Endothelin-1 traps potently reduce pathologic markers back to basal levels in an in vitro model of diabetes

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Abstract

Background Diabetes mellitus is a group of metabolic disorders in which there are high blood glucose levels over a prolonged period. Diabetes is one of many diseases associated with pathologically elevated levels of endothelin (ET)-1. We have recently proposed the development of ET-traps, which are an antibody – based fusion protein that potently bind and sequester pathologically elevated levels of endothelin-1.

Methods We constructed ET-traps that were found to be very potent binders to ET-1, with a K_D of 32.5 pM. We then treated human retinal microvascular endothelial cells (HRMECs), which are an in vitro model of glucose induced cellular damage, with 10 nM ET-1 or high glucose levels (25 mM).

Results In this study, we investigated the effects of our ET-trap constructs on the expression levels of both collagen 4 α 1 and fibronectin, which are both important pathologic markers in diabetes. Treating HRMECs with 10 nM ET-1 or 25 mM glucose significantly induces the expression of the ECM proteins fibronectin and collagen 4 α 1, as is found in chronic diabetic complications; Incubation of the cells with the ET-traps significantly prevented the increased expression of fibronectin and collagen 4 α 1 back to basal levels. This was found with both mRNA and protein expression levels of the two ECM proteins.

Conclusion Our results provide the first evidence of the efficacy of ET-traps in reducing pathologic markers in an in vitro model (of diabetes). Further research is warranted to determine the efficacy of ET-traps as a therapeutic tool for diabetes, which is a major public health burden around the world.

Keywords DM · Diabetes mellitus; ECM · Extracellular matrix; ET-1 · Endothelin-1; ETtr · Endothelin-1 traps; FFP · Fc-fusion protein; FN · Fibronectin

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia due to reduced insulin action. Type 1 diabetes develops over several years and is characterized by a

lack of insulin production due to specific destruction of the pancreatic β cells [1]. Type 2 diabetes on the other hand results from insulin resistance and/or from β -cell dysfunction with a reduced ability of the pancreatic β cells to secrete sufficient insulin to stimulate glucose utilization [2, 3].

An imbalance in the production and degradation of extracellular matrix (ECM) proteins, such as fibronectin (FN) and collagen 4 α 1, is a key feature of chronic diabetes. Previous research has shown that diabetes leads to increased fibronectin in all cases of secondary complications in each of the target organs [4–7].

The mechanism of increased ECM synthesis is important for the development of therapeutics, as ECM deposition is a common occurrence in chronic diabetes [7]. Our previous work has shown high glucose-induced, endothelin-dependent fibronectin synthesis is mediated via NF- κ B and AP-1 [8]. This expression was shown to be dependent on endogenous endothelin (ET) receptor-mediated signaling.

Key points

ET-traps have a very high binding affinity.
ET-traps significantly return to basal levels different pathologic markers both at the mRNA and at the protein level.
ET-traps are non-cytotoxic.

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Our study also showed that inhibition of NF- κ B and AP-1 prevented glucose and endothelin (ET) induced FN production. ET receptor blockade also prevented such changes. The results of this study indicated that glucose-induced increase in FN production in diabetes could be mediated via ET-dependent NF- κ B and AP-1 activation.

ET-1 also increases collagen 4 α 1 accumulation in renal mesangial cells by stimulating a chemokine and cytokine autocrine signalling loop involving MCP-1 (macrophage chemoattractant protein-1) and IL-6 (interleukin-6) [9]. ET-1 levels are in fact increased in patients with diabetes compared with control subjects [10, 11].

Endothelial cell dysfunction is a key pathological element in the development of chronic diabetic complications [12, 13]. Endothelial dysfunction is a systemic pathological state of the endothelium that is broadly defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) the endothelium [14]. Patients with diabetes are associated with endothelial dysfunction. An important effect of endothelial dysfunction is that it leads to increased production and biological activity of the potent vasoconstrictor and pro-inflammatory peptide ET-1 [15]. Based on our previous work [16], we have produced modified, monomeric ET-traps. These are antibody-based fusion proteins, which potentially bind and can sequester increased levels of ET-1 that are prevalent in diabetes [10, 11].

In this study, we investigated the effects of our ET-trap constructs on the expression levels of both collagen 4 α 1 and fibronectin, which are both important pathologic markers in diabetes.

Materials and methods

Creating an Fc-fusion construct

The gene for the fusion protein was designed and optimised for expression in mammalian cells (HEK293) prior to being synthesised. The sequence was then subcloned into a cloning and expression vector for human Fc fusion proteins as previously described [16].

The Fc construct was generated using the services of Absolute Antibody Ltd. (Oxford, UK).

For the binding affinity measurement, we employed the use of the Octet Red96 system [17]. For this, we fixed biotinylated ET-1 (Phoenix Pharmaceuticals) and then probed with our Fc-fusion construct.

Binding affinity measurement

The kinetics of the monomeric Fc-ETtr binding to biotinylated-endothelin-1 was determined using the Octet Red96 system (ForteBio, Menlo Park, CA). The buffer for

the assays was phosphate buffered saline (PBS) with 0.01% (w/v) bovine serum albumin (BSA) and 0.002% Tween-20. The ForteBio Octet analysis software (ForteBio, Menlo Park, CA) was used to generate the sensorgram.

Cell culture

Human retinal microvascular endothelial cells (HRMECs) were obtained from Lonza Walkersville (Walkersville, USA). The culture conditions have previously been described [8]. Briefly, cells were grown in EBM-2 (Lonza Walkersville) containing 1% hEGF, 0.4% hydrocortisone, 1% gentamicin, 10% FBS, 1% vascular endothelial growth factor, 4% human basic fibroblast growth factor (hFGF-B), 1% long R3 insulin-like growth factor 1 (R3-IGF-1), and 1% ascorbic acid. In EBM-2, the glucose concentration was 5 mmol/L. Cells were grown in 25 cm² tissue culture flasks and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells at 80% confluence were growth-arrested by incubation in serum-free medium without growth factors overnight before incubation with specific reagents; ET-1 or glucose with co-treatment of different concentrations of the ET-trap construct. All experiments were carried out after 24 h of 10 nM ET-1 and 48 h of 25 mM glucose incubation with a pre-treatment step of 1 h with different doses of the ET-trap monomer.

Cytotoxicity assessment

The cytotoxic effects of the compounds, ET-1 and glucose, on HRMECs were evaluated using cell proliferation/cytotoxicity reagent (WST-1) from Roche applied sciences. The assay measures cytotoxicity by measuring absorbance of the formazan product at a wavelength of 450 nm. The assay was performed according to the manufacturer's instructions. In brief, cells were seeded onto a 96-well plate and were allowed to attach overnight. The following morning, the full medium was removed and was replaced with 100 μ L of serum-free medium containing various concentrations of D-glucose (5 mM, 25 mM) and other reagents as stated. Following 24 h of incubation, 10 μ L of WST-1 reagent was added to each well. The plates were incubated for 1.5 h at 37 °C to produce a colour reaction. Absorbance was measured with a Multiskan FC Microplate Photometer (Thermo Scientific, Finland) at 450 nm with a reference wavelength of 690 nm. Survival was determined by the difference between the absorbances at 450 nm and 690 nm.

RNA isolation and cDNA synthesis

Trizol reagent (Invitrogen, Burlington, Canada) was used to isolate RNA as previously described [6]. RNA was extracted with chloroform followed by centrifugation to separate the

sample into aqueous and organic phases. RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in diethylpyrocarbonate-treated water. Total RNA (2 µg) was used for cDNA synthesis with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). The resulting cDNA products stored at -20°C .

Real-time RT-PCR

Real-time RT-PCR was performed using the LightCycler (Roche Diagnostics Canada, Laval, Canada), as previously described [18]. For a final reaction volume of 20 µL, the following reagents were added: 10 µL SYBR Advantage qPCR Premix (Clontech, Mountain View, USA), 1 µL each of forward and reverse 10 µmol/L primers (collagen 4α1 and FN), 7 µL H_2O , and 1 µL cDNA template. mRNA levels were quantified by using the standard curve method. Standard curves were constructed by using serially diluted standard template. The data were normalized to β-actin RNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

Primers used:

Human fibronectin primers:

[forward: 5-GATAAATCAACAGTGGGAGC-3,
reverse: 5-CCCAGATCATGGAGTCTTTA-3]

and Human collagen 4α1 primers: [forward:
5-CAAGAGGATTTCAGGTCCA-3,
reverse: 5- TCATTGCCTTGCACGTAGAG-3]

ELISA

For cell protein analyses, total cellular proteins were extracted from human retinal microvascular endothelial cells. The cells were lysed with RIPA buffer and sonicated for 20 s. Protein concentrations were measured using the BCA protein assay kit (Fisher Scientific, Burlington, ON, Canada). Enzyme-linked immunosorbent assay was performed using a commercially available enzyme-linked immunosorbent assay kits for Collagen Type IV Alpha1 (Col 4a1) (Cloud-Clone Corp. USA) and Fibronectin (FN) (R & D Systems, Inc. USA) according to the manufacturer's instructions.

Statistics

All experiments were performed in triplicates. The data are expressed as means $\pm$ SD. Significance was determined using a one-way anova followed by a Tukey's multiple comparisons test between groups.

The differences between groups were considered significant at the level of $p < 0.05$.

Results

The Fc-fused ET-trap construct exhibits a strong binding affinity to ET-1

Based on our previous research [16], we created a monomeric Fc-ETtr construct. A monomer would overcome any issue of intramolecular binding of cysteine residues as previously found [16].

SDS-PAGE analysis confirmed that the construct was successfully expressed as identified by its molecular weight (46.8 kDa).

The construct was expressed and isolated with a purity of $>98\%$. Using this construct, we then proceeded to analyse the binding affinity of the soluble construct.

The binding assay revealed a mean K_D of $3.250\text{E-}11 \pm 2.27\text{E-}12$ M, i.e. a binding affinity of 32.5 pM, with a very slow K_{OFF} rate (Table 1; Fig. 1).

ET-traps and glucose treatment exhibit no cytotoxicity in HRMECs

No difference in viability was observed in cells treated with high glucose (HG, 25 mM) and ET-1 (10 nM) for a period of 48 h. The monomeric ET-trap construct also showed no cytotoxic effects at various concentrations of upto 500 nM for 24 h incubation with 25 mM D-glucose or 10 nM ET-1 (Fig. 2a and b).

ET-traps restore fibronectin and collagen 4α1 mRNA expression to basal levels after ET-1 or high glucose treatment

Endothelial cells (ECs) line the blood vessels and are critical for the function and integrity of the vascular unit. We used human retinal microvascular endothelial cells (HRMECs) as an in vitro model to study the effects of our ET-trap construct. To study the effects of our ET-trap constructs on expression of ECM proteins after ET-1 or high-glucose treatment, we

Table 1 Binding data for the monomeric Fc-ETtr1 construct

Sample	K_D (M)	K_D Error
Fc-ETtr1	2.880E-11	1.266E-12
Fc-ETtr1	2.830E-11	1.251E-12
Fc-ETtr1	3.049E-11	1.383E-12
Fc-ETtr1	3.006E-11	2.755E-12
Fc-ETtr1	4.101E-11	3.651E-12
Fc-ETtr1	3.643E-11	3.329E-12

The binding assay revealed a strong binding of the monomeric Fc-ETtr1 construct; mean binding affinity of 3.250×10^{-11} M, i.e. 32.5 pM ($n = 6$)

Fig. 1 Sensogram of Fc-ETtr1 binding to biotinylated ET-1. Representative plot shown; The binding assay revealed a strong binding of the monomeric Fc-ETtr1 construct; mean binding affinity of 3.250×10^{-11} M, i.e. 32.5 pM ($n = 6$)

Fig. 2 Cell viability data (mean $\pm$ SEM, using WST assay, expressed as absorbance) normalized to NG (5 mM glucose) showing no significant changes in the viability of endothelial cells following incubation with 25 mM glucose (HG), 10 nM endothelin-1 (ET-1) along with simultaneous incubation with various concentration of ET-traps (monomer). Experiment performed in triplicate from three separate experiments, data expressed as mean $\pm$ SD, normalized to NG

performed a dose-response study using 62.5 nM, 125 nM and 250 nM treatments with our ET-traps (Fig. 3a–d).

Based on our previous experiments as FN and collagen 4 α 1 reaches peak at 24 h and after 48 h following ET-1 and glucose treatment respectively, we used these time points for subsequent analyses. Treatment with either 10 nM ET-1 or high glucose treatment (25 mM) produced a significant increase in the expressions of both FN and collagen 4 α 1 (Fig. 3a–d). Treatment with the ET-trap construct gave a significant reduction in expression of both ECM proteins (FN and collagen 4 α 1) back to basal control levels with the 125 nM and 250 nM dose of ET-traps.

ET-traps have a positive effect on fibronectin and collagen 4 α 1 protein expression following ET-1 or high glucose treatment

ELISA analysis revealed that (analogous to the mRNA measurements) treating HRMECs with both 10 nM ET-1 or a high glucose treatment (25 mM) both gave a significant increase in protein expression of fibronectin and collagen 4 α 1. Treatment with various doses of ET-trap constructs prevented such over-expression of both ECM proteins. A significant effect observed on both ECM proteins fibronectin and collagen 4 α 1 with a 125 nM upwards treatment of ET-traps after treatment with 10 nM ET-1 (Fig. 4a and b) and with the 500 nM dose of ET-traps after high glucose treatment (25 mM) (Fig. 4c and d).

Discussion

According to a World Health Organization report, globally, an estimated 422 million adults were living with diabetes in 2014. This figure truly illustrates what a major burden this disease is.

Fig. 3 Real time PCR analysis of fibronectin and collagen 4 α 1 transcripts in HRMECs exposed to 25 mM glucose (HG) or 10 nM endothelin-1 (ET-1). Both transcripts were significantly upregulated in HRMECs after 48 h incubation with HG and 24 h incubation with ET-1 compared to HRMECs treated with normal glucose (5 mM, NG) and were prevented

following incubation with ET-traps (monomer). * Statistically significant compared to NG, ** statistically significant compared to HG or ET-1, experiments performed in triplicate from three separate experiments, data expressed as mean $\pm$ SD, normalized to NG (5 mM glucose)

As introduced earlier, increased expression of extracellular matrix (ECM) proteins are well-established changes in the organs affected in chronic diabetes [6, 8]. Endothelial cells, being the first cell type exposed to high blood glucose levels, play a major role in the pathogenesis of chronic diabetic complications. In this study, we exposed our model (HRMECs) to different doses of ET-traps concomitant to high glucose and ET-1 treatment.

As a first step, we measured the binding affinity of our ET-traps construct, which was found to be very strong at 32.5 pM (Table 1). In addition to a strong binding affinity to ET-1, our ET-traps construct has a very slow K_{OFF} rate (Fig. 1). Both these are important factors when developing a potential therapeutic. Furthermore, cytotoxicity analysis showed that our ET-

trap constructs are not cytotoxic to the cells (Fig. 2a and b). This is also important when developing a potential therapeutic tool.

We have previously demonstrated ET-1 dependent increased ECM protein synthesis in endothelial cells exposed to high glucose and in the organs (retina, kidneys and hearts) of the chronically diabetic animals [6, 8]. Structurally, such changes are manifested as basement membrane thickening, mesangial expansion and focal fibrosis [19]. Upregulations of FN and collagen 4 α 1, two key ECM proteins, are well established in this context [6, 19, 20]. Furthermore, we have previously shown that established endothelin blockers are capable of preventing aforesaid biochemical and structural long-term effects of hyperglycemia [4]. Hence, to examine the efficacy of our ET-traps, we selected FN and collagen 4 α 1. We

Fig. 4 Protein expression analyses (ELISA) of fibronectin and collagen 4α1 in HRMECs exposed to 25 mM glucose (HG) or 10 nM endothelin-1 (ET-1). Both proteins were significantly increased in HRMECs after 48 h incubation with HG and 24 h incubation with ET-1 compared to HRMECs treated with normal glucose (5 mM, NG) and were prevented

following incubation with ET-traps (monomer). * Statistically significant compared to NG, ** statistically significant compared to HG or ET-1, experiment performed in triplicate from three separate experiments, data expressed as mean ± SD, normalized to NG (5 mM glucose)

found that the dose of 125 nM and 250 nM ET-traps gave a significant reduction at the mRNA level of both FN and collagen 4α1 back to basal, untreated levels (Fig. 3). The same effect was observed at the protein levels with a 500 nM dose of ET-traps, which restored basal expression levels of fibronectin and collagen 4α1. We observed this with both the 10 nM ET-1 treatment (Fig. 4a and b), as well as the high glucose treated cells (Fig. 4c and d). These results confirm that our ET-trap construct is able to successfully bind and sequester increased ET-1 levels and ameliorate any associated pathology. Previous work has indicated that a 10 nM dose of ET-1 is optimal in inducing pathologic markers [21]. As previously discussed, ET-1 levels are pathologically elevated in diabetes [10, 11]. Research indicates that high glucose (the primary feature of diabetes) can induce ET-1 levels [22]. Hence, it is fitting that our ET-trap construct also has a positive effect on returning ECM protein levels back to basal levels after high glucose treatment (Figs. 3c and d and 4c and d). Our work shows that interaction of ET-1 with vasoactive factors is involved in mediating glucose-induced increased permeability

in endothelial cells [23]. Data from this study suggest that high glucose-induced increased permeability may be induced through increased ET-1 expression.

Conclusion

In summary, the ET-traps have a positive effect on a pathologic dose of ET-1 or high glucose induced cellular damage in our in vitro model of endothelial cells. The ET-traps gave a significant reduction and return to basal levels of the two ECM proteins (collagen 4α1 and FN) at both the mRNA and protein levels. As a next step, we intend to test the efficacy of ET-traps as a therapeutic tool in a mouse model of diabetes. These in vivo studies will also measure the bioavailability and any toxicity of our ET-traps at different doses.

As ET-1 is implicated in various diseases, including neurodegenerative disorders (like Parkinson's disease and multiple sclerosis), cardiovascular disease and pregnancy disorders

[24–26], further studies could also investigate the efficacy of ET-traps in these areas.

The advantage of ET-traps over current antagonists in clinical use is that we are merely sequestering (pathologically) increased ET-1 levels back to normal physiological levels, without completely blocking the ET-1 activity, which is essential for normal functions.

Author contributions AJ and SC participated in the research design. HY and SC conducted the experiments. AJ and SC contributed new reagents or analytic tools. SC performed the data analysis. AJ, SC and SC wrote or contributed to the writing of the manuscript.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

Declaration statement Any inquiries on data of this study can be directed to Dr. Arjun Jain.

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