Cell matrix contact modifies endothelial major histocompatibility complex class II expression in high-glucose environment

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An estimated 285 million people worldwide are affected by diabetes mellitus (16). With an aging population and the rate of obesity on the rise, the number of people with diabetes is expected to dramatically increase over the next years. This will be accompanied by an increase in diabetes-associated micro- and macrovascular complications such as atherosclerotic heart disease, stroke, blindness, amputations, and kidney disease. Metabolic and hormonal imbalances contribute to the pathogenesis of diabetic vascular diseases, and loss of the modulatory role of the endothelium may be a critical and initiating factor in development of such diseases (37). Fundamental pathogenetic mechanisms in diabetes-associated vascular disease include endothelial dysfunction, accentuated inflammation, and increased oxidative stress. Hyperglycemia and its immediate biochemical sequelae directly alter endothelial function or influence endothelial cell (EC) functioning indirectly by the synthesis of growth factors, cytokines, and vasoactive agents in other cells (36). Hyperglycemia promotes endothelial activation, monocyte chemotactant protein-1, ICAM-1, and VCAM-1 expression in cultured ECs (1, 19). Others and we have demonstrated alteration of the subendothelial basement membrane by a hyperglycemic environment (7, 8, 11, 30, 40).

ECs constitutively express major histocompatibility complex (MHC) class I molecules and upon interferon (IFN)-γ stimulation MHC class II molecules. Together with stimulatory molecules, this enables ECs to act as semiprofessional antigen-presenting cells in activating naïve T lymphocytes. We previously demonstrated that IFN-γ-induced endothelial costimulatory and MHC class II expression is regulated by cell matrix contact (26, 28, 29, 31). We now explore whether this matrix-dependent regulation prevails in a high-glucose environment. In the present study, ECs plated on two-dimensional polystyrene-coated tissue culture plates (TC-EC) or embedded within a three-dimensional (3-D) collagen-based matrices (3D-EC). In the absence of glucose, IFN-γ-induced phosphorylation of JAK and STAT proteins and human leukocyte antigen (HLA)-DR expression were lower in 3D-EC compared with TC-EC. Inversely, the expression of suppressor of cytokine signaling proteins (SOCS)-1 and -3 were significantly higher in naïve 3D-EC compared with naïve TC-EC. IFN-γ-induced upregulation of SOCS proteins was further amplified by the 3-D environment. Glucose significantly augmented IFN-γ-dependent signaling pathways in TC-EC. IFN-γ-induced phosphorylation of JAK and STAT proteins as well as HLA-DR expression by ECs in low- and high-glucose medium was significantly lower in 3-D than in two-dimensional environment. Glucose increased SOCS expression in TC-EC and 3D-EC to the same extent, such that expression levels in 3D-EC exceeded SOCS-1 and -3 expression in 3-D by 1.6–2.5-fold. In conclusion, low- and high-glucose concentrations amplify IFN-γ-induced signaling pathways in TC-EC. Increased SOCS expression raise the threshold for IFN-γ to induce HLA-DR expression in a 3-D environment. This immunoprotective effect is maintained even in states of experimental hyperglycemia.

endothelial cell; ECM; diabetes; immunomodulation

AN ESTIMATED 285 MILLION PEOPLE worldwide are affected by diabetes mellitus (16). With an aging population and the rate of obesity on the rise, the number of people with diabetes is expected to dramatically increase over the next years. This will be accompanied by an increase in diabetes-associated micro- and macrovascular complications such as atherosclerotic heart disease, stroke, blindness, amputations, and kidney disease. Metabolic and hormonal imbalances contribute to the pathogenesis of diabetic vascular diseases, and loss of the modulatory role of the endothelium may be a critical and initiating factor in development of such diseases (37). Fundamental pathogenetic mechanisms in diabetes-associated vascular disease include endothelial dysfunction, accentuated inflammation, and increased oxidative stress. Hyperglycemia and its immediate biochemical sequelae directly alter endothelial function or influence endothelial cell (EC) functioning indirectly by the synthesis of growth factors, cytokines, and vasoactive agents in other cells (36). Hyperglycemia promotes endothelial activation, monocyte chemotactant protein-1, ICAM-1, and VCAM-1 expression in cultured ECs (1, 19). Others and we have demonstrated alteration of the subendothelial basement membrane by a hyperglycemic environment (7, 8, 11, 30, 40).

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We previously demonstrated significantly increased endothelial αβ integrin expression by 3D-EC compared with TC-EC (12). Signaling by αβ integrins is important for outside-in signaling (4, 18, 21) and is involved in endothelial mechanotransduction and arteriogenesis (2, 34). Ligands include fibronectin, fibrinogen, proteolysed collagen, and vitronectin (14). In line with previous results (12), we now also wanted to explore whether effects of glucose environment on endothelial MHC-II expression in Gelfoam can be influenced by blocking αβ integrin binding.

Furthermore, coating of tissue culture polystyrene with collagen type IV was used to mimic physiological basement membrane composition as well as configuration of Gelfoam matrixes (22).

MATERIALS AND METHODS

Human aortic ECs were obtained from Clonetics and grown in optimized endothelial growth medium-2 (EGM-2,Cambrex,MD) supplemented with 5% FBS either on polystyrene-coated tissue culture plates or embedded within Gelfoam sheets (Pfizer) as previously...
described (29). Homogeneous distribution of ECs within the Gelfoam was demonstrated by scanning electron microscopy (data not shown) and biosecretory function through assays for production of a standard panel of factors. Cell viability was determined by trypan blue exclusion and a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes). ECs were left in complete EGM-2 (control), complete EGM-2 with 25 mM glucose, or complete EGM-2 with 50 mM glucose. In some experiments tissue culture plates were coated with collagen type IV (Sigma-Aldrich) or Gelfoam embedded ECs incubated with a human antibody blocking αβ1-integrin binding (Chemicon).

**EC biosecretory function.** Biosecretory function of TC-EC and 3D-EC was compared. Total protein production was determined by BCA protein assay-kit (Pierce, IL). Total glycosaminoglycan (GAG) and heparan sulfate (HS) production was determined using a dimethylmethane blue assay before and after cell-conditioned medium treatment with chondroitinase ABC (0.1 U/sample, Seikagaku America) for 3 h at 37°C to eliminate chondroitin and dermatan sulfate. Prostacyclin (PGI2) concentrations were determined by a 6-keto-prostaglandin F1α enzyme immunoassay system (Amersham Biosciences). Transforming growth factor-β (TGF-β) production was determined using standard ELISA assays (Amersham).

Nitric oxide (NO) production was evaluated by measuring levels of nitrite, the oxidized product of NO, by Griess reaction as previously described (13). Briefly, basal production was measured by subtracting nitrite values at time 0 from cumulative concentrations obtained after 3 h of incubation with low and high glucose.

**RT-PCR.** Total RNA was extracted from TC-EC and 3D-EC after stimulation with IFN-γ for indicated time periods using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Complementary DNA was synthesized using the TaqMan reverse-transcription reagents from Applied Biosystems (Foster City, CA). Real-time PCR analysis was performed with an Opticon Real-Time PCR Machine (MJ Research) using SYBR Green PCR Master Mix Reagent Kit (Applied Biosystems) and primers shown in Table 1. Data from the reaction were collected and analyzed by the complementary Opticon computer software (MJ Research). Relative quantitations of gene expression were calculated with standard curves and normalized to GAPDH.

Flow cytometry. EC monolayers or ECs embedded in Gelfoam were harvested after culture in complete medium stimulated with 1,000 U/ml IFN-γ for 48 h. Media was aspirated and cells were washed with PBS. Monolayers were incubated in 1.0 mM PBS-EDTA for 5 min and disrupted by gentle shaking. Gelfoam were digested with collagenase type I, shown to have no effect on MHC-II surface expression (29). Cell suspensions were washed, and 3 × 10⁵ cells were resuspended in fluorescence-activated cell sorting (FACS) buffer, i.e., PBS containing 0.1% BSA and 0.1% sodium azide, (Sigma Chemicals; St. Louis, MO), ECs were incubated with mouse anti-human leukocyte antigen (HLA)-DP,DQ,DR (clone CR3/43, DakoCytomation, Carpenteria, CA), for 30 min at 4°C, resuspended in FACS buffer and stained with a FITC-labeled rat anti-mouse IgG, (clone A85-1, Pharmingen, San Diego, CA) for 30 min at 4°C. Cells were then washed and fixed in 1% paraformaldehyde, and 10⁵ cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson, San Diego, CA).

**Western blot analysis.** Cell monolayers or cells digested from Gelfoam matrices by collagenase treatment were washed in PBS buffer. Cell lysates were prepared by incubation with lysis buffer containing 20 mM Tris, 150 mM NaCl (pH 7.5), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and protease inhibitor (Roche), and samples were separated on 4–20% Ready Tris-HCl gels (Bio-Rad, Hercules, CA). Proteins were then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) using glycin-Tris transfer buffer. Blot membranes were blocked in Starting Block blocking buffer (Pierce, Rockford, IL) for 1 h. Blocked membranes were next incubated with rabbit anti-human JAK-1, JAK-2, STAT-1 (all phosphorylated and unphosphorylated; Cell Signaling, Danvers, MA) in blocking buffer overnight at 4°C. Membranes were then washed three times at room temperature with wash buffer consisting of PBS with 0.05% Tween 20 and then incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling) in blocking buffer for 2 h at room temperature followed by washing in five changes of wash buffer. For detection, blots were incubated with chemiluminescence substrate (Western Lightning Chemiluminescence Reagent Plus kit, Perkin-Elmer, Boston, MA) according to the manufacturer’s instructions followed by exposure to X-ray film (Kodak X-Omat Blue XB-1).

**Allogeneic T-cell proliferation assay.** Upon confluence, ECs were exposed to 1,000 U/ml IFN-γ for 48 h. Cell growth was subsequently arrested using 50 μg/ml mitomycin C. CD4+ T cells were isolated from fresh human blood using a negative selection kit (Miltenyi). Isolated T cells were labeled with 10 μM carboxyfluorescein succinimidyl ester (Invitrogen) for 10 min at 37°C in phenol red free RPMI-1640 and washed three times. Labeled CD4+ T cells were cocultured at 5 × 10⁵ T cells per 1.25 × 10⁵ ECs or with empty scaffolds/tissue culture plates in RPMI medium without phenol red and maintained for 6 days, and thereafter T cells were pelleted by centrifugation, resuspended in PBS and analyzed by flow cytometry. Proliferation, i.e., division, of T cells resulted in less intense staining per cell and decrease in mean fluorescence intensity of the cells.

**Statistics.** Statistical analyses were performed with Prism (GraphPad) or Excel (Microsoft) software. Data are expressed as means ± SE unless noted. Comparisons between groups were made by ANOVA followed by Tukey’s multiple comparison test. A Spearman correlation determined relations between T-cell proliferation and suppressor of cytokine signaling proteins (SOCS) mRNA expression levels. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**Biochemical activity of ECs.** After 24 h total GAG concentration in conditioned medium from low (3.19 ± 0.2 μg/10⁶ cells) and high glucose (4.21 ± 0.18 μg/10⁶ cells)-treated confluent, TC-EC was significantly higher than in control ECs without glucose (2.63 ± 0.14 μg/10⁶ cells; P < 0.05 vs. 25 mM, P < 0.02 vs. 50 mM glucose). Release of HS by glucose-stressed TC-EC was similar 1.5-fold higher than by control TC-EC (25 mM: 1.62 ± 0.01, 50 mM 2.07 ± 0.02, control: 1.38 ± 0.02 μg/10⁶ cells; P < 0.05 vs. 25 mM, P < 0.05 vs. 50 mM glucose). In contrast, PGI2 concentration in conditioned medium decreased by up to 40% when ECs were exposed to glucose (25 mM: 58.4 ± 4.8, 50 mM 41.8 ± 3.2, control: 69.7 ± 4.1 pg/10⁶ cells; P < 0.05 vs. 50 mM glucose). TGF-β increased 1.3-fold in high-glucose medium (25 mM: 0.9 ± 0.01, 50 mM 1.1 ± 0.02, control: 0.8 ± 0.02 μg/10⁶ cells; P < 0.05 vs. 50 mM glucose). Coating of tissue culture
plates with collagen type IV had no significant effect on biosecretion by naïve and glucose-exposed ECs (data not shown).

There was no difference in concentrations of GAG (2.69 ± 0.12 μg/mL, cells), HS (1.4 ± 0.03 μg/mL, cells), PGL2 (67.4 ± 2.9), and TGF-β (0.8 ± 0.02 μg/mL, cells) in conditioned medium from 3D-EC grown without glucose compared with conditioned medium from TC-EC. Without the exemption of increased HS release in high-glucose environment (1.62 ± 0.02 μg/mL, cells, P < 0.05 vs. 3D-EC control), glucose substitution was without effect on biochemical activity of 3D-EC. Treatment of 3D-EC with αβ3-integrin antibody significantly increased GAG- (2.91 ± 0.20 μg/mL, cells) and HS-release (1.3- and 1.4-fold, respectively (naïve: 103 vs. 3D-EC control) and HS-release (1.7 ± 0.05 μg/mL, cells; P < 0.05 vs. 3D-EC control) but had no influence on PGI2 and TGF-β secretion. Blocking of αβ3-integrin binding amplified release of GAG (3.44 ± 0.2 μg/mL, cells; P < 0.02 vs. 3D-EC control), HS (1.81 ± 0.01 μg/mL, cells; P < 0.05 vs. 3D-EC control), and TGF-β (1 ± 0.03 μg/mL, cells; P < 0.05 vs. 3D-EC control) in high- but not low-glucose environment.

Glucose in low and high concentrations increased NO release by TC-EC 1.3- and 1.4-fold, respectively (naïve: 103 vs. 3D-EC control) and HS-release (1.7 ± 0.05 μg/mL, cells; P < 0.05 vs. 3D-EC control) but remained unchanged on TC-EC in all of the medium environments studied. Upon stimulation with 1,000 U/ml IFN-γ for 4 h in absence or presence of glucose (25 or 50 mM glucose). *P < 0.005 vs. IFN-γ-stimulated TC-EC without glucose; †P < 0.001 vs. IFN-γ-stimulated TC-EC without glucose. TGF-β-stimulated TC-EC without glucose; ‡P < 0.02 vs. IFN-γ-stimulated TC-EC without glucose.

Intracellular signaling is modified by glucose and by cell-matrix contact. We next analyzed intracellular signaling pathways upon IFN-γ stimulation. IFN-γ-induced phosphorylation of JAK1 was higher in TC-EC compared with 3D-EC (P < 0.001). Low and high glucose-induced JAK1 phosphorylation in naïve ECs in both growing conditions. Whereas IFN-γ-induced JAK1 phosphorylation in TC-EC in low- and high-glucose medium to an even higher level, IFN-γ-stimulation

Three-dimensional matrix embedding had a nonsignificant effect on NO release by naïve ECs (113 ± 8 pmol/3 × 10^5 cells/h). Substitution of medium with low and high glucose exerted similar effects on NO release in 3D-EC compared with TC-EC (25 mM: 149 ± 13 pmol/3 × 10^5 cells/h, P < 0.02 vs. naïve 3D-EC, 50 mM: 168 ± 8 pmol/3 × 10^5 cells/h, P < 0.02 vs. naïve 3D-EC). NO release by 3D-EC in high-glucose medium was significantly higher than release by TC-EC in low- and high-glucose medium (P < 0.05 vs. 25 and 50 mm TC-EC). Blocking of αβ3-integrin binding was without effect on NO release in no glucose and low- and high-glucose environment.

Endothelial MHC-II expression is modified by glucose and by cell-matrix contact. There was no significant difference in HLA-DR expression between nonstimulated 3D-EC and TC-EC in all of the medium environments studied. Upon IFN-γ stimulation in control medium without glucose, mRNA expression of HLA-DR was significantly higher in TC-EC when compared with 3D-EC [0.32 ± 0.04 vs. 0.1 ± 0.04 relative units (RU), P < 0.005; Fig. 1A]. Low- and high-glucose medium significantly upregulated IFN-γ-dependent HLA-DR expression in TC-EC (25 mM: 0.71 ± 0.1, 50 mM: 0.97 ± 0.01 RU, P < 0.0001 vs. control; Fig. 1A). In marked contrast, glucose substitution was without significant effect on HLA-DR expression in 3D-EC (25 mM: 0.16 ± 0.04, 50 mM: 0.18 ± 0.08 RU; Fig. 1A).

Protein surface HLA-DR expression tracked mRNA expression patterns with 1.8-fold higher expression in IFN-γ-stimulated TC-EC compared with 3D-EC (68 ± 4 vs. 37 ± 5%, P < 0.001; Fig. 1B). In low- and high-glucose medium, HLA-DR surface expression was further upregulated by IFN-γ in TC-EC (25 mM: 77 ± 3, 50 mM: 86 ± 5%; P < 0.05 vs. 25 mM, P < 0.02 vs. 50 mM glucose; Fig. 1B) but remained unchanged on a low expression level in 3D-EC (39 ± 6% in 25 mM, 39 ± 4% in 50 mM; Fig. 1B).

Coating tissue culture plates with collagen type IV attenuated IFN-γ-induced HLA-DR expression in control TC-EC without glucose and in glucose environment (control: 52 ± 4%, 25 mM: 64 ± 4, 50 mM: 71 ± 3%; P < 0.05 vs. noncoated tissue culture polystyrene; data not shown). Preincubation of 3D-EC with a blocking antibody against human αβ3-integrin limited the 3-D matrix effect on IFN-γ-induced HLA-DR expression by ECs in all culture conditions studied (control: 49 ± 3%, 25 mM: 50 ± 3, 50 mM: 47 ± 4%, data not shown).

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The page contains detailed scientific research on the effects of glucose and high glucose on endothelial cell function, specifically focusing on MHC class II expression and intracellular signaling pathways. It discusses the impact of different glucose concentrations on NO release, HLA-DR expression, and JAK1 phosphorylation in both two-dimensional (2D) and three-dimensional (3D) cultures. The text highlights the differences in cellular responses between TC-EC (tissue culture)- and 3D-EC (3D matrix)-grown cells, with glucose substitution showing varied effects on various biochemical and physiological activities.
Glucose substitution alone resulted in increased phosphorylation of JAK1 in 3D-EC (Fig. 2A).

Phosphorylation of JAK1 upon IFN-γ-stimulation was significantly attenuated when ECs were embedded in 3-D Gelfoam matrixes in all culture conditions tested (P < 0.001). Glucose substitution alone resulted in increased phosphorylation levels of JAK2 in TC-EC but not in 3D-EC (P < 0.001; Fig. 2B).

Upon IFN-γ-stimulation STAT1 phosphorylation in TC-EC significantly exceeded STAT1 phosphorylation in 3D-EC (P < 0.05). Whereas glucose substitution further augmented phosphorylation of STAT1 in TC-EC by IFN-γ, the diabetes-mimicking condition had no influence on cytokine-induced STAT1-phosphorylation when ECs were embedded within Gelfoam matrixes (P < 0.001; Fig. 2C).

**Glucose and cell matrix contact regulate SOCS expression.** Using RT-PCR, we next analyzed expression levels of SOCS-1 and -3. In control medium without glucose, SOCS-1 expression was 2.5-fold higher in unstimulated 3D-EC than in naïve TC-EC (P < 0.002; Fig. 3A). There was no significant difference in SOCS-3 expression between naïve ECs cultured on tissue culture plates or in 3-D matrixes (Fig. 3A). IFN-γ induced upregulation of SOCS protein in TC-EC (Fig. 3A) was significantly amplified by low- and high-glucose environment (P < 0.02; Fig. 3, B and C). Glucose alone had no effect on SOCS-1 and -3 expression in naïve TC-EC (data not shown).

Matrix-embedding significantly enhanced IFN-γ-induced endothelial expression of SOCS (P < 0.005; Fig. 3, B and C). This effect prevailed in low- and high-glucose environment in 3D-EC upon IFN-γ treatment. Again, low and high glucose did not affect SOCS-1 and -3 expression in unstimulated 3D-EC (data not shown).

Coating tissue culture plates with collagen type IV resulted in upregulation of SOCS-1 in IFN-γ-stimulated ECs without reaching the level of SOCS-1 expression in 3D-EC but had no significant effect on SOCS-3 expression (P < 0.05; Fig. 3A). The effect of coating tissue culture dishes with collagen type IV on IFN-γ-induced SOCS-1 expression prevailed in low- and high-glucose medium (Fig. 3B).

Preincubation of naïve 3D-EC with a blocking antibody against human αvβ3-integrin partially reversed the effect of 3-D matrix-embedding on endothelial SOCS-1 and SOCS-3 expression (P < 0.05; Fig. 3A). SOCS-1 induction by glucose was limited by anti αvβ3-integrin treatment (P < 0.05; Fig. 3B), whereas glucose imposed SOCS-3 modulation was not affected.

**Effect of glucose and matrix environment on endothelial induced T-cell proliferation.** As we have previously demonstrated attenuated proliferation of allogeneic T cells when exposed to IFN-γ-stimulated 3D-EC instead of TC-EC (29), we next assayed the effect of low- and high-glucose environment on allogeneic T-cell proliferation by carboxyfluorescein succinimidyl ester staining. Mean fluorescence intensity of T cells is inversely proportional to T-cell proliferation and significantly decreased by glucose exposure of TC-EC (Fig. 4A). The effect of glucose environment on T-cell proliferation was

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**Fig. 2.** IFN-γ induced phosphorylation (p) of JAK-1 (A) and -2 (B) and STAT-1 (C) in human aortic ECs is modified by glucose and by cell-matrix contact. Lysates from TC-EC or 3D-EC were analyzed on Western blots. MW, molecular weight. Treatment of TC-EC and 3D-EC with 100 mM glucose were not included in the analysis because of unphysiological glucose in vivo concentration (STAT-1). Western blots are representative for 3 independent experiments. Densitometer measurements of phosphorylated protein/unphosphorylated protein is shown in the bottom panel. † P < 0.05 vs. 3D-EC; * P < 0.001 vs. 3D-EC; # P < 0.001 vs. 3D-EC without glucose; $ P < 0.05 vs. IFN-γ-stimulated TC-EC with 25 mM glucose.
absent in 3D-EC (Fig. 4A). There was no significant difference in T-cell proliferation when exposed to unstimulated 3D-EC and TC-EC in all of the medium environments studied.

Coating tissue culture plates with collagen type IV significantly attenuated T-cell proliferation when exposed to IFN-γ treated TC-EC without and with glucose medium (Fig. 4B).

Preincubation of 3D-EC with a blocking antibody against human α3β1-integrin partially increased T-cell proliferation when exposed to 3D-EC in all culture conditions studied without reaching significance (Fig. 4C).

An inverse correlation was noted between T-cell proliferation and SOCS-1 ($r = -0.73, P < 0.002$) but not with SOCS-3 expression levels ($r = -0.48, P = 0.64$, data not shown).

**DISCUSSION**

The field of tissue engineering has gained increasing interest over the past decade. Currently matrix and cell-matrix constructs as well as whole organ preparations are tested in clinical trials or readily available for replacing functions and structures of diseased and/or injured tissues. Aiming to mimic the nature of these tissues, recent research has concentrated on the pathophysiology of cell-matrix interactions and the complexity of different cell-tissue interconnections. Others and we have learned that the natural 3-D environment bears fascinating features that are absent or even counterregulated in two dimensions and can thus be of clinical relevance when developing tissue engineered constructs for in vivo applications (17). This holds especially true for issues dealing with immunoproperties of these constructs. Fascinating enough, we could detect reduced immunogenicity of ECs when presented within a 3-D environment compared with the classical two-dimensional mode of culturing (25, 26). Interestingly, these 3-D EC constructs can be implanted in an allogeneic and even xenogeneic environment without the need for immunosuppression (27–29).

A clinical trial with these implants was successfully concluded in patients with end-stage renal disease (ClinicalTrials.gov Identifier: NCT00479180). Among this study population, 60% had diabetes mellitus and 3-D EC constructs provided the same level of effectiveness within this specific subgroup as over the whole study population when looking for patency rates of arteriovenous access sites for hemodialysis use (9). As diabetes mellitus is associated with development of endothelial dysfunction, this prompted us to explore the effects of low- and high-glucose environments on endothelial immunoproperties in three versus two dimensional environments in vitro.

The vascular endothelium represents a critical interface between blood and all tissues. Endothelial dysfunction is one of the early clinical hallmarks of atherosclerotic lesion devel-

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**Fig. 3.** IFN-γ induces suppressor of cytokine signaling proteins (SOCS)-1 and SOCS-3 expression in human aortic ECs. SOCS expression is higher in a 3-D matrix environment and can be further be induced by low and high glucose. A: quantitative mRNA levels demonstrating the time course of SOCS-1 and SOCS-3 induction by IFN-γ (1,000 U/ml) in 3D-EC. 3D-EC incubated with a blocking human α3β1-integrin antibody, TC-EC, or on TC-EC coated with type IV collagen. *P < 0.002 vs. TC-EC; †P < 0.05 vs. 3D-EC and uncoated TC-EC. B: quantitative SOCS-1 mRNA levels in TC-EC or 3D-EC with or without stimulation with 1,000 U/ml IFN-γ for 1 h in absence or presence of glucose (25 or 50 mM glucose). In some experiments, 3D-EC were incubated with a blocking human α3β1-integrin antibody or TC-EC coated with type IV collagen. *P < 0.005 vs. TC-EC; †P < 0.02 vs. 3D-EC; ‡P < 0.05 vs. IFN-γ-stimulated 3D-EC with 25 mM glucose; ‡P < 0.05 vs. TC-EC; #P < 0.05 vs. IFN-γ-stimulated 3D-EC in low- and high-glucose medium. C: Quantitative SOCS-3 mRNA levels in TC-EC or 3D-EC with or without stimulation with 1,000 U/ml IFN-γ for 30 min in absence or presence of glucose (25 or 50 mM glucose). *P < 0.005 vs. TC-EC; †P < 0.02 vs. 3D-EC; ‡P < 0.05 vs. TC-EC; #P < 0.05 vs. IFN-γ-stimulated 3D-EC with 25 mM glucose; §P < 0.05 vs. IFN-γ-stimulated TC-EC with 25 mM glucose.
opment and cardiovascular risk factors including diabetes contribute to its development. In response to inflammatory stimuli, the vascular endothelium expresses a number of adhesion molecules that play key roles in the recruitment of leukocytes to sites of inflammation (20, 33). Furthermore, expression of costimulatory molecules and MHC-I and -II molecules enables ECs to act as antigen-presenting cells capable of activating cells of the adaptive immune system (33).

The importance of EC polarity and interaction with extracellular matrix components as key regulators of EC phenotype and function continue to be elucidated (26, 28, 31). ECs are naturally basement membrane surface adherent, and disruption of extracellular matrix structure, EC-matrix contact, and 3-D architecture are early features of inflammatory diseases and metabolic derangements. Substratum interactions dictate EC control of vascular homeostasis (32). Diabetes mellitus is associated with endothelial dysfunction, and metabolic disarrangements have been linked with alterations in EC binding modalities to the underlying extracellular matrix (e.g., accumulation of advanced glycation end products) (3, 6).

In the present study, we show that glucose influences biochemical activity of ECs by increasing total GAG concentration, HS, and TGF-β, whereas PGI2 secretion by ECs decreased significantly when cells were exposed to low- and high-glucose environment. In marked contrast, matrix embedding prevented the influence of glucose on endothelial biochemical activity with exemption of HS secretion that increased in matrix embedded ECs in glucose environment. Interestingly, glucose enhanced NO release by TC-EC, an effect also seen in 3D-EC with highest NO release by matrix-embedded ECs in high-glucose medium.

As a main result we demonstrated that regulation of MHC-II expression in ECs can indeed be modified by glucose: this regulation is under control of the extracellular matrix environment. Three-dimensional matrix embedding of ECs not only limited IFN-γ-induced MHC-II expression but also attenuated the effect of low- and high-glucose environment on its upregulation. We also demonstrate that hyperglycemia amplifies IFN-γ dependent signaling and downstream expression of MHC II in ECs when cultured on two-dimensional polystyrene-coated culture plates.

In contrast, embedding ECs within 3-D collagen-based matrices prevented the significant downstream signaling activation upon IFN-γ challenge seen in two-dimensional grown ECs. Even more interesting, the short-term effect of low- and high-glucose environment on phosphorylation of JAK and STAT proteins and downstream expression of MHC II upon IFN-γ stimulation was inhibited by the 3-D environment. As a result T-cell proliferation was significantly lower when ECs were presented matrix-embedded compared with their two-dimensional counterparts. Glucose amplified the immune activation in TC-EC but was without significant effect on proliferation of T cells when exposed to 3D-EC in low- and high-glucose medium.

The effect of 3-D matrix embedding on MHC-II expression and intracellular signaling pathways could be partially inhibited by preincubation of ECs with an anti-αβ3-integrin antibody. We have previously demonstrated that αβ3-integrin is significantly higher expressed by 3D-EC compared with TC-EC and mediates effects of the underlying basement membrane as well as of the 3-D environment on immune behavior.
of ECs (12). On the other hand coating tissue culture plates with collagen type IV mimics the basement membrane composition of Gelfoam without its 3-D effect; hence collagen type IV coating partially mimicked the effects of 3-D matrix embedding with increased SOCS1 expression and attenuated MHC-II expression by TC-EC. Coating tissue culture plates with collagen type IV limited T-cell proliferation in naïve and glucose medium whereas blocking αvβ3-integrin binding of ECs to the Gelfoam matrix had no significant effect on T-cell proliferation. While coating of tissue culture plates with collagen type IV and treatment of Gelfoam embedded with ECs counter-regulated some of the immune effects described collagen coating was without significant effect on endothelial bioscertainment and an effect of integrin blocking in the 3-D setting was only seen in high-glucose medium. These observations warrant further analysis.

As one underlying intracellular signaling mechanism for differences in MHC-II expression, we were able to detect elevated SOCS-1 and -3 expression levels in 3D-EC when compared with TC-EC. This elevated baseline expression might indicate a higher threshold at which IFN-γ induces MHC-II expression not only in naïve ECs but also in ECs in states of hyperglycemia. Interestingly, T-cell proliferation associated with SOCS-1 expression levels underlying the importance of intracellular signaling upon cytokine stimulation, surface expression and functional immune properties of ECs in vitro.

Modulation of SOCS expression by glucose has been previously shown with higher expression levels in a variety of human cells (38, 39, 41). To our knowledge, our data presented here within are the first to demonstrate increased SOCS-1 and -3 expression by glucose in TC-EC. This effect was consistently seen in 3D-EC but at a significant higher expression level.

Matrix-embedding presents ECs with the near-physiological state of substrate adherence and confluence. Our results indicate that intact cell-matrix contact may shield ECs from immune recognition and atherosclerotic risk factors like hyperglycemia.

We are aware that our study design bears some limitations: first of all, we only present in vitro data with short-term exposure of ECs to low- and high-glucose environment. The effect of long-term exposure needs to be further elucidated. Additionally, in vivo studies in diabetic animals are needed to study the effects of hyperglycemia on EC phenotype with respect to their underlying basement membrane in more detail. Furthermore, we restricted ourselves to Gelfoam matrices as a model for 3-D tissue engineering. Yet, this model yielded promising results in the past and has been successfully tested in clinical studies in humans.

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