-2 activity due to hypoxia, which further promotes proteolytic degradation of ECM, resulting in the progression of DR.

In vivo, the activity of MMP-2 is regulated by a group of endogenous anti-proteinases which inactivates the enzyme by forming a complex with it [19, 35]. However, such a balance between protease and anti-protease is lost in several disease conditions due to increased activity of proteases or reduced activity of anti-proteases leading to an imbalance between them [36]. This imbalance between protease and anti-protease results in excessive proteolytic activity and crucial tissue damage. This likely points to the therapeutic use of protease inhibitors in diseased conditions [19].

Reduced levels of α1-AT have been reported in subjects affected by T1DM [23]. Elevated blood levels of α1-AT with augmentation therapy have been shown to prevent T1DM development and prolong islet allograft survival [37, 38]. Furthermore, a study conducted by Sandstrom CS *et al.*, showed the association between α1-AT deficiency and T2DM [23]. Experimental studies have suggested α1-AT therapy as a therapeutic approach towards T1DM and T2DM [21, 38, 39]. Studies conducted by Rachmiel M *et al.*, and Weir GC *et al.*, demonstrated the safety and efficacy of α1-AT in T1DM subjects [40, 41]. In the present study, post-treatment with α1-AT, the levels

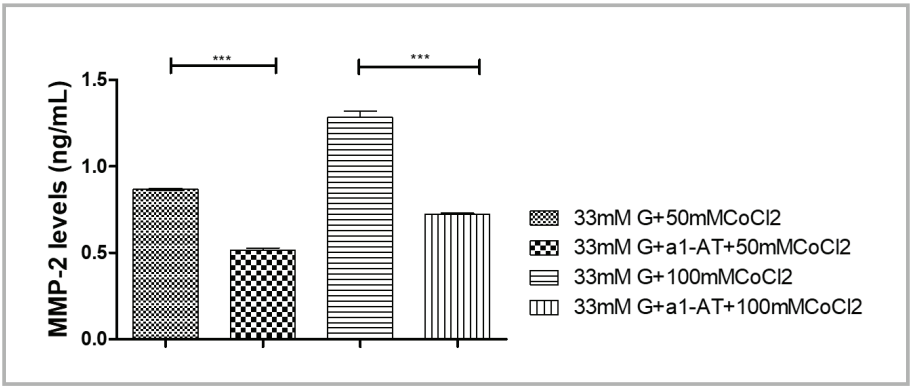

Figure 6: MMP-2 levels in cells treated with 33 mM glucose and different concentrations of $CoCl_2$ without and with α1-AT treatment.

of MMP-2 and VEGF were decreased significantly in cells treated with 15 and 33 mM glucose (with and without hypoxia). Our observation supports the protective role of α1-AT in DR and points to its therapeutic use in DR.

Conclusion

In the present study, we demonstrated that α1-AT reduced VEGF and MMP-2 levels at high glucose concentrations and hypoxic conditions. Hence, the use of α1-AT may be an effective strategy to prevent or hinder the progression of diabetic retinopathy.

Conflict of interest

The authors declare no conflict of interest.

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