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Increased mouse double minute X expression in human placental villous macrophages (Hofbauer cells) in gestational diabetes mellitus

Sefa Arlıer [1, 2], Sadık Kükrer [2], Cevdet Adıgüzel [2]

[1] Department of Obstetrics and Gynecology, Morsani College of Medicine, University of South Florida, Tampa, FL, USA
[2] University of Health Sciences Adana City Training and Research Hospital, Department of Obstetrics and Gynecology, Adana, Turkey

ORCID ID of the author(s)
SA: 0000-0002-0019-8403
SK: 0000-0001-8465-3225
CA: 0000-0002-3003-4573

Corresponding Author
Sadık Kükrer
University of Health Sciences Adana City Training and Research Hospital, Department of Obstetrics and Gynecology, Adana, Turkey (Dr. Mithat Özsan Bulvarı Kışla Mah. 4522 Sok. No:1 Yüreğir/Adana)
E-mail: sadikkukrer@hotmail.com

Ethics Committee Approval
Serial paraffin sections of human placental UC specimens were obtained from the University of South Florida with the protocol approved by the Ethics and Human Investigation Committees of the University of South Florida (approval number: 00015578).
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Gestational diabetes mellitus is a common metabolic problem in pregnancy, and its prevalence ranges between 5-20%. Hofbauer cells are tissue macrophages of the feto-placental component that are raised in the villous tree of the placenta during pregnancy, but their quantity falls off with growing gestational age. We theorize that Hofbauer cells play a significant role in placental pathophysiology in GDM by controlling the MDMX (mouse double minute X/MDM4/HDMX) gene.
Methods: We performed immunohistochemistry on human placental specimens to determine cell-specific expression of MDMX in Hofbauer cells (HC) among the control and GDM (n=8 in each group) groups with matching gestational ages.
Results: Immunohistochemical analysis revealed that MDMXs were secreted by Hofbauer cells in the placental villous tree and compared to the placenta got from normal pregnancies, significantly higher MDMX HSCORE levels were detected in placenta Hofbauer cells (32.8 (24.52) vs. 190.1 (32.54), *P*=0.001) of the GDM group.
Conclusion: We revealed Hofbauer cells to be a source of MDMX secretion in human placenta. MDM2 levels in Hofbauer cells are also increased in GDM. This study found higher levels of MDMX in the Hofbauer cells from GDM placentas, suggesting an induction of MDMX secretion. GDM interaction in placental Hofbauer cells may contribute to GDM-associated feto-placental complications. Further studies are needed to define the significance of this relationship.

Keywords: Gestational diabetes mellitus, Mouse double minute X expression, Hofbauer cells, Placenta

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Introduction

Gestational diabetes mellitus GDM is defined as maternal hyperglycemia due to insulin resistance that develops during pregnancy, causing placental low-grade inflammation resulting in neonatal and maternal mortality and morbidity [1]. GDM prevalence is 11.5% among pregnant women [2], and it is associated with chronic low-grade inflammation of the placenta [2].

Fetal placental macrophages, or Hofbauer cells (HBCs), are present in the placental villous tree from the blastocyst implantation until delivery [2]. Hofbauer cells (HBCs) are characterized by high expression of the surface protein CD163 [3]. Hofbauer cells, located in the chorionic villi of the human placenta, are an essential part in controlling the pregnancy and providing homeostasis that is crucial for fetal growth [2]. It is not clearly explained how Hofbauer cell function varies in healthy pregnancy and specifically in pregnancies complicated by gestational diabetes, preeclampsia, and viral diseases [2].

We hypothesize that the human placenta bares functional, metabolic, and immunological cells in which Hofbauer cells play an essential role by altering many transcriptional gene expressions. MDMX (mouse double minute X/MDM4/HDMX) is the major negative regulator of p53, which regulates apoptosis in the villus tree. P53, the "guardian" of the human genome, is a tumor suppressor that is mutated in all cancers [4]. P53, the tumor suppressor gene, coordinates DNA repair, mitochondrial respiration, cellular metabolism, autophagy, and cellular responses to metabolic and environmental stress. In GDM, p53 is also upregulated in placental villi, in part due to hypoxia, metabolic and oxidative stress. The proteins MDM2, E3 ubiquitin ligase, and MDM4 coordinate the tumor suppressor gene p53 in proliferating cells. MDM2 and MDMX control p53 levels during growth [5]. Previous investigations demonstrate that p53 plays a significant role in the improvement of diabetes mellitus [4]. We hypothesize that the Hofbauer cells play a critical role in placental pathophysiology in GDM by regulating the MDMX gene.

Materials and methods

This prospective experimental laboratory study used placental samples collected from normal pregnant women who had never received steroid treatment, were suitable for their gestational age, and had no pathology, as well as those collected after delivery from patients diagnosed with gestational diabetes mellitus. Serial paraffin sections of human placental specimens were obtained from the University of South Florida per the protocol approved by the Ethics and Human Investigation Committees of the University of South Florida (approval number: 00015578) [6]. Written and verbal informed consent were obtained from each patient. Power Analysis was performed with G*Power 3.1.9.7 software with an α err prob of 0.05, and a power (1-β err prob) of 0.95. At 5% alpha error, 95% power (1-β), d=5.53 (large) and 95% confidence interval (CI), the sample size was calculated as 6. Due to case losses and possible negativities, the study was completed on 16 placental samples. All samples were grouped according to clinical diagnosis: Control (n=8) or GDM (n=8).

Immunohistochemistry

5-µm serial endometrial sections were incubated overnight at 56°C [5]. After deparaffinization, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 15 min for antigen retrieval [5, 6]. The sections were then immersed in 3% hydrogen peroxide (in 1/1 methanol/distilled water) for 10 min to quench the endogenous peroxidase activity [6]. After washing with Tris-buffered saline (TBS; pH: 7.4) ×3 for 5 min, the slides were incubated in a humidified chamber with 5% blocking goat serum (Vector Laboratories, Burlingame, CA) in TBS for 30 min at room temperature [6]. Excess serum was then drained and the slides were incubated with a primary rabbit polyclonal MDMX (1:150; Cell Signaling Technology, Danvers, MA) in 1% normal goat serum overnight at 4°C [6]. The sections were washed ×3 for 5 min with TBS, and then biotinylated goat anti-rabbit IgG (Vector Laboratories) was added at 1:400 dilution for 30 min at RT. The antigen-antibody complex was detected using an avidin-biotin-peroxidase kit (Vector Laboratories) for 30 min at RT. DAB (3, 3-diaminobenzidine tetrahydrochloride dihydrate; Vector Laboratories) was used as the chromogen to visualize immunoreactivity, and sections were slightly counterstained with hematoxylin.

Immunoreactive MDMX levels were semi-quantitatively evaluated using the following intensity categories: 0, no staining; 1+, weak but detectable staining; 2+, moderate, or distinct staining; 3+, intense staining [6]. As defined already, a histological score value (HSCORE) was obtained for each tissue by summing the percentages of cells colored in each intensity group and multiplying this value by the weighted intensity of color, applying the formula HSCORE= Σ Pi (i + l), where i describes the intensity scores and Pi is the corresponding percentage of cells. In each slide, five randomly chosen sections were assessed under the light microscope (x40 magnification), and the percentage of cells for each intensity within these sections was established at various time points by two researchers who were blinded to the type and origin of tissues [7]. The intra-individual and inter-individual coefficients of variation were 10 and 12%, respectively, for the HSCORE evaluation. The average HSCORE of two examiners was used (Figure 1).

Statistical analysis

Results were analyzed using the Mann Whitney U test. Analyses were performed using SigmaPlot version 12.5 (Systat Software, Inc, San Jose, California, USA). A *P*-value of <0.05 was considered statistically significant in binary comparison.

Results

The mean gestational ages in the control and GDM groups were 39.1 (0.5) weeks and 38.8 (0.8) weeks, respectively. The two groups were similar in terms of mean gestational age (*P*=0.89). CA163 immunohistochemical staining revealed that MDMXs were produced by Hofbauer cells in a placental villous tree. MDMX immune activity was detected in high levels in the HBCs of GDM placental samples (Figure 2). This immune reaction was poor in the cytoplasmic region and intensive in the nuclear region of the HBCs. When the intensity of this immunostaining was quantified numerically, the following was found: The HSCORE of the control and GDM group HBCs were

132.8 (24.52), and 190.1 (32.54), respectively (P=0.001). MDMX immunostaining intensity was significantly higher in the GDM group (Figure 2).

Figure 1: Increased MDMX immune reactivity in the GDM placental villous tree. Picture symbolizes histological score (HSCORE) levels for Normal MDMX (n = 8) and GDM MDMX (n = 8) in placental villous tree samples. Bars represent (Mean (SD) •p < .001 compared to Normal and gestational age-matched GDM MDMX.

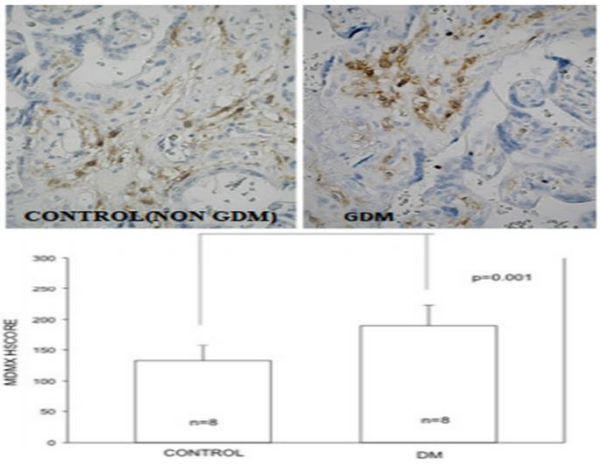
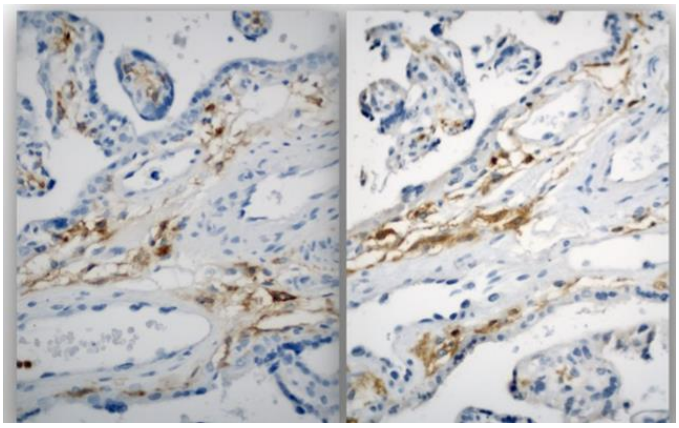


Figure 2: Association of CD 163 immunohistochemical staining with MDMX level in the placental villous tree. Demonstrative micrographs for the immunoreactivity of CD l63 and MDMX in placental villous tree serial sections



Discussion

GDM prevalence is 11.5% in the Asian pregnant population and creates a major obstetric problem, increasing mortality and morbidity in both the pregnant women and the offspring. Differences in diagnostic criteria, screening methods and work environment result in heterogeneity in the prevalence of Gestational Diabetes Mellitus (GDM) [8]. The human placental unit is sensitive to the high maternal glucose level and reacts to the adaptive variations in arrangement and function [9]. GDM is associated with high maternal and placental glucose levels, therefore resulting in a chronic low-grade inflammation of the placental villous tree [1]. An overproduction of pro-inflammatory mediators is found during blastocyst implantation and the developing embryo in the decidua and placenta from GDM. Maternal hyperglycemic environment-induced changes create pro-inflammatory and anti-inflammatory pathways that negatively affect the embryo and placental development in GDM [9–11].

Numerous studies have elucidated the possible role of regulator gene functions in the development and progression of GDM in the human placenta [12–14]. In this study, we researched whether placental villous tree MDMX levels vary in GDM. In addition, we posited that Hofbauer cells play a crucial role in placental pathophysiology in GDM, regulating the MDMX production, and found higher levels of MDMX in Hofbauer cells obtained from the GDM placenta, suggesting the induction of MDMX production by GDM [12]. To address this hypothesis, we identified phenotypic Hofbauer cells in the placental villous tree with surface markers of CD163 in immunohistochemical staining. Our immunohistochemical staining results reveal that MDMXs were mainly produced by the Hofbauer cells in the placental villous tree [15]. Moreover, significantly higher MDMX H scores were detected in the placentas of Hofbauer cells obtained from GDM patients [15]. During placental inflammation, Hofbauer cells produce different regulatory genes, proinflammatory cytokines or mediators that destroy or produce the villous cell boundary and trigger or destroy damaging responses as a continuity of chronic inflammation and anti-inflammatory balance [16].

Macrophages modulate tissue homeostasis and are widely distributed in the placenta during pregnancy, as the initial field of antimicrobial defense [2]. Placental macrophages, identified as Hofbauer cells (HBCs), are found in the villous tree [2]. HBCs are placed in the villous tree of the placenta in both healthy and pathological pregnancies such as those with GDM, preeclampsia and IUGR [2, 3].

Previous research shows that Hofbauer cells have a phenotype related with regulatory and anti-inflammatory reactions in the human placenta [2], and they are believed to play a critical role in regulating the pregnancy and maintaining a homeostatic environment important for fetal development [2, 17]. It is not yet clear how Hofbauer cell function changes in normal pregnancy and exceptionally in GDM, IUGR, preeclampsia, and viral infections [2]. New research recommends that diabetes/hyperglycemia impair the anti-inflammatory profile of the HBCs by arousing these cells to gain an inflammatory capacity [2, 3]. They are defined as anti-inflammatory M2 separated cells, stimulating tolerance and tissue remodeling [2]. They express surface markers such as CD163, CD206, and only intermediate or low levels of MHC-II proteins (MHC-II low). Their primary functions are tissue repair, wound healing and angiogenesis, as well as feto-maternal tolerance induction [3]. HBCs are present in all IUGR pregnancies and in 70% of GDM pregnancies [3].

Feng et al. [18] showed an increased infiltration of the chorionic villi by Hofbauer cells in GDM and inflammatory diseases of the placenta, such as villous inflammation of unknown etiology. Another study described a similar phenomenon in pregnancies complicated with GDM [19]. However, the role of Hofbauer cells is unclear under these conditions.

Previous studies have shown that MDMX are critical regulators of the tumor suppressor p53 and are overexpressed in many human malignancies [4]. It is two negative regulators – the E3 ubiquitin ligase MDM2 and its homologue, MDMX, which strongly regulate the tumor suppressor protein p53 in healthy cells [20]. Under stress conditions, such as DNA breakage, p53 escapes MDM2- and MDMX-induced functional inhibition and degradation and blocks proliferation of injured cells by promoting cell cycle delay, DNA repair, senescence, or apoptosis [20]. Considerable evidence points that stress signals promote phosphorylation of the MDM2 and MDMX, leading to the

activation of p53 [20]. Furthermore, MDMX and MDM2 are major negative regulators of p53 that control apoptosis in the placental villous tree. This regulator gene plays an important part in placental progress, consisting of vasculogenesis and angiogenesis [16]. The vasculogenesis and angiogenesis processes in the villous tree of the placenta are also administered by the expression of vascular endothelial growth factor (VEGF) by HBCs (villous macrophages) and trophoblast cells [16,17]. We investigate what is known about the main origin of this MDMX regulator gene [4]. MDMX is a negative regulator of p53 activity in vivo and in vitro [4]. A new research has established that control of p53 protein action is needed for healthy embryogenesis, tumor suppression, and cellular response to DNA damage [4]. Moreover, destruction of the p53-binding protein MDMX leads to embryo lethality in mid-gestation, a phenotype that is wholly protected by the lack of p53 [4]. Mice with homozygous MDMX and p53 null mutations develop normally [4]. These investigations confirm that MDMX behaves as a crucial negative regulator of p53 in vivo [4]. Recent studies show that increased p53 expression in the placental villi is associated with the placental dysfunction observed in pre-eclampsia and IUGR, suggesting that p53 plays a primary pathogenic role [21]. In contrast, the natural inhibitor of p53, MDMX, is expressed within the HBCs in term pregnancy, likely reflecting a change in the balance of p53 and MDMX with gestation. However, the structural basis of a stress-induced p53 activation remains inadequately known due to a lack of technical means to develop site-specifically phosphorylated MDM2 and MDMX proteins for biochemical and biophysical investigations [20]. Unfortunately, the role of MDMX does not describe the GDM pathophysiology in the human placental unit. Further molecular studies are required to understand the role of MDMX, which is produced by Hofbauer cells (HBCs), in the pathophysiology of GDM on the placenta.

Limitations

We are mindful of the limitations of our research. First, we designed our study on a particularly limited group of patients. In this research, we show that in vitro placenta immunostained HBCs had high MDMX levels. We first concentrated on the information that MDMX gene expression of HBCs has a key position in GDM. We observed that high glucose triggered increased expression of MDMX gene expression. Using immunohistochemical staining, we confirmed the increase in MDMX in response to high glucose in the villous tree of the placenta.

Conclusion

These immunohistochemical studies showed that the MDMX levels in HBCs is significantly lower than in uncomplicated term pregnancies. However, HBC MDMX density was found in all trials. So, there is an important variation in HBCs' MDMX density between GDM and normal gestations. HBCs were identified using immunohistochemistry, with the macrophage marker CD 163. Our investigation sheds light on the fields of molecular researchers on human placenta that can better describe these innate regulatory gene functions. Hofbauer cells are a source of MDMX secretion in the human placenta. Hofbauer cell levels of MDM2 are also increased in GDM. This study found higher levels of MDMX in the Hofbauer cells of the GDM placenta, suggesting the induction of MDMX secretion by GDM interaction. Further studies are needed to define the significance of this relationship.

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