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Reversal of Hyperglycemia-Induced Angiogenesis Deficit of Human Endothelial Cells by Overexpression of Glyoxalase 1 *In Vitro*

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Abstract

Dicarbonyl glycation of RGD and GFOGER sites in type IV collagen has been associated with decreased angiogenesis. In this study, we investigated whether overexpression of glyoxalase 1 to decrease dicarbonyl glycation would prevent the angiogenesis deficit induced by hyperglycemia *in vitro*. Transfection of human microvascular endothelial cells resulted in a four-fold increase in glyoxalase 1 activity compared with controls. Incubation of human microvascular endothelial cells in model hyperglycemia produced a 32% decrease in formation of tube structures that was prevented by glyoxalase 1 overexpression. We conclude that increased protection against dicarbonyl glycation of endothelial cell protein protects hyperglycemia-induced angiogenesis deficit.

Keywords

glyoxalase; angiogenesis; endothelial cells; diabetes; glycation; methylglyoxal

Introduction: Angiogenesis Deficit in Early Stages of Microvascular Complications of Diabetes

Early stages of retinopathy, neuropathy, and nephropathy and impaired wound healing in clinical diabetes are associated with decreased angiogenesis. According to the “ischemia-angiogenesis” hypothesis, the resulting ischemia, arising from decreased blood flow to surrounding tissue, may lead to later excessive angiogenesis in response to the expression of angiogenic growth factors including vascular endothelial-derived growth factor (VEGF), transforming growth factor- β , basic fibroblast growth factor, and connective tissue growth factor (CTGF). In the retina, the process starts in isolated capillaries with the loss of pericytes and endothelial cells (ECs) forming acellular capillaries. The capillary structure is thereby weakened severely and becomes occluded. This spreads to groups of capillaries and arterioles.¹

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Conflict of Interest

The authors declare no conflicts of interest.

Glycation of proteins, forming advanced glycation endproduct (AGE) residues, has been associated with both decreased and increased angiogenesis. Impaired collagenolysis following AGE residue formation has been linked to decreased angiogenesis and induction of increased angiogenic growth factors by AGE-modified proteins has been linked to increased angiogenesis. AGE formation may lead to decreased degradation of vascular collagen despite activation of matrix metalloproteinases,^{2,3} and hence decreased angiogenesis. In one study, collagenolysis was impaired in diabetic mice in ischemia-induced angiogenesis and was restored by treatment with aminoguanidine,² a scavenger of dicarbonyl glycating agents.⁴ Glycation of angiogenic growth factors has also been implicated in the angiogenesis deficit in diabetes.^{5,6} Increased angiogenesis in response to proteins highly modified by AGEs *in vitro* is mediated by induction of VEGF and CTGF expression.^{7,8}

Recent research has shown that protein glycation by α -oxoaldehydes, such as methylglyoxal (MG)—formed mainly by the degradation of cellular glycolytic intermediates—forms quantitatively important AGEs of cellular and extracellular proteins *in vivo*. Glycation by MG is mainly arginine residue-directed.⁹ The plasma concentration of MG is increased in hyperglycemia associated with clinical diabetes,¹⁰ giving rise to increased modification of vascular basement membrane type IV collagen. MG glycation forms arginine-derived hydroimidazolone residues at hotspot modification sites in RGD and GFOGER integrin-binding sites of collagen, causing EC detachment, anoikis, and inhibition of angiogenesis. ECs incubated in model hyperglycemia *in vitro* and experimental diabetes *in vivo* have produced the same modifications of vascular collagen, inducing similar responses.¹¹ Pedchenko *et al.* found similar impaired adhesion of ECs, mesangial cells, and podocytes to extracellular matrix (ECM) proteins glycated by MG.¹² These EC–ECM interactions are essential for angiogenesis.^{13,14}

Glycation of the cellular protein of ECs by dicarbonyls—particularly integrins—may also lead to functional impairment. Glyoxalase 1 (Glo1) is the major cellular enzyme that catalyzes the metabolism of MG and thereby protects against dicarbonyl glycation.¹⁵ To assess the role of dicarbonyl glycation of EC proteins in the hyperglycemia-induced angiogenesis deficit, we overexpressed Glo1 in ECs and investigated the effect on hyperglycemia-induced impairment of angiogenesis *in vitro*.

Methods

Glo1 cDNA in the pUC19 plasmid was provided by Dr. S. Ranganathan (FoxChase Cancer Center, Philadelphia, PA). PCR with primers containing appropriate restriction enzyme sites for the destination plasmid was performed to enable insertion of Glo1 cDNA into pIRES2-EGFP plasmid vector to produce pIRES2-EGFP-Glo1. An analytical digest was performed and indicated correct insertion of the Glo1 cDNA (approximately 622 bp). An empty plasmid served as a control (sham). Human dermal microvascular ECs (HMEC-1 cells) were seeded at 1.25×10^5 cells per well (per 9.5 cm²) in 6-well microplates. After incubation for 24 h, a mixture of DNA/lipofectamine 2000 (1:1) was added and the cells were incubated for a further 24 h. The transfection efficiency was assessed by green fluorescent protein positive fluorescence. Glo1 activity of transfected cells was assayed by the spectrophotometric method¹⁶ and protein content by the Bradford assay. Cells transfected with pIRES2-EGFP-Glo1 or pIRES2-EGFP were grown in model normoglycemia and hyperglycemia (5 mM and 20 mM glucose, respectively) for 3 days and then transferred to 5 mM glucose and plated onto Matrigel (ECM from the Engelbreth-Holm-Swarm tumor; Sigma Poole, UK) and incubated at 37 °C for 24 h. EC tube formation was quantified by image analysis of 100 x phase contrast images of eight random fields using NIH Image

software (developed at the U.S. National Institutes of Health, USA, and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).¹¹

Results

The transfection efficiency of ECs with pIRES2-EGFP-Glo1 and pIRES2-EGFP vectors was 60–70%. ECs transfected with pIRES2-EGFP-Glo1 had a fourfold increase in Glo1 activity compared with controls (447.9 ± 44.8 mU/mg vs. 114.5 ± 20.4 mU/mg; data are expressed as mean $\pm$ SD, $n=3$, $P<0.01$). ECs incubated in hyperglycemia prior to assessment of the formation of tube-like structures showed an angiogenesis deficit that was corrected by overexpression of Glo1. Tube formation after 24 h (total tube length per field, μm ; mean $\pm$ SD, $n=8$) was: sham transfectant cells in normoglycemia 216 ± 61 , Glo1 transfectant cells in normoglycemia 220 ± 59 , sham transfectant cells in hyperglycemia 146 ± 39 ($p = 0.05$, with respect to sham transfectant in normoglycemia), and Glo1 transfectant in hyperglycemia 207 ± 44 ($p = 0.05$, with respect to sham transfectant in hyperglycemia).

Discussion

Impairment of angiogenesis by glycation in diabetes may contribute to tissue ischemia, impairment of wound healing, and coronary collateral vessel development.¹ Decreased formation of tube-like structures by ECs incubated under hyperglycemic conditions and prevention of this by overexpression of Glo1 suggests that: (i) dysfunction of ECs in hyperglycemia may contribute to decreased angiogenesis, and (ii) this effect may be mediated by glycation of cellular proteins by Glo1 substrates—glyoxal and MG. Increased dicarbonyl glycation of ECM proteins is also involved.^{11,17} Arg-261 of integrin $\alpha_v\beta_3$ is a possible site of dicarbonyl glycation leading to impaired engagement of integrins in angiogenesis.¹⁸

We conclude that increased protection against dicarbonyl glycation of EC protein by overexpression of Glo1 protects against hyperglycemia-induced angiogenesis deficit. This implicates dicarbonyl glycation of integrins or related proteins in ECs in mediating decreased angiogenesis in hyperglycemia. Similar processes may contribute to the anti-angiogenic activity of cell permeable Glo1 inhibitors and their antitumor activity *in vivo*.¹⁹

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