Fetal Insulin and IGF-II Contribute to Gestational Diabetes Mellitus (GDM)-Associated Up-Regulation of Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) in the Human Feto-Placental Endothelium

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Context: Gestational diabetes mellitus (GDM)-associated hormonal and metabolic derangements in mother and fetus affect placental development and function. Indeed, in GDM, placentas are characterized by hypervascularization and vascular dysfunction. The membrane-type matrix metalloproteinase 1 (MT1-MMP) is a key player in angiogenesis and vascular expansion.

Objective: Here, we hypothesized elevated placental MT1-MMP levels in GDM induced by components of the diabetic environment. Therefore, we measured placental MT1-MMP in normal *vs.* GDM pregnancies, identified potential functional consequences, and investigated the contribution of hyperglycemia and the insulin/IGF axis.

Design: Immunohistochemistry identified placental cell types expressing MT1-MMP. MT1-MMP was compared between normal and GDM placentas by immunoblotting. Quantitative PCR of MT1-MMP in primary feto-placental endothelial cells (fpEC) and trophoblasts isolated from both normal and GDM placentas identified the cells contributing to the GDM-associated changes. A putative MT1-MMP role in angiogenesis was determined using blocking antibodies for *in vitro* angiogenesis assays. Potential GDM-associated factors and signaling pathways inducing MT1-MMP up-regulation in fpEC were identified using kinase inhibitors.

Results: Total and active MT1-MMP was increased in GDM placentas (+51 and 54%, respectively, $P < 0.05$) as a result of up-regulated expression in fpEC (2.1-fold, $P = 0.02$). MT1-MMP blocking antibodies reduced *in vitro* angiogenesis up to 25% ($P = 0.03$). Pathophysiological levels of insulin and IGF-II, but not IGF-I and glucose, stimulated MT1-MMP expression in fpEC by phosphatidylinositol 3-kinase signals relayed through the insulin, but not IGF-I, receptor.

Conclusions: GDM up-regulates MT1-MMP in the feto-placental endothelium, and insulin and IGF-II contribute. This may account for GDM-associated changes in the feto-placental vasculature. (*J Clin Endocrinol Metab* 97: 3613–3621, 2012)

Gestational diabetes mellitus (GDM) is an increasing problem worldwide and affects about 4–15% of pregnancies (1). GDM may compromise fetal development *in utero* and also prime the offspring for an increased risk of adult diseases (2). The placenta is a fetal organ located between mother and fetus, supplies gases and nutrients to the fetus, and is exposed to both bloodstreams. Thus, the placenta may be affected by GDM-associated metabolic derangements in both the maternal and fetal circulation, resulting in effects on placental morphology

Abbreviations: Ct, Cycle threshold; DMSO, dimethylsulfoxide; EBM, endothelial basal medium; FCS, fetal calf serum; fpEC, feto-placental endothelial cells; GDM, gestational diabetes mellitus; IGF-IR, IGF-I receptor; MMP, matrix metalloproteinases; MT1-MMP, membrane-type MMP1; PI3K, phosphatidylinositol 3-kinase; PkB, protein kinase B; PPP, picropodophyllin.

and function. Despite the profound increase of GDM and the long-term consequences on the fetus, only little is known about how GDM may affect placental structure.

Maternal hyperglycemia is the hallmark of GDM and results also in fetal hyperglycemia, fetal hyperinsulinemia, and other metabolic and hormonal changes in the fetal circulation, such as elevated levels of IGF (3, 4). The maternal and the fetal circulations are in contact with two different placental surfaces. The syncytiotrophoblast is formed by fusion of cytotrophoblast cells, covers the placental villous structure, and is in contact with the maternal blood. The feto-placental vasculature within the placental villi is continuous with the fetal vasculature, lined by endothelial cells, transports the fetal blood and hence is exposed to endocrine and metabolic influences similar to the vasculature of the fetus proper.

Therefore, diabetic derangements will primarily affect these placental surfaces. Indeed, both surfaces are enlarged. The syncytiotrophoblast surface is increased and the villi are hypervascularized resulting in a larger feto-placental endothelial surface. The feto-placental vasculature is characterized by an increased diameter and hypervascularization (5, 6), and signs of endothelial and vascular dysfunction have been described (7).

The morphological and functional changes of the placenta may contribute to the altered fetal development in GDM. Short-term consequences of GDM for the fetus may include excessive fat accretion (8). Recent data show that the diabetic intrauterine environment in GDM can program the fetus and determine the development of the offspring into adulthood. Long-term consequences of GDM include a higher risk for developing metabolic disorders and vascular dysfunction (2). Genetic inheritance of risk factors as well as fetal programming *in utero* contribute to the increased risk (9).

The developmental processes of the placenta, *i.e.* angiogenesis and growth of the feto-placental vasculature as well as invasion and fusion of the cytotrophoblast require proper remodeling and breakdown of extracellular matrix. Key molecules herein are matrix metalloproteinases (MMP) (10). Dysregulation of some members of this protein family has been implicated in various pathologies associated with diabetes (11, 12), and hyperglycemia as well as insulin and IGF have been shown to regulate various MMP (13, 14).

Feto-placental angiogenesis and vascular enlargement as well as trophoblast fusion occur throughout pregnancy until term (15). Therefore, these processes may be affected by the diabetic environment in GDM, which clinically manifests as glucose intolerance in the second half of gestation. However, studies demonstrate hormonal derangements both in mother and fetus already in wk 11–15.

These changes include altered levels of insulin, IGF-I, and leptin (16–18), indicating that placental development may already be affected by these hormonal changes before the onset of GDM.

Membrane-type MMP1 (MT1-MMP) is a membrane-anchored MMP and plays an outstanding role in structural remodeling. Established examples are angiogenesis (19), tube formation (20), and arterial enlargement (21), all of which are key features of endothelial cells. MT1-MMP is synthesized as an inactive, 63-kDa pro-form, transported to the cell membrane, and cleaved at a furin recognition motif into the active 57-kDa enzyme. Apart from the degradation of extracellular matrix components such as collagens, fibronectin, laminin, and vitronectin (22), MT1-MMP activates other MMP, *i.e.* MMP1, MMP2, and MMP13. Moreover, it activates or inactivates various cytokines and chemokines by cleaving their pro-forms, *e.g.* pro-TGF-β and pro-TNF-α, or active forms such as IL-8 and TNF-α (23).

In this study, we tested the hypothesis that GDM is associated with elevated placental MT1-MMP levels in the third trimester. We localized MT1-MMP-expressing cells in the third-trimester placenta, identified the cells accounting for MT1-MMP expression changes, and characterized consequences of the stable alterations identified in GDM. Moreover, we discovered components of the diabetic environment and signaling pathways by which they contribute to the MT1-MMP changes.

Subjects and Methods

Placenta samples

The study was approved by the institutional review board and ethical committee of the Medical University of Graz and of the Medical Faculty, University of Milan. Informed consent of the patients was obtained. After cesarean section, placentas were collected after normal and GDM pregnancies and immediately put on ice. A tissue sample of each placenta was taken within 3 cm of the cord insertion site of each placenta and snap frozen in liquid nitrogen.

Subject characteristics

Control pregnancies (n = 14) were nonsmoking women, with a negative 100-mg oral glucose tolerance test, free of medical or obstetrical disorders. All neonates were delivered by elective cesarean section and had a birth weight between the 10th and 90th percentile.

GDM was diagnosed at 28–32 wk of gestation with a 100-g oral glucose tolerance test with two or more values of the plasma glucose exceeding the fasting value of 5.2 mM, the 1-h value of 10 mM, the 2-h value of 8.6 mM, or the 3-h value 7.7 mM. After diagnosis, all patients were followed according to a clinical protocol (24). The GDM women (n = 9) monitored their capillary glucose levels (memory reflectance meters, Accu-Chek; Roche Diagnostics, Mannheim, Germany) at least four times a day.

TABLE 1. Clinical characteristics of the study subjects

	Control group (n = 14)	GDM group (n = 9)
Mother		
Age (yr)	34 ± 5	37 ± 3
Gestational age (wk)	38.7 ± 0.7	38.6 ± 0.4
Prepregnancy BMI	21.2 ± 3.0 (18.7–27.3)	25.2 ± 5.8 (17.1–32.5)
Postgravid BMI	26.1 ± 4.1 (20.0–35.4)	29.7 ± 7.3 (19.6–36.0)
Fasting blood glucose (mg/dl) (wk 36–40)	Not determined	81.0 ± 8.2
Postprandial blood glucose (mg/dl) (wk 36–40)	Not determined	102.5 ± 11.5
Treatment (n): diet/insulin		7/2
Fetus		
Fetal weight (g)	3357 ± 323	3320 ± 456
Fetal height (cm)	51.5 ± 1.4	50.2 ± 0.8
Fetal ponderal index	24.6 ± 2.4	26.2 ± 3.6
Placental weight (g)	508 ± 97	492 ± 110

Values are presented as mean ± SD. BMI, Body mass index.

They began appropriate diet providing 25–40 kcal/day·kg according to prepregnancy body mass index. Compliance was checked by measuring maternal glycemia and fetal growth every 2 wk. If maternal blood glucose values exceeded mean fasting and preprandial values of 5 mM and postprandial values of 6 mM, insulin therapy was instituted (n = 2). All GDM patients were in good metabolic control with glycosylated hemoglobin below 5% and achieved target preprandial below 5 mM and postprandial below 6.6 mM glucose values until the last glucose control at the day before delivery. None of the women showed signs of hypertension or any other disease. The subject characteristics are shown in Table 1.

Immunohistochemistry

Immunohistochemistry used the Ultra Vision LP detection system (Thermo Scientific, Fremont, CA) with the horseradish peroxidise polymer and the 3-amino-9-ethylcarbazole substrate following manufacturer's instructions. Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were cleared with xylene, and rehydration was followed by antigen retrieval with citrate buffer (pH 6.0). Sections were incubated with monoclonal mouse anti-MT1-MMP (LEM-2/15.8; Millipore, Temecula, CA) in a concentration of 1 μg/ml for 40 min at room temperature. Unspecific mouse IgG (Dako, Glostrup. Denmark) was used as negative control. Samples were counterstained with hematoxylin and mounted with Kaiser's gelatin (Merck, Darmstadt, Germany).

Isolation of trophoblasts and feto-placental endothelial cells (fpEC)

Primary trophoblasts and fpEC from third-trimester placentas were isolated after uncomplicated vaginal delivery from normal and GDM pregnancies as described previously (25, 26).

Trophoblasts were cultured in DMEM (5.5 mM D-glucose; Life Technologies, Inc., Paisley, UK) supplemented with 10% fetal calf serum (FCS) and tested for viability by measuring β-human chorionic gonadotropin levels secreted into the medium (Dade Behring, Deerfield, IL). Purity was determined by immunocytochemical staining for the trophoblast marker cytokeratin 7 (25). Only preparations with a purity of at least 99% and the characteristic kinetics of human chorionic gonadotropin secretion (27) were used. The fpEC were cultured on 1% (vol/vol) gelatin-coated plates using endothelial basal medium (EBM,

Cambrex; Clonetics, Walkersville, MD) supplemented with EGM-MV BulletKit (Clonetics). They were characterized by internalization of acetylated low-density lipoprotein and immunohistochemical staining for the endothelial cell marker von Willebrand factor and absent staining for fibroblast-specific antigen and smooth muscle actin (26). The fpEC were cultured and used for experiments up to passage 5.

Oxygen is a key regulator of placental development particularly in the first trimester of pregnancy. However, preliminary experiments revealed no difference of MT1-MMP expression in fpEC under different oxygen concentrations; thus, all experiments were performed at ambient oxygen tension.

In vitro angiogenesis

In vitro network formation was induced using growth factor-reduced Matrigel basement membrane matrix (BD Biosciences, Bedford, MA) as recommended by the manufacturer. Primary fpEC were preincubated (30 min at 37 C) in EBM (2% FCS) containing 15 μg/ml nonspecific mouse IgG1 (BD PharMingen, Bedford, MA) or 15 μg/ml MT1-MMP blocking antibodies (LEM-2/15.8 and LEM-2/63.1; Millipore). Then 8000 cells per well were added onto polymerized Matrigel in 96-well plates and incubated for 12 h at 37 C to allow for capillary-like network development. Quantitative analysis measured the total tube length and number of branching points of the network using AngioJ-Matrigel assay plug-in (28), which was developed and adapted for the ImageJ software (National Institutes of Health, Bethesda, MD) by Dr. Diego Guidolin (University of Padova, Padova, Italy). Each experiment was run in triplicate and independently repeated four times with different cell isolations.

Immunoblotting

Tissue was homogenized and cells were washed and lysed in buffer containing 10 mM Tris (pH 7.4), 1% sodium dodecyl sulfate, 1 mM Na-orthovanadate, and Complete protease inhibitor (Roche) mixed with an equal volume of Laemmli sample buffer (Sigma Chemical Co., St. Louis, MO). Before electrophoresis, samples were centrifuged and boiled for 5 min at 99 C. Equal amounts of protein were used for SDS-PAGE on a 10% gel (Pierce, Rockford, IL). After electroblotting according to the manufacturer's instructions, membranes were stained with PonceauS to check for blotting efficiency and densitometrically scanned. Then they were blocked for 1 h with 5% (wt/vol) nonfat
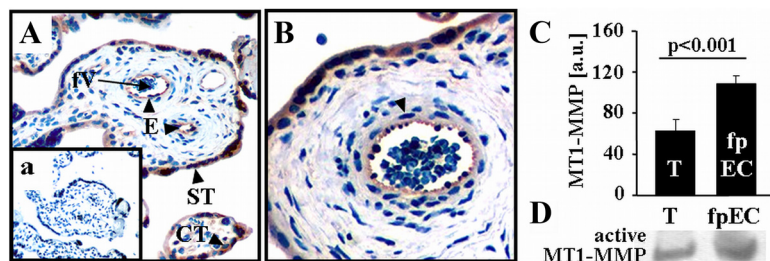
FIG. 1. Identification of placental cells expressing MT1-MMP in the normal third-trimester placenta. Immunohistochemical staining of active MT1-MMP demonstrated high expression in the syncytiotrophoblast and cytotrophoblasts and in the endothelium of the feto-placental vessels (fV). A, overview of MT1-MMP staining in a placental villous cross-section with staining in the syncytiotrophoblast (ST), the cytotrophoblast (CT), and the feto-placental endothelium (E). Magnification, ×200. The *inset* (a) shows the negative control using unspecific mouse IgG. B, Higher magnification more clearly shows the staining of endothelial cells (×400) marked with an *arrowhead*. C, Quantification (immunoblotting) of MT1-MMP protein in isolated trophoblasts (T) *vs.* isolated fpEC revealed expression in both cell types. D, One representative immunoblot of five. Data are presented as mean ± SEM.

dry milk (Bio-Rad, Hercules, CA) and 0.1% (vol/vol) Tween 20 (Sigma) in 0.14 mol/liter Tris-buffered saline (pH 7.3) at room temperature, which was also used for subsequent washings and as an antibody diluent. The membranes were incubated overnight at 4 C with polyclonal antibodies against MT1-MMP (hinge region; Chemicon, Bedford, MA; 0.5 μg/ml), β-actin (Amersham, Buckinghamshire, UK; 150 ng/ml), phospho-ERK1/2$^{Tyr202/204}$ (no. 9101; Cell Signaling, Danvers, MA; 1:1000), or phospho-protein kinase B (PkB)^{Ser473} (no. 4050; Cell Signaling; 1:1000). After washing, membranes were incubated with the secondary antibody (Bio-Rad; 1:1000) for 1 h at room temperature. Immunolabeling was visualized using the SuperSignal chemiluminescent substrate for horseradish peroxidise substrate system (Pierce). Membranes were exposed to Hyperfilm (Amersham) and densitometrically scanned using a digital camera and the AlphaDigiDoc 1000 software within the linear range of film and camera. Signals were normalized to β-actin or to the total protein amount in the respective molecular weight range of MT1-MMP.

Real-time PCR

One microgram of total RNA isolated with TriReagent (MRC, Cincinnati, OH) was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA (100 ng) was subjected to real-time PCR using FAM-labeled TaqMan gene expression assays (*MMP14*, Hs00237110_m1; *RPL30*, Hs00265497_m1) and TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ). Components were mixed according to the manufacturer's instructions and amplified in 20 μl total volume/well (96-well plates; Roche) using an ABI7900 (Applied Biosystems) real-time cycler. Cycle threshold (Ct) values were automatically generated by the software (SDS version 2.2), and relative gene expression was calculated by the standard $2^{-\Delta\Delta Ct}$ method (29) using gene expression of ribosomal protein L30 (*RPL30*) as reference. Statistical analyses used the ΔCt values.

Insulin/IGF and glucose treatment of fpEC

For the different treatments 40,000 cells per well were seeded in gelatin-coated 24-well plates and cultured in EBM supplemented only with 2% (vol/vol) FCS. After 24 h, medium was replaced by the same medium supplemented with insulin (0.1 and 1 nM), IGF-I (6.7 and 13 nM), IGF-II (22 and 44 nM), or D-glucose (17 and 25 mM). The basal glucose concentration of EBM was 5.5 mM, and the insulin concentration in EBM supplemented with 2% FCS was 0.002 nM. To identify receptors and pathways involved, inhibitors for the IGF-I receptor (IGF-IR) [picropodophyllin (PPP), 100 nM], phosphatidylinositol 3-kinase (PI3K) (wortmannin; 100 nM), or the ERK1/2 pathway (U0126; 10 μM) were added 1 h before treatment. IGF-I and IGF-II were purchased from R&D Systems (Minneapolis, MN) and insulin and the inhibitors from Calbiochem (Darmstadt, Germany).

To confirm that the concentrations of wortmannin and U0126 indeed blocked insulin and IGF-II-induced phosphorylation of PkB and ERK1/2, respectively, signaling experiments using antibodies against phospho-ERK1/2$^{Tyr202/204}$ and phospho-PkB^{Ser473} were performed. The fpEC were seeded as described above, serum starved overnight, pretreated with wortmannin (100 nM), U0126 (10 μM), or dimethylsulfoxide (DMSO) alone for 1 h, and stimulated with insulin or IGF-II (both 10 nM). After 15 min, cells were harvested and used for immunoblotting.

Data and statistical analysis

For data analysis, the immunoblots obtained from the insulin/IGF and glucose treatment of fpEC were band intensity harmonized for inter-blot variation. Each immunoblot replicate contained the same sequence of untreated (control) and treated (insulin, IGF-I, IGF-II, glucose, and inhibitor) samples, but from different cell isolations. The average signal of each blot was adjusted to 100. This allowed calculation of mean ± SD for controls and treatments as well as statistical analysis.

Statistical analysis used Sigma Stat version 3.1 (Jandel Scientific, San Rafael, CA) software. After testing for normal distribution (Kolmogorov-Smirnov), Student's *t* test was used. For testing the effect of different insulin, IGF-I, and IGF-II concentrations, one-way ANOVA and Dunn's method as *post hoc* test was performed. For testing the effect of the inhibitors, two-way ANOVA and Holm-Sidak *post hoc* test was used. Significances were accepted at a level of $P < 0.05$.

Results

Localization of MT1-MMP in the placenta in the third trimester of gestation

Immunohistochemistry was used to identify the cell types expressing MT1-MMP in the normal third-trimester placenta. The syncytiotrophoblast, subjacent cytotrophoblasts, and the feto-placental endothelium showed intensive and specific MT1-MMP staining (Fig. 1, A and B). Immunoblotting revealed that MT1-MMP protein was also expressed in primary trophoblasts and fpEC isolated from third trimester placentas with 90% higher levels ($P < 0.001$) in fpEC (110 ±

18 arbitrary units) compared with trophoblasts (58 ± 28 arbitrary units) (Fig. 1, C and D).

MT1-MMP expression in normal and GDM placentas

MT1-MMP protein is expressed in the third-trimester placenta. Comparison of MT1-MMP in placentas from GDM *vs.* normal pregnancies by immunoblotting revealed about 50% higher levels ($P < 0.05$) in total MT1-MMP in GDM. When pro- (63 kDa) and active (57 kDa) MT1-MMP were analyzed separately, only the approximately 55% increase in the active form was significant ($P = 0.03$) (Fig. 2, A and B).
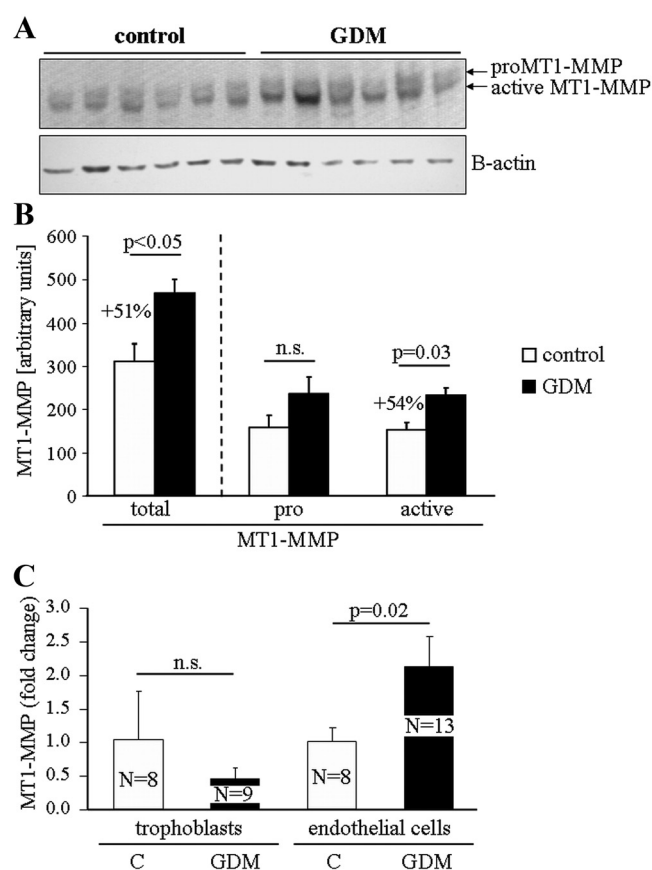
FIG. 2. MT1-MMP expression in placental tissue and cultured placental cells from normal and GDM pregnancies. MT1-MMP protein was measured in third-trimester placental tissue from normal (n = 14) and GDM (n = 9) pregnancies. Panel A, Representative immunoblot for MT1-MMP and β-actin. Panel B, Total (63 kDa) and active (57 kDa) MT1-MMP are lower in control than in GDM placentas. Data are presented as mean ± SEM. Panel C, MT1-MMP mRNA expression in fpEC and trophoblasts isolated from normal and GDM placentas as determined by quantitative RT-PCR. Cells were isolated after normal [control (C)] and GDM pregnancies. Trophoblasts were cultured for 48 h to allow them to regenerate after isolation. The fpEC were cultured and expanded to passages 4–5 to obtain sufficient cells for quality control and RNA isolation. Ribosomal protein L30 was used as an internal control. Data are presented as mean of the fold changes ± SEM, and ΔCt values were used for statistical analysis. Sample size for trophoblasts and fpEC is indicated in the *bars*.

MT1-MMP levels in fpEC and trophoblasts in normal *vs.* GDM pregnancies

Furthermore, we aimed to identify whether trophoblasts, fpEC, or both account for the increased MT1-MMP levels in GDM. Therefore, primary fpEC and trophoblasts were isolated from normal and GDM pregnancies, and their MT1-MMP expression was compared using quantitative RT-PCR (Fig. 2C). The only significant change was up-regulation of MT1-MMP in fpEC in association with GDM (fold change 2.1; $P = 0.02$).

MT1-MMP function in *in vitro* angiogenesis of fpEC

To identify potential consequences of the up-regulated MT1-MMP in the feto-placental endothelium, we determined the role of MT1-MMP in fpEC. Angiogenesis as a key process of endothelial cells was chosen as an endpoint, and *in vitro* angiogenesis assays were performed with and without inhibition of MT1-MMP action. Because chemical MMP inhibitors often interact also with other proteases and are thus not absolutely specific, we used blocking antibodies. To increase credibility of the results, two different clones of blocking antibodies were used. Unspecific mouse IgG of the same isotype was added to the controls. Addition of both MT1-MMP blocking antibodies significantly reduced the total tube length by 20.0 and 22.5% (Fig. 3A) as well as the number of branching points by 21.0 and 24.8%, respectively (Fig. 3B).

Regulation of MT1-MMP expression in fpEC by insulin, IGF-I, IGF-II, and glucose

We further aimed to identify potential factors of the diabetic environment to account for MT1-MMP up-regulation in the feto-placental endothelium in the third trimester. The concentrations of several metabolites are altered in the fetal compartment resulting from maternal GDM, *e.g.* glucose, amino acids, and nonesterified fatty acids (24, 30, 31). In GDM, the fetus may be exposed to hyperglycemia, which may result in a deregulation of the insulin/IGF axis. High glucose as well as insulin/IGF are known to alter, directly or indirectly, expression of some MMP in different cell types (13, 14). Therefore, fpEC were cultured in presence of insulin, IGF-I, IGF-II, or glucose. Only the 57-kDa band of the active MT1-MMP was detected when isolated cells were analyzed by immunoblotting. Insulin and IGF-II increased MT1-MMP expression in fpEC by 22 ± 18 and 157 ± 26%, whereas IGF-I and glucose had no effect (Fig. 4, A and B). This insulin and IGF-II effect was blocked by pretreatment with the PI3K inhibitor wortmannin but not by the ERK1/2 pathway inhibitor (U0126). Wortmannin indeed blocked insulin- and IGF-II-stimulated phosphorylation of PkB at
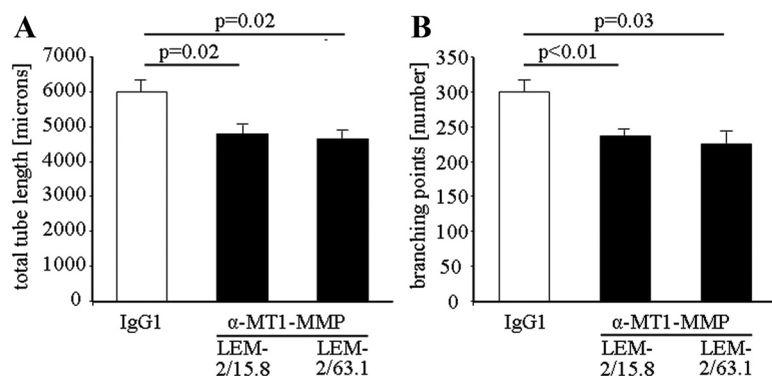
FIG. 3. *In vitro* angiogenesis of primary fpEC in presence of MT1-MMP blocking antibodies. *In vitro* angiogenesis was quantified by determining the total tube length (A) and the number of branching points (B). Nonspecific mouse IgG1 was added to the controls in the same concentration as the blocking antibodies LEM-2/15.8 and LEM-2/63.1 (15 μg/ml). Data are presented as mean ± sem of four independent experiments, each performed in triplicate.

Ser473, whereas U0126 fully abolished any ERK1/2 phosphorylation at Tyr202/204 (Fig. 4C). To identify which receptor signals the IGF-II effect, the IGF-IR inhibitor PPP was added. Currently, no inhibitor for the insulin receptor is available. The blocking effect of PPP on IGF-IR signaling was demonstrated by the inhibition of IGF-stimulated *in vitro* angiogenesis of fpEC in presence of PPP (data not

shown). PPP did not abolish the IGF-II-induced MT1-MMP up-regulation; thus, the insulin receptor, but not the IGF-IR, accounted for the IGF-II effect (Fig. 4, D and E), which, like the insulin effect itself, involves pathways downstream of PI3K.

Discussion

The present study reveals two major findings. 1) GDM affects the fetal environment in such a way that MT1-MMP, a key player in vascularization, angiogenesis, and arterial enlargement, is up-regulated. 2) MT1-MMP is elevated only in the feto-placental endothelium, suggesting the diabetic environment in the fetal rather than maternal circulation induces its up-regulation.

One of the potential functions of MT1-MMP in endothelial cells is its contribution to angiogenesis, which was shown by MT1-MMP overexpression in bovine aortic en-
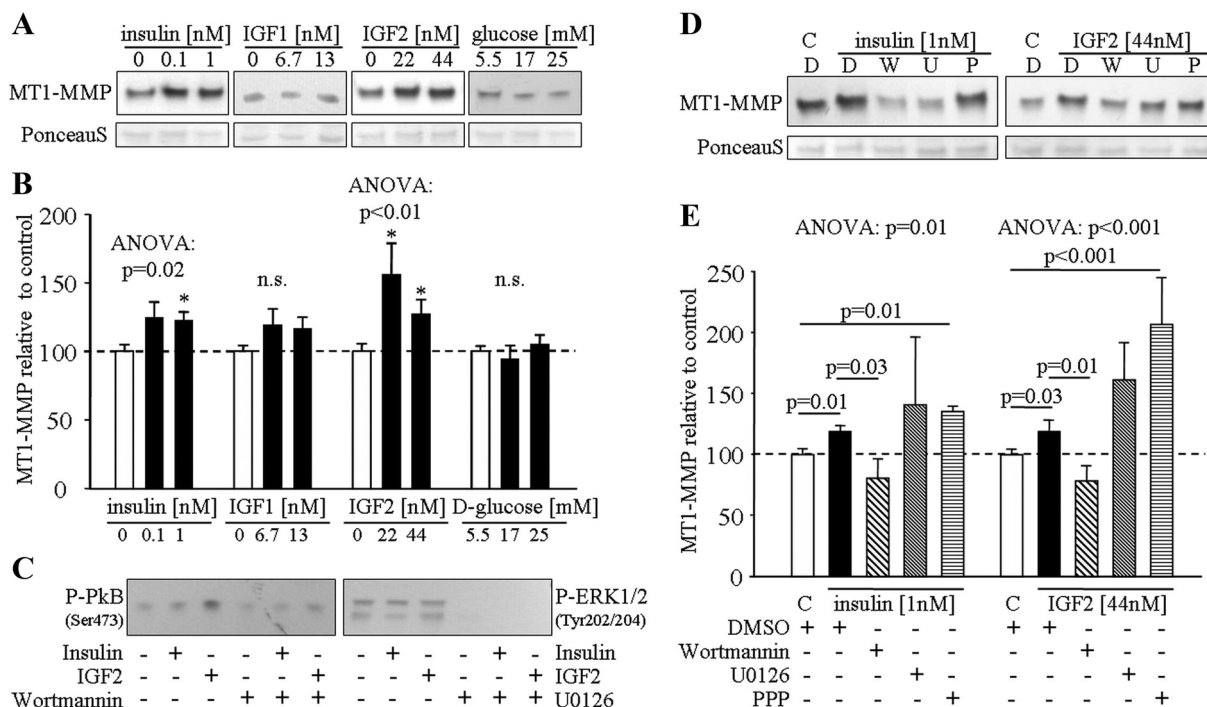

FIG. 4. Effect of insulin and IGF on MT1-MMP expression in fpEC. The fpEC were isolated from normal third-trimester placentas treated for 48 h with insulin, IGF-I, IGF-II, and D-glucose. Immunoblotting determined their effect on MT1-MMP protein levels. Because 2% FCS results in maximal insulin and IGF levels of 2% of the physiological concentration, only the supplemented concentrations are indicated. A, Representative immunoblot and PonceauS staining of the membrane. B, Data expressed relative to the controls. To identify signaling receptors and pathways involved, cells were pretreated for 1 h with the PI3K inhibitor wortmannin, the ERK1/2 pathway inhibitor U0126, and the IGF-IR inhibitor PPP; DMSO alone was added to the controls. Data are expressed relative to untreated controls (set to 100%). C, Representative immunoblots to test for inhibitory effect of wortmannin (100 nM) on phosphorylation of PkB and U0126 on phosphorylation of ERK1/2 in isolated fpEC after insulin and IGF-II treatment (both 10 nM) for 15 min. D, Representative immunoblot and PonceauS staining of the membrane. E, Data expressed relative to DMSO-treated controls (set to 100%). Data are presented as mean ± sem; n = 10. The statistical tests used the raw data. C, Control; D, DMSO; P, PPP; U, U0126; W, Wortmannin.

dothelial cells (32). Angiogenesis is a key function of fpEC in the placenta throughout pregnancy (15) and might involve MT1-MMP. This hypothesized role of MT1-MMP in fpEC was indeed verified by reduced two-dimensional network formation using MT1-MMP blocking antibodies. *In vitro* angiogenesis is a useful tool to determine and compare the effect of treatments on individual endothelial cell isolations, in which the untreated cells serve as internal control. However, basal *in vitro* angiogenesis varies profoundly between different cell isolations, thus precluding its comparison between fpEC from normal *vs.* GDM pregnancies.

The observed changes represent long-term effects of maternal GDM on the feto-placental endothelial cells because changes were still present after five passages in culture. It was already shown that endothelial cells isolated form diabetic donors remember their endothelial dysfunction in culture (33). GDM can induce epigenetic changes in various fetal organs (9), including the placenta (34). However, whether this is the underlying cause for the stable up-regulation of MT1-MMP in fpEC isolated from GDM pregnancies will be the focus of additional studies. Because hyperglycemia and insulin/IGF regulate some MMP (13, 14), the effect of hyperglycemia and the insulin/IGF axis on MT1-MMP expression of fpEC *in vitro* was determined. The insulin, IGF-I, and IGF-II concentrations were chosen to mimic the physiological and pathological concentrations of the growth factors in cord plasma of normal and GDM pregnancies (3, 4). The glucose levels chosen are commonly used for experiments mimicking hyperglycemia (35). Both insulin and IGF-II stimulated MT1-MMP production in endothelial cells. This effect was dependent on the PI3K/PkB pathway. The IGF-IR inhibitor PPP did not abolish the IGF-II effect. This strongly suggests that the IGF-II signal is predominantly relayed by the insulin receptor of which particularly the isoform A (IR-A) is a signaling receptor for IGF-II (36). The contribution of the IGF-IR, if any, will be rather minor, which is supported by an absent MT1-MMP increase by IGF-I treatment, a growth factor that exclusively binds to the IGF-IR (Fig. 5). The role of PI3K/PkB signaling in the regulation of MT1-MMP expression is in line with data from tumor cells, in which mammalian target of rapamycin was identified as a downstream link between PI3K and MT1-MMP regulation (37).

The study is limited by the fact that tissues and cells were obtained at the end of pregnancy, and any extrapolations to earlier stages have to be made with caution. It may well be that placental development is altered already before GDM clinically manifests in the second half of gestation because some metabolic alterations occur already in earlier phases of gestation as indicated by higher levels of
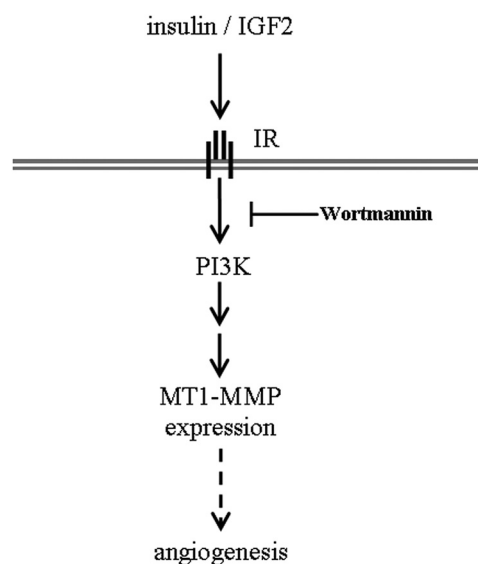
FIG. 5. Signaling pathway by which insulin and IGF-II up-regulate MT1-MMP expression in human placental endothelial cells. The IGF-IR and the insulin receptor isoform A (IR-A) are the predominant signaling receptors for IGF-II (36). Because the IGF-II effect was blocked by the IGF-IR inhibitor PPP, the observed IGF-II effects are most likely mediated by the insulin receptor isoform A. Up-regulation of MT1-MMP by both insulin and IGF-II requires P13K signaling.

maternal glucose, insulin, and growth factors already in the first trimester (17, 18). Furthermore, we previously demonstrated that placental MT1-MMP is sensitive toward the alterations of insulin and IGF-II levels in T1D already during the first trimester (13). Because placental vascularization is most pronounced in the third trimester (15), it is likely to be sensitive to changes in the *in utero* environment, which will manifest as hypervascularization found in all studies at the end of diabetic pregnancies. Placental vascularization may thus be affected by the diabetic environment in the first and third trimester.

The study clearly shows that not only the GDM-associated changes in the maternal environment but also the resulting derangements in the fetal circulation can induce modifications in the placenta. Besides insulin and IGF-II as identified here, other alterations including fetal hypoxia and hyperleptinemia as well as elevated fetal fibroblast growth factor 2 levels may contribute (38). On the basis of the present data, it is tempting to speculate that the MT1-MMP elevation in placental endothelial cells of GDM pregnancies may lead to morphological and functional changes also in the fetus vasculature. Hence, this study fuels into the growing body of evidence that maternal diabetes contributes to fetal endothelial and vascular dysfunction. This notion is supported by studies using animal models as well as human feto-placental and umbilical endothelial cells and vessels (39, 40). Investigating these endothelial and vascular changes may help in understanding the impact of diabetes on endothelial cell function.

Acknowledgments

References

1. **American Diabetes Association** 2010 Standards of medical care in diabetes–2010. Diabetes Care [Erratum (2010) 33:692] 33(Suppl 1):S11–S61
2. **Burguet A** 2010 Long-term outcome in children of mothers with gestational diabetes. Diabetes Metab 36:682–694
3. **Roth S, Abernathy MP, Lee WH, Pratt L, Denne S, Golichowski A, Pescovitz OH** 1996 Insulin-like growth factors I and II peptide and messenger RNA levels in macrosomic infants of diabetic pregnancies. J Soc Gynecol Investig 3:78–84
4. **Simmons D, Breier BH** 2002 Fetal overnutrition in polynesian pregnancies and in gestational diabetes may lead to dysregulation of the adipoinsular axis in offspring. Diabetes Care 25:1539–1544
5. **Mayhew TM, Sørensen FB, Klebe JG, Jackson MR** 1994 Growth and maturation of villi in placentae from well-controlled diabetic women. Placenta 15:57–65
6. **Jirkovská M, Kubínová L, Janácek J, Moravcová M, Krejcí V, Karen P** 2002 Topological properties and spatial organization of villous capillaries in normal and diabetic placentas. J Vasc Res 39:268–278
7. **Sobrevia L, Abarzua F, Nien JK, Salomon C, Westermeier F, Puebla C, Cifuentes F, Guzman-Gutierrez E, Leiva A, Casanello P** 2011 Review: differential placental macrovascular and microvascular endothelial dysfunction in gestational diabetes. Placenta 32(Suppl 2):S159–S164
8. **Verhaeghe J, Van Bree R, Van Herck E, Laureys J, Bouillon R, Van Assche FA** 1993 C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: correlations with birth weight. Am J Obstet Gynecol 169:89–97
9. **Seki Y, Williams L, Vuguin PM, Charron MJ** 2012 Minireview: epigenetic programming of diabetes and obesity: animal models. Endocrinology 153:1031–1038
10. **Kessenbrock K, Plaks V, Werb Z** 2010 Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67
11. **Chung AW, Yang HH, Sigrist MK, Brin G, Chum E, Gourlay WA, Levin A** 2009 Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc Res 84:494–504
12. **Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H** 2002 Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia 45:1011–1016
13. **Hiden U, Glitzner E, Ivanisevic M, Djelmis J, Wadsack C, Lang U, Desoye G** 2008 MT1-MMP expression in first-trimester placental tissue is upregulated in type 1 diabetes as a result of elevated insulin and tumor necrosis factor-α levels. Diabetes 57:150–157
14. **Death AK, Fisher EJ, McGrath KC, Yue DK** 2003 High glucose alters matrix metalloproteinase expression in two key vascular cells: potential impact on atherosclerosis in diabetes. Atherosclerosis 168:263–269
15. **Mayhew TM** 2002 Fetoplacental angiogenesis during gestation is biphasic, longitudinal and occurs by proliferation and remodelling of vascular endothelial cells. Placenta 23:742–750
16. **D'Anna R, Baviera G, Cannata ML, De Vivo A, Di Benedetto A, Corrado F** 2007 Midtrimester amniotic fluid leptin and insulin levels and subsequent gestational diabetes. Gynecol Obstet Invest 64:65–68
17. **Qiu C, Vadachkoria S, Meryman L, Frederick IO, Williams MA** 2005 Maternal plasma concentrations of IGF-1, IGFBP-1, and C-peptide in early pregnancy and subsequent risk of gestational diabetes mellitus. Am J Obstet Gynecol 193:1691–1697
18. **Riskin-Mashiah S, Damti A, Younes G, Auslender R** 2010 First trimester fasting hyperglycemia as a predictor for the development of gestational diabetes mellitus. Eur J Obstet Gynecol Reprod Biol 152:163–167
19. **Genís L, Gálvez BG, Gonzalo P, Arroyo AG** 2006 MT1-MMP: universal or particular player in angiogenesis? Cancer Metastasis Rev 25:77–86
20. **Stratman AN, Saunders WB, Sacharidou A, Koh W, Fisher KE, Zawieja DC, Davis MJ, Davis GE** 2009 Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices. Blood 114:237–247
21. **Sho E, Sho M, Singh TM, Nanjo H, Komatsu M, Xu C, Masuda H, Zarins CK** 2002 Arterial enlargement in response to high flow requires early expression of matrix metalloproteinases to degrade extracellular matrix. Exp Mol Pathol 73:142–153
22. **Barbolina MV, Stack MS** 2008 Membrane type 1-matrix metalloproteinase: substrate diversity in pericellular proteolysis. Semin Cell Dev Biol 19:24–33
23. **Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM** 2004 Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. Proc Natl Acad Sci USA 101:6917–6922
24. **Cetin I, de Santis MS, Taricco E, Radaelli T, Teng C, Ronzoni S, Spada E, Milani S, Pardi G** 2005 Maternal and fetal amino acid concentrations in normal pregnancies and in pregnancies with gestational diabetes mellitus. Am J Obstet Gynecol 192:610–617
25. **Cervar M, Blaschitz A, Dohr G, Desoye G** 1999 Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages. Cell Tissue Res 295:297–305
26. **Lang I, Pabst MA, Hiden U, Blaschitz A, Dohr G, Hahn T, Desoye G** 2003 Heterogeneity of microvascular endothelial cells isolated from human term placenta and macrovascular umbilical vein endothelial cells. Eur J Cell Biol 82:163–173
27. **Polliotti BM, Abramowsky C, Schwartz DA, Keesling SS, Lee GR, Caba J, Zhang W, Panigel M, Nahmias AJ** 1995 Culture of first-trimester and full-term human chorionic villus explants: role of human chorionic gonadotropin and human placental lactogen as a viability index. Early Pregnancy 1:270–280
28. **Guidolin D, Vacca A, Nussdorfer GG, Ribatti D** 2004 A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. Microvasc Res 67:117–124
29. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402–408
30. **Schaefer-Graf UM, Meitzner K, Ortega-Senovilla H, Graf K, Vetter K, Abou-Dakn M, Herrera E** 2011 Differences in the implications of maternal lipids on fetal metabolism and growth between gestational diabetes mellitus and control pregnancies. Diabet Med 28:1053–1059
31. **Ortega-Senovilla H, Schaefer-Graf U, Meitzner K, Abou-Dakn M, Graf K, Kintscher U, Herrera E** 2011 Gestational diabetes mellitus causes changes in the concentrations of adipocyte fatty acid-binding protein and other adipocytokines in cord blood. Diabetes Care 34:2061–2066

32. **Jeong JW, Cha HJ, Yu DY, Seiki M, Kim KW** 1999 Induction of membrane-type matrix metalloproteinase-1 stimulates angiogenic activities of bovine aortic endothelial cells. Angiogenesis 3:167–174

33. **Lehle K, Haubner F, Münzel D, Birnbaum DE, Preuner JG** 2007 Development of a disease-specific model to evaluate endothelial dysfunction in patients with diabetes mellitus. Biochem Biophys Res Commun 357:308–313

34. **Bouchard L, Hivert MF, Guay SP, St-Pierre J, Perron P, Brisson D** 2012 Placental adiponectin gene DNA methylation levels are associated with mothers' blood glucose concentration. Diabetes 61:1272–1280

35. **Pandey V, Chaube B, Bhat MK** 2011 Hyperglycemia regulates MDR-1, drug accumulation and ROS levels causing increased toxicity of carboplatin and 5-fluorouracil in MCF-7 cells. J Cell Biochem 112:2942–2952

36. **Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R** 2009 Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev 30:586–623

37. **Zhang D, Brodt P** 2003 Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion via PI 3-kinase/Akt signaling. Oncogene 22:974–982

38. **Hiden U, Desoye G** 2010 The placenta in diabetes in pregnancy. In: McCance DR, Maresh M, Sacks DA, eds. Diabetes in pregnancy. 1st ed. Chichester, UK: Wiley-Blackwell; 26–33

39. **Duong Van Huyen JP, Vessières E, Perret C, Troise A, Prince S, Guihot AL, Barbry P, Henrion D, Bruneval P, Laurent S, Lelièvre-Pégorier M, Fassot C** 2010 In utero exposure to maternal diabetes impairs vascular expression of prostacyclin receptor in rat offspring. Diabetes 59:2597–2602

40. **Leiva A, Pardo F, Ramírez MA, Farías M, Casanello P, Sobrevia L** 2011 Fetoplacental vascular endothelial dysfunction as an early phenomenon in the programming of human adult diseases in subjects born from gestational diabetes mellitus or obesity in pregnancy. Exp Diabetes Res 2011:349286