MT1-MMP expression in first trimester placental tissue is up-regulated in type 1 diabetes as a result of elevated insulin and TNF-α levels

Ursula Hiden, Ph.D.¹, Elisabeth Glitzner B. Sc.¹, Marina Ivanisevic, M.D.², Josip Djelmis, M.D.², Christian Wadsack, Ph.D.¹, Uwe Lang, M.D.¹, Gernot Desoye, Ph.D.¹

¹Department of Obstetrics and Gynecology, Medical University of Graz, Austria
²Department of Obstetrics and Gynecology, University Hospital, Petrova, Zagreb, Croatia

Running Title: MT1-MMP is elevated in the 1st trimester placenta in T1D

Address for correspondence:
Ursula Hiden, M.Sc., Ph.D.
Department of Obstetrics and Gynecology, Medical University of Graz
Auenbruggerplatz 14, 8036 Graz, Austria
E-mail: ursula.hiden@klinikum-graz.at

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Abstract

Objective. In pre-gestational diabetes the placenta at term of gestation is characterized by various structural and functional changes. If similar alterations occur in the first trimester has remained elusive. Placental development requires proper trophoblast invasion and tissue remodeling, processes involving matrix-metalloproteinases (MMPs) of which the membrane-anchored members (MT-MMPs) such as MT1-MMPs are key players. Here we hypothesize a dysregulation of placental MT1-MMP in the first trimester of type 1 diabetic pregnancies induced by the diabetic environment.

Research Design and Methods. MT1-MMP protein was measured in first trimester placentas of healthy (n=13) and type 1 diabetic (n=13) women. To identify potential regulators first trimester trophoblasts were cultured under hyperglycaemia and various insulin, IGF1, IGF2 and TNF-α concentrations in presence or absence of signaling pathway inhibitors.

Results. MT1-MMP was strongly expressed in first trimester trophoblasts. In type 1 diabetes placental pro-MT1-MMP was up-regulated, whereas active MT1-MMP expression was only increased in late first trimester. In isolated primary trophoblasts insulin, IGF1, IGF2 and TNF-α up-regulated MT1-MMP expression, whereas glucose had no effect. The insulin effect was dependent on PI3-kinase, the IGF1 effect on MAP-kinase and the IGF2 effect on both.

Conclusions. This is the first study to reporting alterations in the first trimester placenta in type 1 diabetes. The up-regulated MT1-MMP expression in type 1 diabetes may be the result of higher maternal insulin and TNF-α levels. We speculate that the elevated MT1-MMP will affect placental development and may thus contribute to long term structural alterations in the placenta in pre-gestational diabetes.
Despite improvement in the quality of metabolic control over the past decades maternal pre-gestational diabetes mellitus, in particular type 1 diabetes mellitus (T1D), is still often associated with a range of maternal and fetal complications (1). In addition to the effects on fetal growth the structural and functional development of the placenta is affected. In contrast to the well-known placental changes at term of diabetic pregnancies (2-4), the effect of pre-gestational diabetes on the placenta in the first trimester has remained elusive. We have recently proposed that a diabetic insult early in pregnancy will alter long term placental development and thus result in the observed changes at term (5).

Key processes early in placental development involve implantation and placentation. These require proliferation, migration and invasion of trophoblast cells as well as extensive uterine tissue remodeling (6; 7). Trophoblast invasion is a process that is tightly controlled in time and space in a paracrine and autocrine manner. A multitude of factors has been implicated in its control including the non-classical MHC class I molecule HLA-G (8), which is expressed on a specialized trophoblast subpopulation, the extravillous trophoblast.

Matrix metalloproteinases (MMPs) have been implicated in tissue remodeling. They form a large family of proteolytic enzymes capable of degrading extracellular matrix (ECM). MMPs are members of the metzincin group of proteases, which have a conserved Met residue and a zinc ion at their active site (9). Mammalian MMPs share a conserved domain structure that consists of a catalytic domain and an auto-inhibitory pro-domain. When the pro-domain is destabilized or removed by proteolytic cleavage the active site becomes available for substrates (10). Various MMP family members are highly expressed in the first trimester placenta (11; 12).

New emerging data show that the membrane-anchored subfamily of MMPs, i.e. MT-MMPs, are major modifiers of the pericellular environment and key regulators of tumor cell behavior (13). To date, the MT-MMP family includes six members i.e., MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP (13). MT1-MMP plays an outstanding role in tissue remodeling, migration and invasion (7; 14; 15). Apart from its role in extracellular matrix breakdown and activation of other metalloproteinases i.e., MMP2 and MMP13, MT1-MMP is also able to activate or inactivate several cytokines and chemokines by cleaving their pro-forms e.g., tumor necrosis factor-alpha (TNF-α) or active forms such as of interleukin-8 (IL-8), growth-regulated protein alpha (GRO-α) and gamma (GRO-γ) (16; 17). The resulting active cytokines may affect further placental development.

MT1-MMP levels can be regulated at various stages including transcription, translation, activity and degradation (13; 18). MT1-MMP is synthesized as an inactive, 63 kDa zymogen (pro-MT1-MMP) and, after transport to the cell membrane, is cleaved into the 57 kDa active enzyme at a furin recognition motif. Shedding of the membrane-anchored MT1-MMP results in degradation products among which the soluble 50 kDa fragment remains active whereas both 44 and 32 kDa products are proteolytically inactive (18). In the human placenta in early gestation, MT1-MMP is highly expressed by various trophoblast subpopulations such as extravillous invading and non-invading cytotrophoblasts as well as the proliferating cytotrophoblasts of cell islands (11).

MMPs in general are dysregulated in various diabetes-associated complications such as
MT1-MMP is elevated in the 1st trimester placenta in T1D nephropathy, retinopathy and vascular complications (19-22). Notably, MMP2 is implicated in diabetes-induced changes (23-25) and is also dysregulated in the rat placenta in diabetes (26). MT1-MMP is the only MT-MMP member that has been reported to be dysregulated in diabetes (19; 21; 22).

The overall hypothesis tested in this study predicted changes in placental MT-MMP expression associated with pre-gestational diabetes. After identification of MT1- and MT2-MMP as the only membrane-type MMPs present in the placenta we focused on MT1-MMP because of its pleiotropic effects. It is predominantly found in the extravillous trophoblast (11). Therefore, we determined differential expression of MT-MMPs in the HLA-G expressing invasive versus the HLA-G negative, non-invasive trophoblast subpopulations. We further hypothesized that potential changes in MT1-MMP synthesis are accounted for by the diabetic environment. In order to identify the diabetes-associated factors causing MT1-MMP dysregulation, in vitro experiments were performed using isolated primary trophoblasts from the first trimester of pregnancy. They were treated with insulin, TNF-α and glucose, factors with elevated concentrations in the maternal circulation in diabetes and their effect on MT1-MMP expression was determined. Since IGF1 and IGF2 share some signaling pathways with insulin they were included in the in vitro experiments.

Research Design and Methods

First trimester placental samples
After pregnancy termination for psycho-social reasons (IR) or missed abortions (MA), tissue samples (Table 1) were collected in Medium 199 supplemented with penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, USA), washed immediately in PBS and snap-frozen. The gestational age was calculated from the last menstrual period and corrected after endovaginal ultrasound examination using published charts (27). The study was approved by the institutional review board and ethical committee of the Medical University of Graz and informed consent of the patients.

Isolation of first trimester trophoblasts
Primary trophoblasts were isolated from first trimester placetas after pregnancy terminations for psycho-social reasons as described previously (28). The cells thus obtained constitute a mixture of both trophoblast subpopulations i.e., villous and extravillous trophoblasts. All cell preparations were subjected to rigorous immuno-cytochemical characterization (28). Trophoblasts were tested for viability by measuring human chorionic gonadotropin (hCG) levels secreted into the culture medium (Dade Behring, Deerfield, IL). Only preparations with a purity ≥ 99% and the characteristic kinetics of hCG secretion (29) were used.

Cell Culture
Primary trophoblasts were cultured in gelatine-coated plates with DMEM (Gibco) supplemented with 2% (v/v) FCS in a humidified atmosphere of 5% CO2 at 37°C. For growth factor and glucose treatment, isolated trophoblasts were seeded in gelatine-coated 24 well plates (50,000 cells per well) and cultured in DMEM with 2% (v/v) FCS. After 24 h medium was replaced by fresh medium supplemented with insulin (0.1, 1 nM; Calbiochem, Merck, Darmstadt, Germany), IGF1 (50, 100 ng/ml; R&D Systems, Minneapolis, MN), IGF2 (165, 300 ng/ml; R&D Systems), TNF-α (10, 25 ng/ml; Sigma) and glucose (25 mM; Sigma) and the cells were cultured for further 48 h. The experimental levels of insulin as well as IGF1 and IGF2 were chosen as to lie within the (patho)-physiological range to
avoid low-affinity binding to others but their specific receptor (30). The concentrations of insulin (1 nM) and glucose (25 mM) were chosen as to lie about two to three-fold higher than the maternal in vivo concentrations attained either post-prandially (glucose) or after pharmacological administration (insulin) (31). The IGF1 level (85 ng/ml) parallels the maternal IGF1 levels in the first trimester of gestation (32). IGFBP2 was used in the concentration range that showed the highest effect on trophoblast invasion in vitro (between 125 and 625 ng/ml) (33). The concentration of TNF-α (25 ng/ml) is a standard concentration used to study the effect of TNF-α (34).

In experiments with inhibition of PI3-kinase or MEK1, cells were pre-treated for 5 h with either Wortmannin (100 nM; Calbiochem) or U0126 (10 µM; Calbiochem) dissolved in DMSO before being stimulated with 1 nM insulin for 48 h. In un-stimulated control cells and insulin-stimulated cells, the same volume of DMSO without inhibitors was added as vehicle control.

**Separation of HLA-G positive and HLA-G negative first trimester trophoblasts**

Immediately after isolation first trimester trophoblasts were separated into subpopulations expressing or lacking surface HLA-G. Trophoblasts were incubated with immuno-magnetic beads (Dynabeads M-450) conjugated with anti-HLA-G antibody (MEM-G/9; Abcam, Cambridge, UK). The unbound, HLA-G devoid, cells and the cells bound to the beads were separated by applying a magnet above the tube. The two cell populations thus obtained were subsequently cultured in DMEM supplemented with 10% (v/v) FCS.

**Microarray analysis of placental primary cell RNA**

Total RNA from ten first trimester trophoblast preparations isolated from different placenta was pooled and 5 µg RNA prepared for hybridization as described previously (35). For expression analysis cRNA was hybridized against Affymetrix (Santa Clara, CA, USA) HU133A-chips according to the manufacturer’s instructions. Raw data were normalized globally and processed with Microarray Suite, version 5.0 and Data Mining Tool (Affymetrix) software. Annotations were obtained from NetAffx (Affymetrix) and the data screened for membrane anchored matrix metalloproteinases (MT-MMPs).

**Isolation of RNA and RT-PCR for various MT-MMPs in first trimester trophoblasts**

Total RNA was isolated from first trimester trophoblasts with Trizole (MRC, Ohio). Primers (Table 2) for RPL30 and the MT-MMPs (MT1-MMP, MT2-MMP, MMP16, MMP17, MMP24, MMP25) were designed using the public web-page Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and purchased from Ingenetix (Vienna, Austria). The primer-pairs included splicing sites within the amplicon. Because of its stable expression within the different placental cell types the mRNA-amount of the ribosomal protein L30 (RPL30) was used as an internal control (35). Two hundred ng of total RNA was used for the one step RT-PCR kit from Quiagen (Hilden, Germany) according to the manufacturer’s instructions. For RPL30 24 cycles and for all MT-MMPs 27 cycles were used. For all primer pairs the annealing temperature was 60°C. PCR-products were electrophoresed on 3% agarose gels, documented with the Eagle-Eye™ system (Stratagene, CA) and quantified with the AlphaDigiDoc 1000 (Alpha Innotech, CA) software. In order to validate primer pairs, RT-PCRs were carried out using human RNA from brain, lung and colon as positive controls. These gave distinct bands with
sizes corresponding to the calculated size of the amplicon in at least one organ. In preliminary experiments using first trimester trophoblast RNA the optimal RT-PCR cycle number for MT1-MMP, MT2-MMP and L30 was determined to lie within the linear range of the amplification.

Preparation of proteins and western blot analysis
Tissue was homogenized and cells were lysed in buffer containing 0.01 mol/l tris pH 7.4, 1% SDS, 1 mmol/l Na-orthovanadate and Complete protease inhibitor (Roche) mixed with an equal volume of Laemmli sample buffer (Sigma). Prior to electrophoresis, samples were centrifuged and boiled for 5 min at 99°C. Equal amounts of protein, determined according to Lowry were used for SDS-PAGE on a 10 % gel (Pierce, Rockford, IL). After electroblotting membranes were blocked for 1 h with 5 % (w/v) non-fat dry milk (BioRad, Hercules, CA) and 0.1 % (v/v) Tween-20 (Sigma) in 0.14 mol/l tris-buffered saline, pH 7.3, at room temperature. This solution was used for subsequent washings and as a diluent for the antibodies. The membranes were incubated overnight at 4°C with antibodies against MT1-MMP (Chemicon, Millipore, Bedford, MA; 1 : 2000) or β-Actin (Amersham, Little Chalfont, UK, 1:10,000). After washing, membranes were incubated with the adequate secondary antibody (BioRad; 1:1000) for 1 h at room temperature. Immuno-labeling was visualized using the SuperSignal CL-HRP Substrate System (Pierce). To allow comparison between gels an internal standard sample was prepared as lysate from one first trimester placental tissue to which all optical densities within each blot were normalized. 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.

Statistical analysis
Statistical analysis used Sigma Stat 3.1 (Jandel Scientific, San Rafael, CA) software. After testing for normal distribution (Kolmogorov-Smirnov), the Mann-Whitney U-test for non-parametric data or a Students t-test was used to test for differences in the amounts of pro-, active and total MT1-MMP protein and mRNA of various MT-MMPs. To test for treatment effects Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn’s Method as post-hoc test was used. Correlations were analyzed by Person Product Moment Correlation. Significances were accepted at a level of $P < 0.05$.

Results
Expression of various MT-MMPs in first trimester trophoblasts
Among all six MT-MMPs surveyed by microarray analysis only MT1-MMP and MT2-MMP were expressed in isolated primary trophoblasts from the first trimester of gestation (Table 3). This was further confirmed by RT-PCR, which demonstrated a similar amount of MT1-MMP and MT2-MMP expression (Figure 1A, B). Thus, MT3-MMP - MT6-MMP were either absent or present at levels below the sensitivity threshold of both methods i.e., microarray and RT-PCR. After separation of the trophoblasts in subpopulations according to their surface HLA-G expression, the HLA-G positive trophoblasts representing the more invasive phenotype of trophoblasts had a 33 % ($p=0.0004$) higher expression level of MT1-MMP (Figure 1C, D).

MT1-MMP protein expression in first trimester placental tissue of healthy and T1D women
In first trimester placenta complicated by T1D, MT1-MMP expression was
MT1-MMP is elevated in the 1st trimester placenta in T1D

increased (p<0.0001) by 100% (Figure 2). When the two bands of active (57 kDa) and pro-MT1-MMP (63 kDa) were analyzed separately, an up-regulation of both, zymogen (96 %; p=0.0001) and active proteinase (111 %; p=0.04) was observed. The ratio between active and pro-MT1-MMP did not differ between both groups. No other bands at lower molecular weight i.e., 50, 44 and 32 kDa were detected, which would represent processed MT1-MMP species.

When the data were stratified according to the gestational age i.e., early (≤ week 8) and late (> week 8) first trimester, active MT1-MMP levels were lower by 60% (p=0.026) in the control samples from late as compared to early first trimester (Figure 3). Because the T1D samples did not show this change their MT1-MMP levels were higher by 72% (p=0.0018) after week eight in the first trimester. No change between early and late first trimester could be found in total and pro-MT1-MMP expression (not shown). No difference in MT1-MMP was found between the miscarriages (MA) and the interruptions (IR) within and between both study groups (not shown).

Neither total nor active or pro-MT1-MMP levels correlated with maternal HbA1c values. However, the daily insulin dose of the T1D subjects correlated (r=0.626, p=0.04) with the total MT1-MMP protein levels (not shown).

Regulation of MT1-MMP expression in first trimester trophoblasts by insulin, IGF1, IGF2, glucose and TNF-α

When primary first trimester trophoblasts were analyzed by western blotting only the 57 kDa band of active MT1-MMP could be detected. Insulin, IGF2 and TNF-α increased MT1-MMP expression in a dose-dependent manner (ANOVA) (Figure 4). IGF1 up-regulation was only significant with Student’s t-test. Glucose had no effect. In the culture supernatant none of the shedded MT1-MMP species (50 and 44 kDa) could be detected by immunoblotting (not shown). The 113% induction (p=0.05) caused by insulin was attenuated by inhibition of the PI3-kinase (PI3K) pathway, but not affected by inhibition with U0126 of MEK1, a central kinase of the ERK1/2 MAP-kinase (MAPK) pathway (Figure 5). The IGF1-induced stimulation was only inhibited by U0126, whereas the IGF2-effect was abolished by inhibition of both the PI3K and the ERK1/2 pathway. The effects of insulin, IGF1, IGF2 and TNF-α did not differ between isolated primary trophoblasts from early vs. late first trimester (not shown).

Discussion

This study identified MT1-MMP and MT2-MMP as the only members of the membrane-anchored family of MMPs expressed at high mRNA levels in the first trimester trophoblast of the human placenta.

The prominent expression of MT1-MMP early in gestation suggests a major role in processes involved in early placental development. This notion is further supported by its predominant presence in the HLA-G expressing trophoblast subpopulation, which represents the invasive extravillous trophoblast. The high MT1-MMP expression in the HLA-G positive trophoblasts is in accordance with results of in-situ hybridization in first trimester placental tissue (11).

During the first trimester of pregnancy the amount of active MT1-MMP notably decreased in the non-diabetic control group. The underlying mechanism is unclear. The decrease of active, but not of total or pro-MT1-MMP in the late first trimester suggests a change in pro-MT1-MMP activation. This may result from a reduction of expression or activity of furin or the pro-protein convertase PCSK6 that both can activate pro-MT1-MMP (36). The
absence of different MT1-MMP levels or effects in the isolated cells from different gestational weeks indicates stability of the intrinsic responsiveness of the cells during the first trimester and suggests changes in placental environment to contribute to the decrease in tissue MT1-MMP expression.

The period in gestation from week 6 to week 12 is characterized by an increase in oxygen tension in the intervillous space (37). Analysis of the MT1-MMP promoter sequence found no potential binding site of hypoxia inducible factor 1 (HIF-1) that could directly up-regulate MT1-MMP expression. However, furin expression is up-regulated under hypoxic conditions by HIF-1 (38). Hence, as a hypothesis, lower furin expression under normoxic conditions in the late first trimester may result in lower levels of cleaved, active MT1-MMP. This decrease of MT1-MMP synthesis was absent in the T1D group.

To the best of our knowledge this is the first study carried out on placental tissue from first trimester diabetic pregnancies. Therefore, it is unknown if the oxygen tension in the intervillous space is lower in T1D than in non-diabetic pregnancies. Moreover, it is also unknown if utero-placental function associated with MMP activity such as trophoblast migration, invasion or uterine tissue remodeling is altered.

In other tissues MT1-MMP expression is decreased (19; 21) or increased (22; 39) in diabetes. Hence, the ultimate effect of the diabetic environment on MT1-MMP expression strongly depends on the specific tissue as well as on the proportion of factors dysregulated in this pathology such as glucose, insulin and TNF-α.

Miscarriage is a pregnancy problem that may have several underlying causes including inadequate trophoblast invasion (40). Pregestational diabetes is associated with an increased risk for spontaneous abortions (41). Given the prominent role that is attributed to MT1-MMP it was of special interest that placental MT1-MMP expression did not differ between both groups regardless of presence or absence of maternal diabetes. This strongly suggests that MT1-MMP dysfunction is not involved in the pathogenesis of miscarriages.

Even in the first trimester the placenta is a complex tissue comprising several cell types. Here only total tissue samples were analyzed and hence the differences between T1D samples and controls cannot be directly attributed to a specific cell type. However, as in-situ hybridization did not detect MT1-MMP mRNA in the villous stroma in the first trimester placenta (11) we conclude trophoblast cells to account for the observed changes of MT1-MMP in placental tissue. Therefore, primary trophoblasts were used for the further in vitro experiments.

In first trimester trophoblasts in vitro only one MT1-MMP species was detected which corresponded to active MT1-MMP. This may be the result of high trophoblast expression of furin and PCSK6 found by microarray analysis (Hiden and Desoye, unpublished). In the absence of other potential substrates such as ECM in cell culture the high proteolytic activity against pro-MT1-MMP may result in full activation of the enzyme.

This study did not measure maternal IGF1, IGF2 and TNF-α levels, because restrictions from the ethical committee did not permit measurements other than the HbA1c values. Hence, the concentrations for the in vitro experiments were chosen from published values for first trimester levels of IGF1, IGF2 and TNF-α in maternal T1D. Under this condition maternal IGF1 and IGF2 serum levels are unchanged (42). To our knowledge no published data about serum TNF-α levels of T1D women in the first trimester are available, but TNF-α is elevated in non-pregnant T1D patients (43).
Furthermore, rodents have increased uterine TNF-α expression throughout diabetic pregnancy (44). The increase of MT1-MMP expression in T1D was paralleled by up-regulation of MT1-MMP in isolated primary trophoblasts after treatment with insulin and TNF-α, both factors with elevated concentrations in the diabetic environment. Insulin shares some intracellular signaling pathways with IGF1 and IGF2. Therefore, both IGFs were included in the study despite their lack of concentration change in the first trimester T1D pregnancies. Since glucose had no effect on MT1-MMP expression, insulin and the pro-inflammatory TNF-α are likely candidates to account for the observed changes in placental MT1-MMP expression in T1D. The promoter of MT1-MMP includes a binding site for the nuclear transcription factor SP-1 and several TIE-like (TGF-β1 inhibitory element like) sequences (45). Both insulin as well as TNF-α can activate SP-1 (46; 47) and thereby could stimulate MT1-MMP expression. IGF1 and IGF2 also up-regulated MT1-MMP expression in trophoblasts. The stimulatory IGF1 effect, however, was only significant with Student’s t-test. We assume that ANOVA missed the concentration effect as a result from the already maximum stimulation of MT1-MMP with the lower IGF1 concentration (50ng/ml). None of the treatments resulted in the occurrence of an additional MT1-MMP species indicating that enzyme processing was not affected. The key role of insulin in regulating placental MT1-MMP production as found in vitro is corroborated further by the correlation of total MT1-MMP protein with the daily insulin dose of the T1D subjects.

Insulin can activate two major signaling pathways i.e., the MAP-kinase and the PI3-kinase pathway, respectively. Both pathways can transcriptionally activate MT1-MMP expression in various cells (48; 49). When PI3-kinase was inhibited by Wortmannin the notably strong effect of insulin was reduced. In contrast, the IGF1 effect was diminished by inhibition of the ERK1/2 pathway. The IGF2 effect was absent after inhibition of both pathways, which may be accounted for by the ability of IGF2 to bind to and activate the IGF1-receptor (IGF1-R) as well as the short insulin receptor isoform (50). Thus, the induction of MT1-MMP synthesis by IGF2 may be mediated by the IGF1-R and ERK1/2 as well as by the insulin receptor and PI3K. The IGF1 effect in trophoblasts was different from tumor cells (49) in which IGF1 stimulated MT1-MMP expression via the PI3K/Akt pathway, which may reflect changes in tumor cell signaling resulting from malignant transformation. Thus, transcriptional activation of MT1-MMP in trophoblasts can be accomplished by signaling through different pathways i.e., the PI3K-kinase and the ERK1/2 pathway.

The consequences of the diabetes-associated alterations in MT1-MMP expression and processing are unknown. Among all MMPs, MT1-MMP has a notably broad range of substrates including ECM components such as fibronectin, collagen I-III, laminin 1 and 5, pro-MMPs such as proMMP2, proMMP13 as well as cytokines and chemokines including proTNF-α, IL-8 as well as GRO-α and -γ (16; 17). Therefore, one can picture several scenarios by which higher levels of MT1-MMP may affect placental development: 1) MT1-MMP degrades extracellular matrix; 2) other MMPs are activated, which subsequently cleave extracellular matrix components; both mechanisms may directly affect villous differentiation and development; 3) over-activation (proTNF-α) or enhanced degradation and, hence, inactivation (IL-8, GRO-α and -γ) of cytokines and chemokines involved in trophoblast function (16) may further indirectly
modify cellular processes relevant for placental development.

We propose that some of the well-described structural alterations of the placenta at the end of a T1D pregnancy may begin already in the first trimester of pregnancy. Obviously, tight glycaemic control in the first gestational weeks by insulin treatment does not prevent all diabetes-associated changes in placental development as dysregulation of placental MT1-MMP expression is dependent on insulin levels.

Acknowledgements

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168, 2001
Tables

Table 1. Characteristics of study subjects

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<td>Interrupted pregnancies (IR)</td>
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<tr>
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<td>HbA$_{1c}$ (%)</td>
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<tr>
<td>Mean ± SD</td>
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HbA$_{1c}$ values of the control group were not determined (n.d.). The cut off HbA$_{1c}$ value for non-diabetic pregnant women as established by the local clinical laboratory was 6 %.
**Table 2.** Primers used for RT-PCR.

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<td>MT6-MMP</td>
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Table 3. Microarray analysis of MT-MMP family members expressed in first trimester trophoblasts. Total RNA from ten preparations isolated from ten different placentas was used. Expression of some metalloproteinases was not detectable (ND).

<table>
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</table>
Figure Legends

Figure 1. Expression of MT-MMP mRNAs in human first trimester trophoblasts. RT-PCR revealed that MT1-MMP and MT2-MMP are expressed (A). No bands were detected for MT3-, MT4-, MT5- and MT6-MMP (not shown). Mean mRNA expression levels did not differ between MT1-MMP and MT2-MMP (B). MT1-MMP mRNA is present in HLA-G positive (+) and HLA-G negative (-) trophoblasts (C) with higher expression in the HLA-G positive trophoblast subpopulation (D). The ribosomal protein L30 (RPL30) was used as an internal control; n = 4 trophoblast preparations from different placentas.

Figure 2. MT1-MMP protein expression (mean ± SEM) in first trimester placental tissue from control (n=13) and type 1 diabetic (T1D; n=13) pregnancies. Six representative western blots are shown (A). Prior to data analysis all samples were normalized to the internal control (placenta extract). The graph (B) displays the expression of total MT1-MMP and separate expression of the pro-MT1-MMP zymogen (63 kDa) and the active MT1-MMP (57 kDa).

Figure 3. Box plot depicting expression of active MT1-MMP protein (57 kDa) in early (≤ week 8; n=6) and late (> week 8; n=7) first trimester placental tissue of type 1 diabetic (T1D) and healthy (control) women.

Figure 4. (A) Representative western blot of active MT1-MMP (57 kDa) in first trimester primary trophoblasts (n=5 preparations from different placentas) stimulated for 48 h with insulin (I; 1 nM), TNF-α (25 ng/ml), IGF1 (100 ng/ml), IGF2 (300 ng/ml) and glucose (G; 25 mM) as compared to the untreated control (C). The graphs (B-E) show MT1-MMP protein expression (mean ± SEM) in trophoblasts treated with different concentrations of insulin (0.1; 1 nM), TNF-α (10; 25 ng/ml); IGF1 (50, 100 ng/ml); IGF2 (165; 300 ng/ml). The data are expressed relative to the controls (= 100%). Statistical tests used the raw data. * indicates a significant change (p<0.05) vs. controls using post hoc test, § indicates a significant change using Student’s t-test.

Figure 5. Induction of active (57 kDa) MT1-MMP in primary first trimester trophoblasts after 48 h treatment with insulin (1 nM), IGF1 (100 ng/ml) and IGF2 (300 ng/ml) in the presence or absence of the PI3K inhibitor Wortmannin (100 nM) or the MEK1 inhibitor U0126 (10 µM). The graphs (A-C) display the protein expression (n=4 trophoblast preparations from different placentas; mean ± SEM) compared to the untreated control. P-values refer to differences vs. controls.
Figure 1

MT1-MMP is elevated in the 1st trimester placenta in T1D
MT1-MMP is elevated in the 1st trimester placenta in T1D

Figure 2
MT1-MMP is elevated in the 1st trimester placenta in T1D

Figure 3
MT1-MMP is elevated in the 1st trimester placenta in T1D

Figure 4

A

B

ANOVA \( p < 0.030 \)

\[ \begin{array}{l}
\text{control} \\
\text{insulin [0.1 nM]} \\
\text{insulin [1 nM]}
\end{array} \]

ANOVA \( p < 0.049 \)

C

\[ \begin{array}{l}
\text{control} \\
\text{TNF-\( \alpha \) [10 ng/ml]} \\
\text{TNF-\( \alpha \) [25 ng/ml]}
\end{array} \]

\( \star \)

D

ANOVA \( p < 0.099 \)

\[ \begin{array}{l}
\text{control} \\
\text{IGF1 [50 ng/ml]} \\
\text{IGF1 [100 ng/ml]}
\end{array} \]

\( \star \)

MT1-MMP

ANOVA \( p < 0.002 \)

\[ \begin{array}{l}
\text{control} \\
\text{IGF2 [165 ng/ml]} \\
\text{IGF2 [300 ng/ml]}
\end{array} \]
MT1-MMP is elevated in the 1st trimester placenta in T1D

Figure 5

A

B

C

MT1-MMP is elevated in the 1st trimester placenta in T1D