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Diabetes-Relevant Regulation of cultured Blood Outgrowth Endothelial Cells

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Abstract

Many cell and tissue abnormalities in diabetes mellitus are mediated by auto- and paracrine TGF β which is induced by high ambient glucose and glycated proteins. In most cell types TGF β reduces cell proliferation and enhances apoptosis which are mediated through the TGF β type I receptor, Alk5. In contrast, early diabetic microangiopathy is characterized by endothelial cell proliferation. Endothelial cells are unique in expressing a second TGF β type I receptor, Alk1, as well as the co-receptor, endoglin which increases the affinity of the ligand to Alk1. In differentiated blood outgrowth endothelial cells from normal subjects Alk1 and endoglin are constitutively expressed. Incubation with high glucose (HG) and glycated albumin (gAlb) induces Alk5 and raises TGF β secretion 3-fold without affecting Alk1 or endoglin levels. This diabetic milieu accelerates cell proliferation, at least in part, through TGF β /Alk1-smad1/5 and probably involving VEGF as well as pro-migratory MMP2 downstream of Alk1. In contrast, HG/gAlb also increases caspase-3 activity (suggesting increased apoptosis) in part but not entirely using a TGF β /Alk5-smad2/3 pathway. The findings support pleiotropy of TGF β in endothelial cells including proliferative effects (through Alk1-smad1/5) and proapoptotic signals (through Alk5-smad2/3).

Keywords

Alk1; Alk5; TGF β ; Endothelial Cell; Apoptosis; Angiogenesis

Introduction

Diabetes mellitus causes accelerated vascular pathologies including peripheral vascular disease, coronary artery disease and strokes as well as microangiopathies in different vascular beds. In some micro vessels diabetes causes endothelial cell proliferation and abnormal angiogenesis. For example, proliferation of endothelium is observed in early diabetic glomerulopathy, proliferate diabetic retinopathy and rubeosis iridis with formation of micro aneurysms.

In most short-term (1–2 weeks) experiments, incubation of cultured cells with high glucose causes negative growth cues, reduced proliferation and increased apoptosis with a net effect of reductions in cell numbers (Baumgartner-Parzer et al., 1995; Kamal et al., 1998; Risso et

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al., 2001). These antiproliferative in-vitro effects of high glucose are thought to be mediated by autocrine TGF β (McGinn et al., 2003). However, in-vivo studies in early experimental diabetes in rodents have provided evidence for predominant proliferation and neo-angiogenesis of micro vessels, such as glomerular capillaries predating onset of diabetic nephropathy which develops late in these models. Early after induction of diabetes in mice the glomerular capillary length and anatomical surface area increase, on average by 56 % and 61 %, respectively (Nyengaard and Rasch, 1993). Moreover, cultured micro vascular endothelial cells that were exposed to glycated proteins in-vitro (perhaps a more valid experimental design) proliferate and gain expression of an angiogenic program, similar as can be observed in-vivo in early diabetes (Okamoto et al., 2002). In 'ex-vivo' experiments Münzel and associates showed a proliferation response in primary cultures of venous endothelial cells that were outgrown from diabetic patients and incubated with serum from diabetic subjects (Munzel et al., 2007).

The diabetic milieu (i.e., hyperglycemia and increased levels of glycated proteins) appears to induce both proliferative and anti-proliferative responses as well as increased or decreased incidence of apoptosis in a cell type and context dependent manner. TGF β has emerged as a major mediator of these glycemia effects.

TGF β induces its cellular responses through specific receptors, namely TGF β type I (TBRI) and type II receptors (TBRII). The type I receptor, activin-like kinase-5 (Alk5) is widely expressed in all TGF β -responsive cell types. TBRII has high affinity for the ligand and its binding activates this receptor initiating recruitment and cross-activation of the type I receptor (Wieser et al., 1995; Wrana et al., 1994). Recent crystallographic studies have shown that the high efficiency receptor complex actually is a multimer of two molecules each, TBRI, TBRII and TGF β (Groppe et al., 2008). The TBRI, Alk5 activates its specific intracellular substrates, smad2 and -3 which undergo nuclear translocation and form transcriptional complexes at specific regulatory gene elements with smad4 and other co-regulators. The complex of TBR II and TBRI is both required and sufficient to induce cellular responses to TGF β (Wieser et al., 1995). Most cell types express the TBRI, Alk5, and TGF β causes growth inhibition, apoptosis and transcriptional activation of several extra cellular matrix proteins and pro-fibrogenic regulators (Carcamo et al., 1994).

In epithelial cells high glucose and glycated albumin induce TGF β which mediates many of their effects. In epithelial cells, these actions are antiproliferative and pro-apoptotic. In contrast, in fibroblasts high glucose is a proliferative agent and TGF β is the main, autocrine mediator (Han et al., 1999). The growth and activation responses to TGF β in fibroblasts are transmitted through the TGF β - smad2/3 pathway as well as by two alternative pathways involving (a) PAK2 and Abl and (b) Akt, tuberlin, mTOR, and p70S6K; both alternative pathways are downstream of PI3K which functions as a branch point in alternative TGF β signaling (Wang et al., 2008; Wang et al., 2005; Wilkes et al., 2005). The canonical and the alternative TGF β -pathways in fibroblasts are downstream of Alk5/TBRII receptors.

In endothelial cells TGF β also activates two pathways albeit the alternative pathways differ from fibroblasts. Endothelial cells express two type I TGF β receptors, Alk5 and Alk1, leading to formation of Alk1/TBRII and/or Alk5/TBRII receptor complexes. Moreover, endothelial cells express abundantly the co-receptor endoglin. This protein associates preferably with the Alk1/TBRII complex and increases TGF β efficacy towards binding to and/or activating this complex compared to the alternative receptor containing Alk5 (Goumans et al., 2003; Lebrin et al., 2004; Lee et al., 2008).

We tested the hypothesis that human endothelial cells when exposed to a diabetes-relevant milieu respond with an increase in TGF β levels. This cytokine induces both, proliferative cues

through activation of Alk1-smad1/5 and proapoptotic signals through Alk5-smad2/3. This hypothesis was tested in blood outgrowth endothelial cells.

Materials & Methods

Blood Outgrowth Endothelial Cells (BOECs)

The studies involving human subjects were approved by the institutional review board at the Los Angeles Biomedical Research Institute and were performed after obtaining written, informed consent. Endothelial cells were outgrown from venous blood from normal subjects (n=6). Fifty ml of heparinized blood was separated by centrifugation through a Histopaque 1077 gradient (Sigma, St. Louis, MO). Mononuclear cells at the gradient-plasma interface were washed four times, seeded in collagen I – coated flasks and incubated with ECGM-2 medium containing hydrocortisone (0.2 µg/ml), EGF (5 ng/ml), VEGF (500 pg/ml), FGF2 (10 ng/ml), R3-IGF-I (20 ng/ml), heparin (22.5 µg/ml), ascorbic acid (1 µg/ml) and 2% FCS (PromoCell, Heidelberg, Germany). Media were changed daily and non-attaching cells were removed. Most cells that initially attached subsequently detached during about 2 weeks. At three weeks only very few cells remain attached to the coated surface, about 10 cells per 25 cm² flask. After about 6 weeks these cells had proliferated and were detached with trypsin/EDTA and subcultured in fibronectin-coated (50 µg/ml) flasks. Media were then exchanged every two days. Cells differentiate from a progenitor to an endothelial phenotype after 3 passages as indicated by their expression of the endothelial markers vWF, VE-cadherin, KDR, Tie2 and loss of the progenitor marker CD34 (see below). Passage 4 and 5 cells were used in individual experiments. Each experiment was performed at least in triplicate using cells from different normal subjects.

Experiments & Assays

For individual experiments BOECs grown to about 70% confluence in multiwell plates or on cover slips were incubated in supplement-free EBM-2 medium containing 0.1 % FCS.

Expression of Alk1, Alk5 and secreted TGFβ-Activity under Diabetes Conditions

Cells in 6-well plates were incubated with normal glucose (5 mM) or high glucose (25 mM) plus glycated albumin (gAlb, 400 µg/ml, Sigma) for 72 hrs. Conditioned media were collected, cleared by centrifugation and assessed for TGFβ bioactivity. Cells were washed with PBS and lysed with 2x reducing Laemmli buffer for subsequent Western blot analysis of Alk1, Alk5 and endoglin.

Active TGFβ was measured with a bioassay as previously described from this laboratory (Wang et al., 2000). Briefly, Mink lung epithelial cells stably expressing a minimal PAI-1 promoter/luciferase reporter (kindly provided by Dr. Dan Rifkin, New York, NY) were plated in 96-well plates in DMEM/F12 containing 10% FCS at 30,000 cells per well. Cells were washed with serum free medium containing 0.1% BSA and then incubated with cleared media (diluted 1:10), 50 µl/well for 12 hrs at 37 °C. Wells were washed with PBS and cells were lysed with 100 µl/well of lysate buffer. Luciferase activity in lysates was measured luminometrically using commercially available reagents (Luciferase Assay System, Promega, Madison, WI).

Activation of Erk and Effect of the Alk5 Inhibitor, SB431542

Additional experiments were performed to examine the effects of the Alk5 kinase inhibitor SB431542 on Alk5 levels and on the phosphorylation-activation of smad1/5, smad2/3 and Erk1/2. Cells were incubated in supplement-free media with normal or high glucose/gAlb in the presence or absence of the small molecule Alk5-inhibitor, SB431542, 8 µM (Sigma), for

72 hrs. Cells were then lysed and Western blot analysis for Alk1, Alk5, phospho- and total smad1/5, phospho- and total smad2/3 and phospho- and total Erk1/2 were performed.

Endothelial Cell Proliferation upon High Glucose/gAlb

To examine the proliferative response to high glucose/gAlb and to assess the requirement of Alk5 activity, BOECs were incubated with normal or high glucose/gAlb for 72 hrs in the presence or absence of SB431542. In aliquot cells PCNA levels were examined by Western blot analysis. Cell proliferation was assessed in cells that were plated in fibronectin-coated 96-well plates and incubated with the same additives for 72 hrs (n=24 each). Thereafter, cells were lysed and proliferation was assessed with the CyQuant reagent (Molecular Probes, Eugene, OR) and the manufacturer's procedure.

In additional experiments cells in 6-well plates cells were incubated with normal or high glucose/gAlb or with recombinant human TGF β (100 pg/ml, R&D-Systems, Minneapolis, MN) for 72 hours and levels of VEGF were assessed by Western blot analysis of cell lysates.

Apoptosis Pathway Activation

In endothelial cells high glucose and glycated albumin have been shown to increase the incidence of apoptosis and to activate apoptosis pathways that lead to increased activity of the execution caspases (Ho et al., 2000). We examined the activity of the execution caspase, caspase-3, by Western blot analysis with an active caspase-3 – specific antibody in BOECs that were incubated for 72 hrs with normal or high glucose/gAlb in the presence or absence of the Alk5 inhibitor, SB431542.

Caspase-3 activity was also assessed in a functional assay that had been previously described from this laboratory (Mitu et al., 2007). Briefly, cells were lysed in buffer containing 50 mM PIPES, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, and 1 mM DTT, pH 7.0. Cleared lysates were adjusted to a total protein concentration of 4 μ g/ μ l. Assays were performed in 96-well plates containing 50 μ l/well of cell lysates (or lysate buffer as blank), and 2x assay buffer (250 mM HEPES, 1 mM EDTA, 50 mM KCl, 2 mM DTT, 0.01% CHAPS, pH 7.4), 50 μ l/well were added. The mixture was shaken at room temperature for 4 min and 10 μ l/well of the caspase-3 - specific substrate, Ac-Asp-Gly-Val-Asp-pNA (Ac-DEVD-pNA, Bachem, Torrance, CA), in 5% NaHCO₃ was added. Plates were mixed and incubated for 6 hrs at 37 °C. Optical density was measured at 405 nm in a multiwell plate reader (Molecular Devices, Sunnyvale, CA). Results were expressed in percent of mean controls.

Assessment of Collagenase in BOECs that were incubated with rhVEGF or Diabetes Conditions

Angiogenesis requires increased collagenase activity (Stetler-Stevenson, 1999). We assessed the activation of the predominant matrix metalloprotease, MMP2, in BOECs that were grown on fibronectin support and then incubated with rhVEGF (1 ng/ml, R&D Systems) or normal or high glucose/gAlb with or without SB431542 for 72 hrs. Pro- and active MMP2 were assessed by Western blotting of heparin-affinity precipitates from cell lysates and conditioned media. Briefly, cells were lysed in RIPA buffer containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Lysates and media were cleared by centrifugation and 50 μ l of a 1:1 slurry of heparin sepharose (Pharmacia/Amersham/GE Biosciences, Piscataway, NJ) were added to 1.0 ml of lysate or 2.0 ml of cleared medium from each well of a 6-well plate. The mixtures were incubated on a rocker platform at 4 °C for 2 hrs. Precipitates were collected by centrifugation and washed 3x with RIPA buffer (lysates) or cold PBS (media) each containing protease inhibitors. Washed precipitates were taken up in 2x Laemmli buffer for subsequent Western blot analysis.

Source of Primary Antibodies

The following antibodies were used in the various experiments and assays: anti-VE-cadherin (sc-9989; Santa Cruz Biotechnology, Santa Cruz, CA); anti-CD34 (sc-65261, Santa Cruz); anti-CD133 (sc-23797, Santa Cruz); anti-Alk1 (sc-19546, Santa Cruz); anti-Alk5 (sc-398, Santa Cruz); anti-pSmad1/5 (sc-12353R, Santa Cruz); anti-smad1/5 (sc-6031, Santa Cruz); anti-pSmad2/3 (sc-11769, Santa Cruz); anti-smad2/3 (sc-6202, Santa Cruz); anti-endoglin (sc-20632, Santa Cruz); anti-Erk1/2 (sc-94, Santa Cruz); anti-PCNA (sc-56, Santa Cruz); anti-VEGF (sc-507, Santa Cruz); anti-MMP2 (sc-10736, Santa Cruz); anti-gapdh (Fitzgerald Industries, Concord, MA); anti-vWF (AB7356, Chemicon, Temecula, CA); anti-active caspase-3 (No. 9661, Cell Signaling Technology, Beverly, MA); anti-pErk1/2 (No. 9102, Cell Signaling).

Statistical Analysis

Group means were compared by ANOVA and Newman-Keuls multicomparison test. A probability of less than 5% ($p < 0.05$) was defined to indicate statistical significance of difference.

Results

Characterization of BOECs

Cells from passages 4 and 5 which are used in the current experiments express the endothelium specific cell-cell adhesion molecule, VE-cadherin which is expressed at cell-cell junctions (Figure 1). Cells also express von Willebrand Factor, vWF, in a punctuate, perinuclear enriched, distribution corresponding to Weibel-Palade bodies which are known to store this protein (Figure 1). Cells were negative for the progenitor-defining marker CD34 and the early progenitor marker CD133 (Figure 1). Western blotting confirmed that BOECs expressed the VEGF receptor II, KDR, as well as the angiopoietin receptor, Tie2 (data not shown). Thus, the cells that were outgrown and passaged in-vitro are indeed differentiated endothelial cells.

Expression of TGF β type I Receptors, Secretion of Active TGF β and Smad Pathway Activation under Diabetes Conditions

The alternative TBRI, Alk1 is constitutively expressed in BOECs and its levels are not changed during incubation with high glucose/gAlb. Similarly, the co-receptor, endoglin, is also expressed and its levels are unaffected by the diabetes conditions (Figure 2a). In contrast, the 'classic' TBRI, Alk5, is not or only minimally expressed during incubation with normal glucose but becomes induced by high glucose/gAlb (Figure 2a). Active TGF β accumulates in the medium and is increased about 3-fold after 72 hrs of incubation under diabetes-mimicking conditions (Figure 2b).

Incubation of BOECs with HG/gAlb induces phosphorylation of smad1/5, the substrate of activated Alk1 as well as the Alk5 substrate, smad2/3 (Figure 3). The levels of phospho-smad2/3 are reduced by co-incubation with the Alk5-kinase inhibitor SB431542 which does not affect pSmad1/5 levels (Figure 3). Levels of total smad2/3 or smad1/5 do not change during either incubation condition. Overall, these findings indicate that in cultured human endothelial cells the diabetic milieu induces TGF β and activates both the Alk1-smad1/5 and the Alk5-smad2/3 pathways. Moreover, SB431542 reduces the activity of the TGF β -Alk5-smad2/3 pathway in the presence of HG/gAlb but does not affect the Alk1-smad1/5-pathway.

Diabetes Conditions reduce Erk Phosphorylation through Alk5

Incubation of cells with high glucose/gAlb reduces the levels of phosphorylated Erk1/2 (Figure 3). Since this activity of the diabetes-mimicking milieu is substantially inhibited by SB431542,

it is fair to conclude that the down-regulation of Erk1/2 activity is mediated by TGF β through Alk5 rather than Alk1 (Figure 3).

The Diabetic Milieu accelerates BOEC Proliferation

In BOECs, incubation with high glucose/gAlb accelerates cell cycle transition as indicated by the increase in levels of proliferating cell nuclear antigen, PCNA, a nuclear protein within the active DNA polymerase Δ complex (Figure 4a). This increase in PCNA is further amplified by co-incubation with SB431542 which inhibits the activity of the Alk5 kinase. Moreover, high glucose/gAlb modestly but significantly raises the proliferation of BOECs, on average by 15 % (Figure 4b). Importantly, inhibition of Alk5 by SB431542 amplifies the proliferative response to high glucose/gAlb significantly (Figure 4b). At the same time high glucose/gAlb as well as rhTGF β induce VEGF (Figure 4c), the major growth and angiogenesis factor in endothelial cells. The increase in VEGF levels that is induced by HG/gAlb is not blocked by co-incubation with the Alk5-kinase inhibitor, SB431542. These data suggest that TGF β induces endothelial cell proliferation through an Alk1-smad1/5-VEGF pathway.

High Glucose/gAlb raise Caspase-3 Activity through Alk5

Other investigators have previously shown that a diabetic milieu raises the incidence of cell apoptosis and that this effect depends, in part, on a decline in Erk1/2-activation (Figure 3) but may also be mediated to some extent through TGF β (Yang et al., 2008). Here we demonstrate that high glucose/gAlb induces activation of caspase-3 and raises active caspase-3 levels in blood outgrowth endothelial cells (Figure 5). Active caspase-3 levels as well as caspase-3 activity are less induced during co-incubation with the Alk5 inhibitor, SB431542, suggesting that glucose/gAlb-induced TGF β and its activation of Alk5 contributes to this high glucose effect (Figure 5). Thus, in totality, this data indicates that Alk5 plays an important but not exclusive role in high glucose/gAlb – induced caspase-3 activation (and apoptosis).

Pro-Angiogenic Activation of Collagenase is Mediated by Alk1

Diabetes mellitus is associated with neo-angiogenesis in some tissues and several factors contribute to this effect. Among major angiogenesis mechanisms are proliferation of endothelial cells as well as activation of matrix metalloproteases. Given that high glucose/gAlb induces VEGF (likely via TGF β -Alk1; Figure 4c) we also examined the levels of pro- and active MMP2 under the current control and diabetes-mimicking conditions. Incubation of BOECs with rhVEGF induces a decrease in pro-MMP2 and an increase in active MMP2 levels in cell lysates (Figure 6a). Quantitative analysis of the change in the ratio of active/total MMP2 indicates a 3.5-fold increase by VEGF (Figure 6b). In conditioned media from cells that were incubated with VEGF only the 64 kDa band of active MMP2 is visible in Western blots. This is consistent with the fact that only the activated MMP2 is secreted by the cells (Figure 6a). As compared to normal glucose, incubation of BOECs with high glucose/gAlb activates MMP2 as indicated by the disappearance of the 72 kDa pro-MMP2 band (Figure 6c). In addition, active MMP2 is secreted into the medium under these latter conditions but not in cells incubated with normal glucose (Figure 6c). This transition of pro- into active MMP2 is not at all inhibited by SB431542 suggesting that this effect of high glucose/gAlb is not Alk5 rather than Alk1 dependent.

Discussion

Proliferation of endothelial cells and increased angiogenesis is a hallmark early in diabetes mellitus. In some vascular beds such as renal glomeruli increased angiogenesis contributes to an enlargement of the glomerular surface area and, hence, contributes to early hyperfiltration but may also give rise to subsequent pathology such as micro aneurysm formation. Activation of endothelial cell proliferation in normal organisms occurs after local injury. During wound

healing TGF β levels increase in the wound and induce VEGF which promotes angiogenesis and formation of granulation tissue (Tonnesen et al., 2000). Long-term tissue hypoxia promotes angiogenesis and neovascularization which, however, is primarily driven by hypoxia-inducible factor-1 α (HIF1 α). Similar mechanisms provide angiogenesis, survival and growth advantages to some malignant tumors (Liao and Johnson, 2007). In early diabetes hypoxia-dependent mechanisms do not appear to play a major role vis-à-vis induction of TGF β expression and secretion which occurs in many different cell types and tissues in diabetic hyperglycemia.

TGF β is an important mediator of tissue fibrosis as well as endothelial proliferation. This pleiotropic cytokine induces several cellular pathways cell type and context dependently. In endothelial cells TGF β induces complex responses which can include the production of extra cellular matrix proteins and other fibrosis-stimulating regulators (Kose et al., 2007). Other effects of this growth factor include growth cues. These different responses are explainable by the expression of two different type I receptors, Alk1 and Alk5 and the presence of endoglin, which associates with Alk1 and steers TGF β activity to this latter receptor by enhancing its affinity (Lebrin et al., 2004; Lee et al., 2008). The present studies specifically examined the involvement of Alk1 and Alk5 in diabetes mellitus – simulating effects of high glucose and glycated albumin on endothelial cell proliferation.

The current experimental studies confirm previous reports that TGF β induces endothelial cell proliferation (Lebrin et al., 2004). The mechanisms of diabetes-induced BOEC-proliferation involve activation of the Alk1-smad1/5 pathway (rather than Alk5-smad2/3), and VEGF. Current findings are compatible with the notion that endothelial cell proliferation in the diabetes-mimicking milieu is regulated, at least in part, through the axis TGF β -Alk1-smad1/5-VEGF. It is independent of Erk which is, in fact, inactivated by glycemia probably through a TGF β /Alk5 mechanism as suggested from current experimental findings. Another novel finding by the present studies is that high glucose/gAlb induces Alk5 levels. The mechanisms of this effect of the diabetic environment were not further explored.

Activation of Alk1 and Alk5 by high glucose/gAlb through TGF β lead to opposing effects in endothelial cells: Proliferation and angiogenesis downstream of Alk1 and antiproliferative cues and pro-apoptotic activation of caspase-3 through Alk5 as shown in the present studies. The in-vivo validity of the present findings are supported by recent studies by other investigators who demonstrated in diabetic smad-3 knock-out mice that the development of glomerular fibrosis, a TGF β – Alk5 – smad3 mediated effect of diabetes, is reduced but the glomerular capillary growth and endothelial proliferation are similar compared to diabetic wild-type animals (Wang et al., 2007). Goumans and associates recently showed elegantly that Alk1 activation not only leads to opposite TGF β effects than activation of Alk5 in endothelial cells but also antagonizes Alk5-smad2/3 signaling (Goumans et al., 2003). These differential effects are similarly induced by high glucose in conjunction with glycated albumin as shown in the current studies.

In summary, in the present studies we examined the role of Alk1 and Alk5 type I TGF β receptors in high glucose/glycated albumin – induced responses in human blood outgrowth endothelial cells. The diabetic milieu induces TGF β and also leads to increased expression of Alk5 whereas Alk1 and the co-receptor endoglin are constitutively expressed and levels remain unchanged. Both the smad2/3 and the smad1/5 TGF β -pathways downstream of Alk5 and Alk1, respectively, are activated by HG/gAlb in cultured endothelial cells. Diabetes induces both endothelial cell proliferation through Alk1-smad1/5 and VEGF as well as pro-apoptosis activity through Alk5. High glucose/gAlb also down-regulates Erk phosphorylation through Alk5 which, however, has no implication for endothelial cell growth regulation but may contribute to increased apoptosis. Other high glucose/gAlb induced effects include activation of MMP2 which is a pre-requisite for endothelial cell migration and, hence, angiogenesis. It

is possible that the TGF β pathway through Alk1-smad1/5-VEGF contributes to MMP2 activation in the diabetic milieu. From these observation and published findings from other laboratories we develop a model for endothelial cell responses to a diabetic environment that is depicted in Figure 7.

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Figure 1.

Characterization of differentiated normal human outgrowth endothelial cells, passage 4. Cells are positive for VE-cadherin (red) and von Willebrand Factor (vWF, green) but are negative for the endothelial progenitor markers CD34 and CD133. Nuclei are counter-stained with DAPI (blue).

Figure 2.

a) Incubation of BOECs with growth factor-free, low serum medium containing high glucose and glycated albumin (HG/gAlb) induces expression of Alk5 whereas Alk1 and endoglin are constitutively expressed and remain unchanged. b) HG/gAlb raises accumulation of active TGFβ in media; *p<0.05.

Figure 3.

HG/gAlb induces phosphorylation of smad1/5 as well as smad2/3 indicating activation of the Alk1-smad1/5 and the Alk5-smad2/3 TGF β -pathways. Alk5 pathway activation is blocked by the Alk5 kinase inhibitor, SB431542. HG/gAlb reduces Erk1/2 phosphorylation which is reversed by co-incubation with SB431542 suggesting that this effect of HG/gAlb depends on the TGF β -Alk5-smad2/3 pathway.

Figure 4.

a) HG/gAlb raises the levels of PCNA which is moderately enhanced by inhibition of Alk5 with SB431542. b) HG/gAlb accelerates cell proliferation which is enhanced by blocking Alk5 with SB431542. c) Both HG/gAlb and TGF β each induce VEGF. Induction of VEGF by HG/gAlb is not blocked by co-incubation with SB431542 suggesting independence from the Alk5-smad2/3 pathway. *p<0.05 vs. normal glucose (NG) control; **p<0.05 vs. HG/gAlb.

Figure 5.

a) HG/gAlb induces activation of Caspase-3 as shown in Western blots using an antibody that specifically recognizes active (cleaved) caspase-3. b) Quantitation of densitometric data from active Caspase-3 Western blot analyses. c) Results of an independent caspase-3 activity assay using a specific peptide substrate. SB = SB431542; * $p < 0.05$ compared to normal glucose (NG) controls; ** $p < 0.05$ vs. HG/gAlb.

Figure 6.

a) Western blot analysis of MMP2 in cell lysates (top) or media (bottom) after incubation with VEGF. The 72 kDa band represents pro-MMP2; the 64 kDa band represents mature, active MMP2. b) Quantitative analysis of the ratio of densitometric units of active/total MMP2 in cell lysates. c) MMP2 Western analysis in cell lysates and media after incubation with HG/gAlb in the presence or absence of the Alk5 inhibitor, SB431542. Findings indicate that the cleaving (activation) of pro-MMP2 by HG/gAlb is independent of Alk5.

Figure 7.
Working model of dual actions of high glucose/albumin through Alk1- and Alk5-containing TGFβ receptor complexes.