Studies on differentiation of uterine natural killer cells at the feto-maternal interface in perforin-deficient, β_2-microglobulin-deficient and diabetes mice

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LIST OF ABBREVIATIONS

β_2m	beta-2-microglobulin
$\beta_2m^{-/-}$	beta-2-microglobulin-deficient
Bwt	body weight
CSF	colony stimulating factor
CTL	cytotoxic T Lymphocytes
DAB	diaminobenzidine
dl	deciliter
°C	Degree Celsius
cDNA	complementary DNA
EDTA	ethylenediamine-tetraacetic acid
EVT	extravillous cytotrophoblasts
HLA	human leukocyte antigen
h	hour (s)
IFN-γ	interferon-gamma
IL15	interleukin-15
iNOS	inducible nitric oxide synthase
NOS	nitric oxide synthase
KIR	killer inhibitory receptor
KOH	potassium hydroxide
LIF	leukemia inhibitory factor
mRNA	Messenger Ribonucleic acid
MHC	major histocompatibility complex
Mg	milligram (s)
mM	milimolar
ml	milliliter (s)
min	minute (s)
$MgCl_2$	Magnesium chloride
MLAp	mesometrial lymphoid aggregate of pregnancy
ug	microgram (s)
µl	microliyre (s)
μm	micrometer
NaCl	sodium chlolide
NK	natural killer

PAS	periodic acid Schiff
PlGF	placenta growth factor
$P^{-/-}$	perforin-deficient
pre-NK	progenitor NK
real-time PCR	real-time Polymerase Chain Reaction
rmp	revolutions per minute
SEM	scanning electron microscopy
ssDNA	single-stranded Deoxyribonucleic Acid
STZ	streptozotocin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
uNK	uterine natural killer
VEGF	vascular endothelial growth factor

GENERAL INTRODUCTION

Mother's immunology tolerance to a semi-allogenic fetus has been puzzling for immunology. The endometrium is ready to respond to potential pathogen challenges, and yet able to control immune responses to allow the development of a semi-allogenic fetus. However, alternate viewpoint to gaining support is that the maternal immune system may work together with fetal derived cells to create hospitable environments in which the fetus can develop.

Natural killer (NK) cells are a part of the innate immune system and defend against allogenic cells under stress such as a virally infected or tumor cell [1, 2]. It is well known that NK cells are present in the uterus during pregnancy, called uterine natural killer (uNK) cells [3-6]. uNK cells are bone marrow-derived leukocytes, but their immediate precursors may migrate from the spleen [7- 9]. These cells become progenitor NK (pre-NK) cells in the secondary lymphoid tissues and then migrate to the various tissues of the body to further differentiate [7]. Once these small agranular pre-NK cells home to the uterus, they begin their maturation process into large granulated cells, i.e., uNK cells [10]. The process of pre-uNK cell recruitment into the uterus and uNK cell maturation within the uterus has been shown to involve interleukin-15 (IL15) [11, 12]. In mice and rat, a few uNK cells are present in the endometrium before implantation, but their numbers rapidly increase between day 5-11 of pregnancy [6, 10, 13, 14]. uNK cells proliferate, gain increasing numbers of cytoplasmic granules, and form a transient lymphoid structure as the mesometrial lymphoid aggregate of pregnancy (MLAp), metrial gland, or decidualized mesometrial triangle [15]. After day 15 of pregnancy, uNK cells are decreased in number and size, their morphology shows pyknosis and vacuolation, indicative of apoptosis [13, 14, 16, 17].

uNK cells are large up to 50 μm in diameter, containing numerous large cytoplasmic granules up to 5 μm in diameter in mice, achieving a frequency of up to 20% in the metrial gland [6]. The cytoplasm of some uNK cells contains extensive deposits of glycogen, moderate amounts of rough endoplasmic reticulum and Golgi bodies. Some uNK cells are binucleate and at certain stages of pregnancy may undergo mitosis. uNK cells are characterised by their cytoplasmic granules which contain lytic molecules such as perforin and granzymes A and B, matrix components including osteopontin, and vasoactive factors such as inducible nitric oxide synthase (iNOS) and endothelial (e) NOS [18-20]. These cells are also characterized by the large granules stained by zurophilic and periodic acid Schiff (PAS), and are localized in the metrial gland and the decidual basalis of each placenta [21, 22].

uNK cells can produce various cytokines such as interferon-gamma (IFN-γ), colony stimulating factor (CSF), leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) [23-25]. They express Thy 1.1, asialo-GM1, IL-15Rα, and at least two members of the CD94/NKG2 C-type lectin–like family of class I MHC receptors, NKR-P1 (NK1.1) and Ly49G2 (LGL-1), and can lyse YAC-1 target cells [18, 23, 26, 27]. In human uNK cells express CD56 (polysialylated neural cell adhesion molecule), members of the CD94/NKG2 and killer inhibitory receptor (KIR) 2D class I MHC receptor families, and can lyse K562 target cells [28, 29]. Although the conceptus is a semi-allogenic graft that expresses both maternal and paternal antigens, it is not rejected by the maternal immune system under normal conditions [30]. uNK cells are present in the uterus during pregnancy [21, 27, 31, 32, 33,], thus they play an

important role in normal pregnancy to development of the placental, fetal growth, angiogenesis and immune responses at the feto-maternal interface [8, 34, 35]. By mid-gestation, most of murine uNK cells (65%) are intimately associated with endometrial vessels and 10% are intravascular [36], suggesting that uNK cells can directly influence the vascular smooth muscle, endothelium, and possibly conducted responses in these vessels.

OBJECTIVE

There are several evidences that uNK cells have an important role in maintenance placental growth, fetal growth, angiogenesis and immune responses at the feto-maternal interface during normal pregnancy in primate and rodent. However, factors affecting uNK cell performance remain to be fully determined. I addressed here about such factors. The topics in this study are summarized as follows;

Chapter 1

1). Perforin is known to be associated with the cytoplasmic granules of cytotoxic T Lymphocytes (CTL) and NK cells, and to be related to uNK cells apoptosis during pregnancy. Hence, perforin may affect function of uNK cells. This study was undertaken to understand effects of perforin on proliferation, differentiation and function of uNK cells during pregnancy, using perforin-deficient ($P^{-/-}$) mice.

2). β_2-microglobulin (β_2m) is a subunit of MHC class I molecule. uNK cell activity is involved in MHC non-restrictive immune responses at the feto-maternal interface, and trophoblast MHC class I molecules and uNK cell receptors are important in the control of implantation. Thus, the effect of β_2m on proliferation and differentiation of uNK cells is investigated, using β_2-microglobulin-deficient ($\beta_2m^{-/-}$) mice.

Chapter 2

1). Diabetes is well known to cause reproductive abnormalities during pregnancy. However, it is unknown whether diabetes can influence uNK cells during pregnancy. Also, little information is about the affect of diabetes on reproductive performance. Thus, this study was undertaken to understand the influences of diabetes to uNK cell function and PlGF expression at the feto-maternal interface, using KK/TaJcl mice (Type-II diabetes) and streptozotocin-induced diabetes mice (Type-I diabetes).

2). uNK cells are increased in number and reach at the maximum level until day 12 of pregnancy. After on day 15, the granules disappear from the cytoplasm of uNK cells, and finally the uNK cells disappear from the endometrium. Such a loss of uNK cells in late pregnancy is considered to be due to apoptosis by Fas/Fas-ligand system. Thus, this study was undertaken to evaluate affects of diabetes to proliferation and apoptosis of uNK cells in diabetes mice.

CHAPTER 1

Uterine natural killer cells in perforin and β_2-microglobulin deficient mice

ABSTRACT

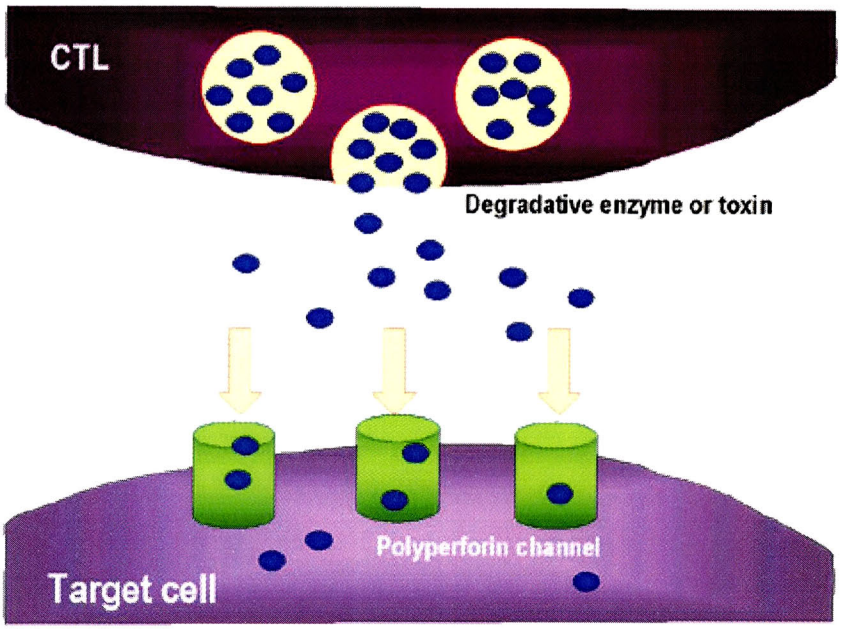
Uterine natural killer (uNK) cells have roles for immune responses at the feto-maternal interface in mice. We studied the effects of perforin and β_2-microglobulin (β_2m) on proliferation and differentiation of uNK cells in pregnancy, using perforin-deficient ($P^{-/-}$) mice and β_2-microglobulin-deficient ($\beta_2m^{-/-}$) mice. The cell population of uNK cells in the metrial gland and decidua basalis of $P^{-/-}$ mice was tended to be higher than the control B6 mice. The cell population of uNK cells in the decidua basalis of $\beta_2m^{-/-}$ mice was significant increased when compare to B6 mice at Day 10 of pregnancy. The cell population of uNK cells in the metrial gland of $\beta_2m^{-/-}$ mice was significantly increased at Day 12 of pregnancy comparing to B6 and $P^{-/-}$ mice, while the cells population of uNK cells in $\beta_2m^{-/-}$ mice was increased significantly from Day 10 toward Day 12 in the metrial gland and uNK cells in $\beta_2m^{-/-}$ mice was decreased from Day 10 toward Day 12 in the decidua basalis. On the other hand, the cell population of uNK cells in the decidua basalis of $\beta_2m^{-/-}$ mice was tended to be lower than B6 and $P^{-/-}$ mice. These results indicate that β_2m may be involved in proliferation of uNK cells in the metrial gland, and that β_2m may affect the maturation of uNK cells in the decidua basalis.

INTRODUCTION

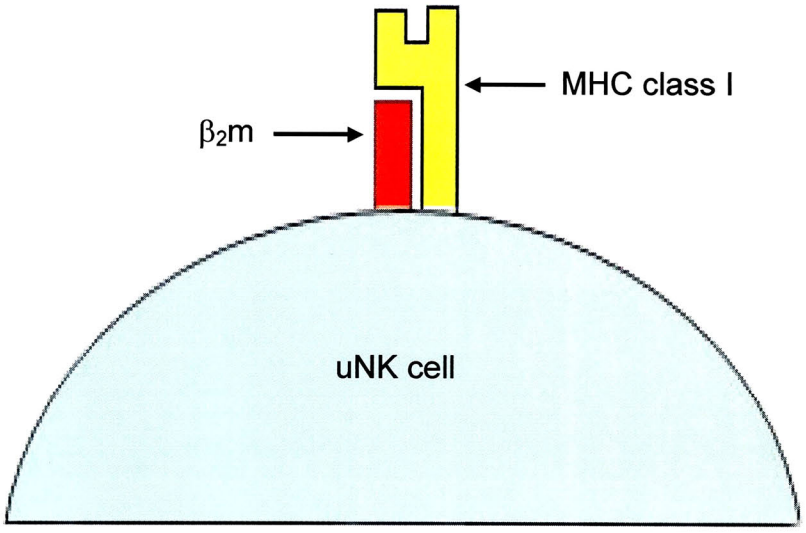
In the murine pregnant uterus, uterine natural killer (uNK) cells are the major leukocytes in the decidua basalis [6]. uNK cells play an important role in pregnancy success by intercepting and killing aberrant placental or embryonic cells that invade the uterine tissue [18]. In addition, uNK cells express abundant quantities of pore forming protein, termed perforin [12, 18, 23, 37], which polymerize on the target cell membrane to form transmembrane pores that are involved in target cell death (Text. 1.) [37]. Perforin is known to be associated with the cytoplasmic granules of cytotoxic T Lymphocytes (CTL) and NK cells [38-40]. Even though uNK cells show cytotoxic activities in vitro, clear cytotoxic figures mediated by perforin has not been shown in vivo. Recently, it has been revealed that uNK cells play an important role for placental angiogenesis in mice [8, 34, 36]. uNK cells are a member of NK cell lineage and share many characteristics with peripheral blood NK cells and splenic NK cells. The uNK cell activity is involved in major histocompatibility complex (MHC) non-restrictive immune responses at the feto-maternal interface [5, 41, 42]. The MHC class I molecule consists of heavy α chain associated noncovalently with a light chain, termed 2 microglobulin (Text. 2.), that is involved in rejection of transplanted tissues or organ. MHC class I molecule expresses on most cells.

In humans, human leukocyte antigen (HLA)-G and E have been suggested as an important factor for a successful pregnancy because of its restricted expression on extravillous cytotrophoblasts (EVT) and its capability of modulating the function of uNK cells such as cytokine production via NK cell receptors [43].

Also, it has been proposed that interaction between trophoblast MHC class I molecules and uNK-cell receptors could be important for the control of implantation [44]. In this chapter, effects of beta-2-microglobulin (β_2m), which is a subunit of MHC class I, and perforin on proliferation and differentiation of uNK cells are investigated to reveal the function of uNK cells in pregnancy by using perforin deficient ($P^{-/-}$) and beta-2-microglobulin deficient ($\beta_2m^{-/-}$) mice.



Text. 1. Perforin is a cytolytic mediator produced by CTL, and is stored in and released by cytoplasmic granules. The CTL may also release degradative enzymes and toxins which travel through the perforin chanels and damage the target cell.



Text. 2. β_2-m is a protein found on the surface of many cells, a component of MHC class I molecules.

MATERIALS AND METHODS

Two kinds of deficient ($P^{-/-}$ and $\beta_2m^{-/-}$) and their background C57BL/6 (B6) (each n=6) mice were used in this study. B6 mice were purchased from the Japan Clea (Osaka, Japan). $P^{-/-}$ mice described by Kagi et al. [38] were given from Osaka Medical College and $\beta_2m^{-/-}$ mice were given from the Central Institute for Experimental Animals, Japan. All mice were used at 12 weeks old, according to the guideline of the committee on animal welfare in Yamaguchi University. Mice were housed under the condition of 12h light and 12h dark cycle, water and food ad libitum. The day when the vaginal plug was detected was defined as Day 0 of pregnancy. Uteri were sampled at Day 10 and 12 of pregnancy. Uteri were fixed with Bouin's solution and embedded in paraffin wax by the conventional

procedure. Sections of 4 μm thickness were stained by PAS to observe histological structure of the placenta. uNK cells can be readily identified due to PAS positive granules. Using 3 mice from each group, uNK cells with PAS positive granules and other cells without PAS positive granules were counted in three randomly selected fields on three non-serial sections from each sample. The cell population of uNK cells (%) was calculated in the decidua basalis and the metrial gland of each mouse model. Mean values of the cell population of uNK cells from each selected field were compared among animal groups by ANOVA. $P<0.01$, or $P<0.05$ was considered as statistically significant.

RESULTS

The placental morphology at Day 10 and 12 in B6, $P^{-/-}$ and $\beta_2m^{-/-}$ mice showed the similar structures. Placentas at Day 12 of all models were completely developed and the metrial gland was larger than that in Day 10 (Fig. 1.1, a-f). uNK cells were found only in the metrial gland (Fig. 1.2) and the decidua basalis, but there were no uNK cells in the placental labyrinth. Even though the population of uNK cells both at Day 10 and Day 12 in the metrial gland and the decidua basalis of $P^{-/-}$ and $\beta_2m^{-/-}$ mice was tended to be higher than B6 mice, but only at Day 12 in the decidua basalis of $\beta_2m^{-/-}$ mice to be decreased than B6 and $P^{-/-}$ mice. Significant difference was recognized in the decidua basalis between B6 and $\beta_2m^{-/-}$ mice ($P<0.05$) at Day 10, and in the metrial gland between B6 and $\beta_2m^{-/-}$ mice ($P<0.01$), $P^{-/-}$ and $\beta_2m^{-/-}$ mice at Day 12 ($P<0.01$). uNK cells in $\beta_2m^{-/-}$ mice was increased significantly from Day 10 toward Day 12 in the metrial gland

($P<0.01$) and uNK cells in $\beta_2m^{-/-}$ mice was decreased from Day 10 toward Day 12 in the decidua basalis ($P<0.05$) (Table. 1.1).

Table 1.1 The population (%) of uNK cells at Day10 and 12 of pregnancy.

Day / region	B6 (%) (n=3)	$P^{-/-}$ (%) (n=3)	$\beta_2m^{-/-}$ (%) (n=3)
Day 10			
MG	16.84 ± 0.04	17.60 ± 0.03	19.26 ± 0.02[d]
DB	14.46 ± 0.08[a]	20.03 ± 0.06	23.42 ± 0.1[a, d, e]
Day 12			
MG	19.00±4.69[b]	23.83±6.12[c]	36.96±8.63[b, c, d]
DB	19.53±9.41	20.03±8.47	16.06±6.94[e]

All values are means ± SD. Significant difference between the same superscripted alphabets, a,e ($p<0.05$), b,c,d ($p<0.01$). DB=decidua basalis, MG=metrial gland.

DISCUSSION

The number of uNK cells was increased until Day 12 to 14 of pregnancy in mice and their function could be enough for placental growth and fetal survival [17, 45]. It has been reported that the lack of perforin expression in the uterine uNK cells does not measurably restrain successful pregnancy and reproduction, i.e, proferin-deficient mothers were able to give birth to the same number of syngeneic or semi-allogeneic litters of similar sizes as control mice [45, 46]. Our result also showed that although the cell population of uNK cells in $P^{-/-}$ mice was tended to be higher than B6 mice at Day 12, there is no significant difference between these 2 groups. This suggests that perforin could have house keeping properties in uNK cells without effects on their development. Otherwise, it may not be essential for successful pregnancy.

This result clearly showed that the cell population of uNK cells in $\beta_2m^{-/-}$ mice was increased in the metrial gland at Day 12 when compared to B6 and $P^{-/-}$mice. β_2m is a component of MHC class I molecule which is necessary for transport and surface expression of functional class I molecules of MHC in mice [47]. In human, coincubation of NK cells with class-I-deficient target cells results in the induction of NK cell death and inhibition of NK cell cytotoxic activity [6], suggesting that MHC class I antigens regulate the function of NK cell [41, 48]. Thus, the function of MHC class I antigens on target cells may be important for survival and function of the activated NK cells. The trophoblast cells in the human placenta express HLA-G specific to invasive cytotrophoblast [49]. In the cattle, trophoblast cells express MHC class I antigen in the intercotyledonary and arcade region during the

second half of pregnancy [21]. Fetal trophoblast cells may be susceptible to inhibition by maternal NK cells, because the trophoblast cells lack expression of the classical MHC class I molecules. The deficiency of β_2m affect the expression of MHC class I molecule on the trophoblast cells, so that uNK cells could be increased in number at the feto-maternal interface. Indeed, uNK cells can proliferate only in the metrial gland but not in the decidua basalis, and its peak is on Day 12 [6]. Furthermore, uNK cell can differentiate into the matured type only in the decidua basalis [5, 17, 44]. Thus, it is likely that due to β_2m deficiency uNK cell can more proliferate in the metrial gland but less differentiate in the decidua basalis.

In conclusion, the present results indicate that β_2m may be involved in proliferation of uNK cells in the metrial gland. In addition, β_2m may affect the differentiation of uNK cells in the decidua basalis.

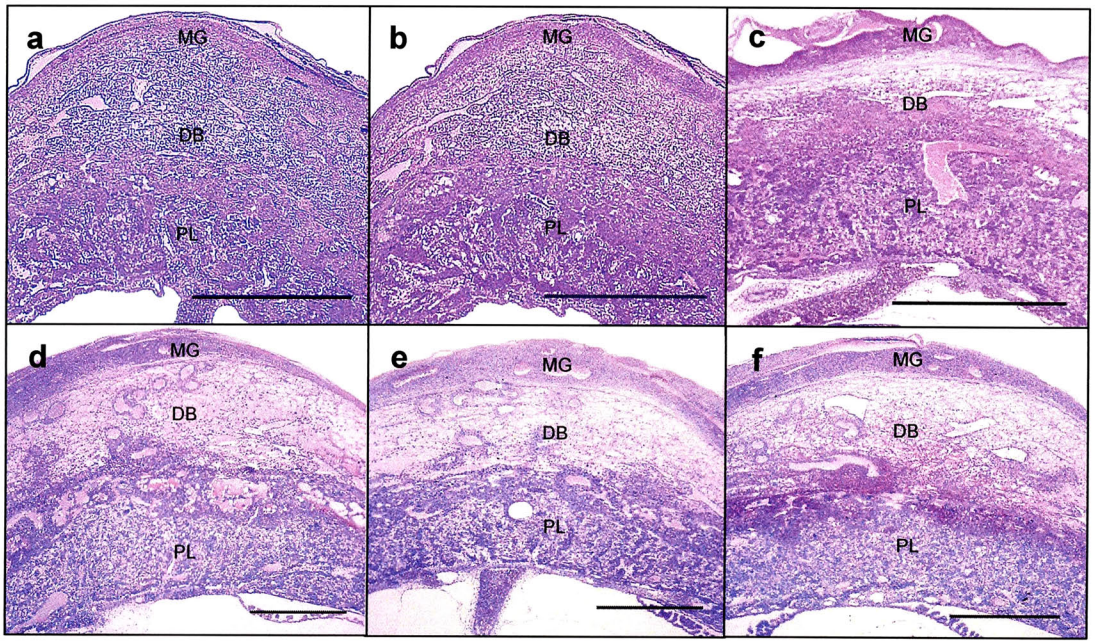


Figure 1.1 Placentas in B6 (a), $P^{-/-}$ (b), and $\beta_2m^{-/-}$ (c) at Day 10 and B6 (d), $P^{-/-}$ (e) and $\beta_2m^{-/-}$ (f) mice at Day 12 of pregnancy. The placental morphology shows the similar structures in 3 groups. DB=decidua basalis, MG=metrial gland, PL=placental labyrinth. PAS staining. Bar= 1 mm.

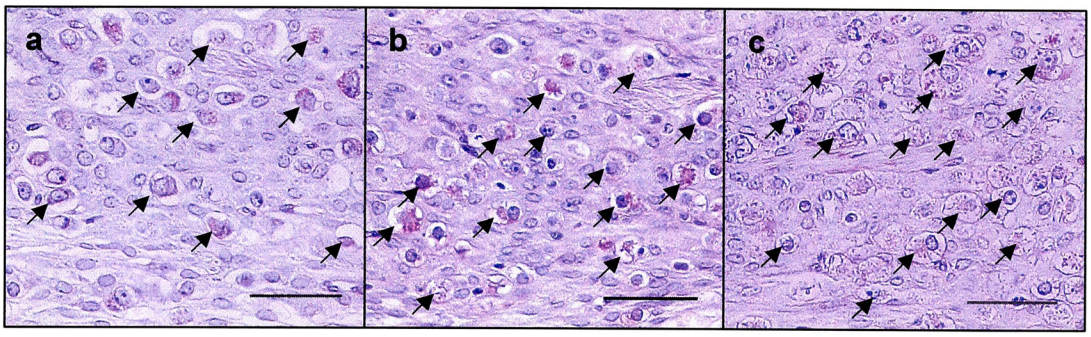


Figure 1.2 uNK cells in metrial gland at Day 12 of pregnancy. More uNK cells are found in the metrial gland of $\beta_2m^{-/-}$ (c) than B6 and $P^{-/-}$ mice (a, b). PAS staining. Bar=50 μm.

CHAPTER 2

Reproductive performance in diabetes mice with a special reference to uterine natural killer cells and placental growth factor

ABSTRACT

To determine the effect of diabetes on reproductive performance, two kinds of diabetes mice, i.e., KK/TaJcl mice with Type-II diabetes and streptozocin-induced diabetes mice with Type-I diabetes, were used in this study. Particular attention was paid to uterine natural killer (uNK) cells and placental growth factor (PlGF). The number of fetuses, the fetal weight and placental weight in both diabetes mice was significantly decreased when compared to controls. Surprisingly, uNK cells in both diabetes mice persisted in the metrial gland even at the term of pregnancy. The cell density (cells/mm^2) of uNK cells in the metrial gland of KK and STZ mice was significantly increased when compared to control mice. In addition, immunohistochemical detection revealed that anti-single-stranded DNA (ssDNA) negative reactions in the uNK cells nuclei in KK and STZ mice. Although PlGF expression in both diabetes mice was significantly decreased, PlGF protein did not change. These results show that diabetes condition affected reproductive performance, particularly uNK cell behavior, but not PlGF production. Persistence of uNK cells in both diabetes mice during late pregnancy may be due to inhibition of apoptosis by Fas/Fas-L system.

INTRODUCTION

Diabetes is a syndrome characterized by disordered metabolism and abnormally high blood sugar (hyperglycaemia), causing many complications. Among such complications, diabetes in a pregnant mother can cause reproductive abnormalities, abortion, congenital anomalies, alterations of fetal growth, neonatal morbidity and mortality [50, 51]. Diabetes is mainly classified 2 types; Type 1 diabetes is an autoimmune disease, is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to a deficiency of insulin. The main cause of this beta cell loss is a T-cell mediated autoimmune attack [52]. Type 2 diabetes is the most common form of diabetes, is a metabolic disorder that is primarily characterized by insulin resistance, relative insulin deficiency, and hyperglycemia. It is often managed by increasing exercise and dietary modification, although medications and insulin are often needed, especially as the disease progresses [53]. However, there is little information about the influences of diabetes on reproductive performance, placental morphology and immune responses at the feto-maternal interface.

It is well know that uterine natural killer (uNK) cells have critical functions in pregnancy through maintenance of decidual health, the appropriate vascularization of implantation, and promotion of placental growth [54, 55]. In addition, the uNK cells can produce several cytokines and growth factors including placental growth factor (PlGF) [56, 57]. PlGF is a member of the vascular endothelial growth factor (VEGF) [56, 58]. PlGF is homodimeric glycoprotein sharing 53% sequence homology at the amino acid level with VEGF

[58, 59], having crucial roles not only in the differentiation and migration of both endothelial and trophoblast cells [59], but also in placental growth [56, 60, 61] and fetal development during mid-late pregnancy [54]. However, it is not know whether diabetes may affect or alter the role and function of uNK cells at the feto-maternal interface. Thus, we studied here the influences of diabetes on reproductive performance by using two kinds of diabetes mice; one is KK/TaJcl mice, a mouse model with Type-II diabetes, and the other is streptozotocin-induced diabetes mice, a mouse model with Type-I diabetes. Particular attention was paid to uterine natural killer (uNK) cells and placental growth factor (PlGF).

MATERIALS AND METHODS

Experimental animals: Three groups of female mice were used; Control ICR mice, KK/TaJcl (KK) mice and streptozotocin (STZ)-induced diabetes mice. They were purchased from the Japan Clea (Osaka, Japan). Mice of 12-14 weeks old and approximately 30g Bwt were used in this study, according to the guideline of the committee on animal welfare in Yamaguchi University. Mice were housed under the condition of 12h light and 12h dark cycle, water and food ad libitum and 25 °c room temperature. STZ-induced diabetes mice were produced from ICR mice given a single intraperitoneal injection (0.1 ml) of STZ (200 mg/kg body weight) (Sigma, St. Louis) in 0.9% sodium chloride (NaCl). Six days after STZ injection, diabetes was confirmed by measurement of blood glucose using Glucose C2 (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and we designed mice

with the blood glucose of over 250 mg/dl as STZ mice. STZ mice are known as a model mouse with Type-I (insulin-dependent) diabetes. KK mice with genetic background of Type-II (insulin-independent) diabetes had blood glucose of approximately 180 mg/dl. Control ICR mice were given 0.1 ml of 0.9% NaCl. Level of blood glucose in each group tested is summarized in Table 1. Diabetic and control female mice were mated overnight with non-diabetic male ICR mice. The first day when the vaginal plug was detected was designed as Day 1 of pregnancy. The placenta and fetus were removed and rapidly weighed. The placenta was processed for morphological analysis, stained by periodic acid Schiff (PAS). The uNK cells can be readily identified due to their PAS-positive granules. The cell density (cells/mm^2) of uNK cells was calculated in the metrial gland of each mouse model. Mean values of the cell density of uNK cells from each selected field were compared among each animal model and each stage of pregnancy.

Scanning electron microscopy (SEM): All animals were anesthetized, perfused through the abdominal aorta by saline solution keeping temperature at 35-37 °C, then fixed by 2.5% glutaradehyde, and finally injected with a Mercox resin (Dainippon ink Co, Japan). The placentas were removed, put into water bath at 40-60 °C and incubated overnight. The placentas were transferred into 2-5% KOH for 3 h to 1 week at room temperature, dried up at room temperature and observed by SEM (H-3000. Hitachi Co, Japan).

Immunohistochemistry: To detect aopotosis cells by the monoclonal antibody to single-stranded DNA (ssDNA). The sections were incubated in 0.2 mg/ml saponin and 20 ug/ml Proteinase K at room temperature for 20 min, washed in

distilled water, transferred to 50% formamide (v/v distilled H_2O) in water bath at 56-60 °C for 20 min, and then transferred into ice-cold PBS for 5 min. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 min. The sections were blocked by 3% non-fat dry milk for 20 min at 37°C. The sections were incubated into 100 ul of anti-ssDNA antibody (4.5 ml of 1% non-fat dry milk in PBS to the vial containing 50 ug) (Biomol Research Laboratories Inc, USA), and then incubated into 100 µl of peroxidase-conjugated anti-mouse IgM (Vector Lab, USA), for 30 min. The samples were visualized with diaminobenzidine (DAB).

Immunoblotting: The placentas were homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT, 1.5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, complete protease inhibitor, 1% Triton X-100), centrifuged at 15,000 rpm for 30 min and supernatant was taken as the loading sample. One-hundred μg of each protein sample was resolved by 12% SDS-PAGE and transferred to PDVF membrane. The membrane was blocked with 5% nonfat dry milk powder in 0.1% tween-20 for 2 hr, incubated with anti mouse-PlGF goat IgG (dilution 1:300, Santa Cruz Biotechnology, CA, USA) overnight at 4°C, and then incubated by anti-goat IgG (Vector Lab, USA). Followed by incubation with VECTASTAIN ABC reagent (Vector Lab, USA), the samples were visualized with DAB. Beta-actin protein was used as an internal standard.

Quantitative real-time PCR: Total RNA was extracted from placentas using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). One μg of total RNA was utilized for reverse transcription to cDNA. For quantification of PlGF expression, the SYBR Green PCR Master Mix (Applied Biosystems, USA) was used. PlGF primers are designed as forward (5'-TGCTGGTCATGAAGCTGTTC-3') and

reverse primer (5'-GGACACAGGACGGACTGAAT-3') [60]. Data were quantified using StepOne Software for StepOne and StepOnePlus Real-Time PCR system (Applied Biosystem, USA).

Statistical analysis: Student's *t*-test was used to determine significance of differences between groups. $P<0.01$ was considered to be significant.

Table 2.1 Level of blood glucose in control, KK and streptozotocin mice used in this study.

Experiment group	control	KK	STZ
Blood glucose (mg/dl)	114.4±41.9 (22)	182.5±18.1* (26)	425.3±76.2*# (25)

Values are the mean ± SD. Parentheses=number of female mice.

*P<0.01 vs control, #P<0.01 vs KK.

RESULTS

The body weight in STZ mice was significantly lower than that of the controls, and also the fertile rate of STZ mice was very low; 16 STZ mice out of 42 STZ mice with a vaginal plug (approximately 38.1%) were non-pregnant, while all the control and KK mice with a vaginal plug became pregnant. Gross anatomy of the uterus on Day 12 of pregnancy, showing the number of fetuses in both KK and STZ mice is tended to be decreased when compared to controls mice (Fig. 2.1). The number of fetuses in KK and STZ mice was significantly decreased when compared to control mice. Although STZ mice had more fetuses than KK mice, there was no significant difference in the fetal numbers between 2 groups (Fig. 2.2, a). The fetal weight (Fig. 2.2, b) and the placental weight (Fig. 2.3) in both diabetes mice were significantly decreased when compared to controls mice on Day 12 and Day 18 of pregnancy, except in KK mice was tended to be higher than control mice on Day 12 of pregnancy, but were not significant different during 2 groups. The morphology of placentas on Day 12 in KK and STZ mice was different from control mice, especially the decidua basalis in KK and STZ mice was larger in area than that of control mice (Fig. 2.4, a-c). The metrial gland and the spiral artery showed the same morphology in 3 groups (inset of Fig. 2.4, a-c). The spiral artery in STZ was the same structure to that of control mice on Day 12 of pregnancy (Fig. 2.5).

On Day 18 of pregnancy, the uNK cells in metrial gland were degenerated in control mice, while uNK cells in KK and STZ mice still remained in the metrial gland (Fig. 2.4, d-f). The cell density (cells/mm^2) of uNK cells in the metrial gland of KK

and STZ mice was significantly increased when compared to control mice on Day 18 of pregnancy ($P<0.01$) (Table. 2.2). The immunohistochemical detection revealed that anti-ssDNA positive reactions were seen in the uNK cell nuclei in control mice, but that negative reactions in the uNK cell nuclei in KK and STZ mice (Figs. 2.6, a-c).

The Western blot analysis showed that PlGF protein in both diabetes mice did not change when compared to the control mice on Day 12 and Day 18 of pregnancy (Fig. 2.7, a and 2.8, a), although the real-time PCR analysis indicated that PlGF expression in STZ was significantly decreased on Day 12 of pregnancy (Fig. 2.7, b), and that PlGF expressions in both diabetes mice were significantly decreased on Day 18 of pregnancy (Fig. 2.8, b).

Table 2.2 The cell density (cells/mm^2) of uNK cells in the metrial gland on Day 18 of pregnancy. The cell density of uNK cells in KK and STZ is increased significantly comparing to control mice.

Day	CONT (cells/mm^2)	KK (cells/mm^2)	STZ (cells/mm^2)
D18	277.78 ±160.03	716.67 ±156.12*	666.67 ±171.39*
	(4)	(4)	(4)

Values are the mean ± SD. Parentheses=number of female mice.

*P<0.01 vs control.

DISCUSSION

This study clearly showed that diabetes condition during pregnancy induces fetal loss and decreases of both the placental and fetal weights. It is known that diabetes in pregnant mothers causes abnormalities of intrauterine metabolic and hormonal milieu [50], so that fetal loss occurs in those mothers [50, 51, 62]. The spiral artery is of great importance to blood supply to the placental labyrinth. The study using uNK cell-deficient mice has clearly indicated that uNK cell-derived IFN-γ is essential to triggering pregnancy-induced spiral artery modification [63, 64]. INF-γ induces gene transcription in uterine stromal cells, vascular smooth muscle cells and endothelial cells of the spiral artery [64]. IFN-γ mRNA has been documented in healthy human and murine implantation sites [65, 66]. In the human the relationship between extravillous cytotrophoblasts (EVT) and spiral arteries is thought to be essential to successful spiral artery remodeling. However, in mice the spiral artery remodeling is independent of trophoblast [67]. My result shows that the spiral arteries showed the same morphology and structure in the diabetes and control groups, suggesting that diabetes condition could not affect the spiral artery remodeling, although diabetes is known to affect the vascular architecture in various tissue [62]. Nevertheless, since all the spiral arteries observed in this study were in the placenta with an alive fetus, we cannot rule out the possibility that diabetes affected the activity of INF-γ secretion by uNK cells in other regions.

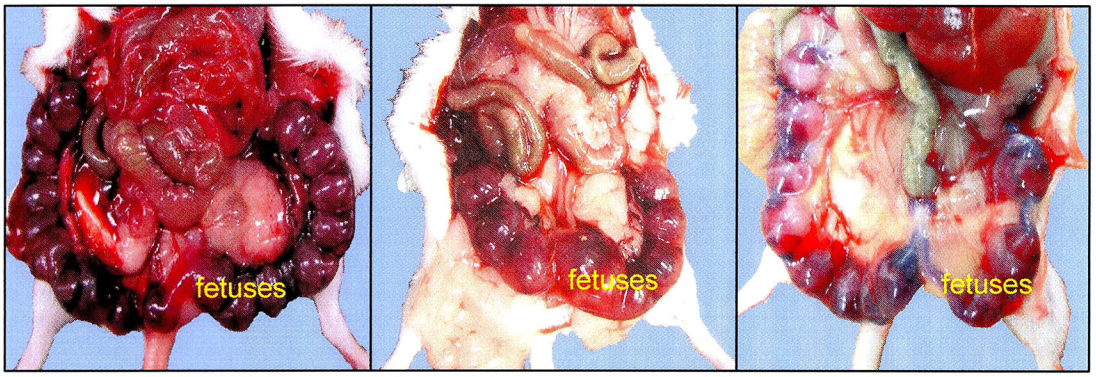
Surprisingly, this study clearly indicated that the uNK cells still remained in the metrial gland on Day 18 of pregnancy in KK and STZ mice, then we was

detected apoptosis by ssDNA were seen negative reactions in the uNK cell nuclei in KK and STZ mice. Normally, uNK cells are present at the metrial gland and decidua basalis until Day 15 of pregnancy, then degenerate during late gestation, finally disappear from the endometrium near the full term of pregnancy [6, 18]. Apoptosis by Fas/Fas-ligand (Fas-L) system can account for this disappearance of uNK cells in peripartum uteri [13, 68]. Indeed, it is reported that, in *lpr/lpr* mice deficient in *Fas* antigen, uNK cells persist until the full term of pregnancy [68]. Thus, diabetes may affect Fas/Fas-L system during pregnancy, so that uNK cells in the pregnant diabetes mice could persist on Day 18 of pregnancy. Similarly, since the decidual cells are also known to undergo apoptosis through Fas/Fas-L system during late pregnancy [56, 69, 70], it is likely that the decidua basalis in the pregnant diabetes mice could have larger area on Day18 of pregnancy.

PlGF is a N-glycosylated homodimeric protein, potentiating the angiogenesis activity of VEGF [71]. Prominent production of PlGF by trophoblast and the presence of Flt-1 receptors on trophoblast suggest that PlGF may act in an autocrine manner to modulate the trophoblast differentiation [72]. The uNK cells can also produce PlGF [54, 55, 60, 61], suggesting that uNK cells may support the differentiation of endothelial cells together with VEGF. The present study also showed that PlGF protein did not change under diabetes condition, although PlGF mRNA expression was decreased. This result would correspond to the fact that the spiral artery did not change nor alter in morphology under diabetes condition, suggesting that diabetes could not affect PlGF production. Abnormally abundant uNK cells may compensate the lack of PlGF secreted by trophoblast. Indeed, it is reported that uNK cells in IL-2 receptor β-chain overexpressed transgenic mice

(Tg2Rβ) [69, 70] and in IGF-I overexpressed mice [73], could alter in quality and behavior due to unusual condition. Similar changes or alterations on uNK cells may occur under diabetes condition. Further studies on the molecular basis are needed to determine the alterations of uNK cells function and cytokine networks under diabetes condition.

In conclusion, the present study shows that diabetes condition affects reproductive performance, apoptosis by Fas/Fas-L system occured on uNK cells during late pregnancy and particularly uNK cell behavior, but not PlGF production.



CONT KK STZ

Figure 2.1 Gross anatomy of the uterus on Day 12 of pregnancy, showing that the number of fetuses in both KK and STZ mice is tended to be decreased when compared to controls mice.

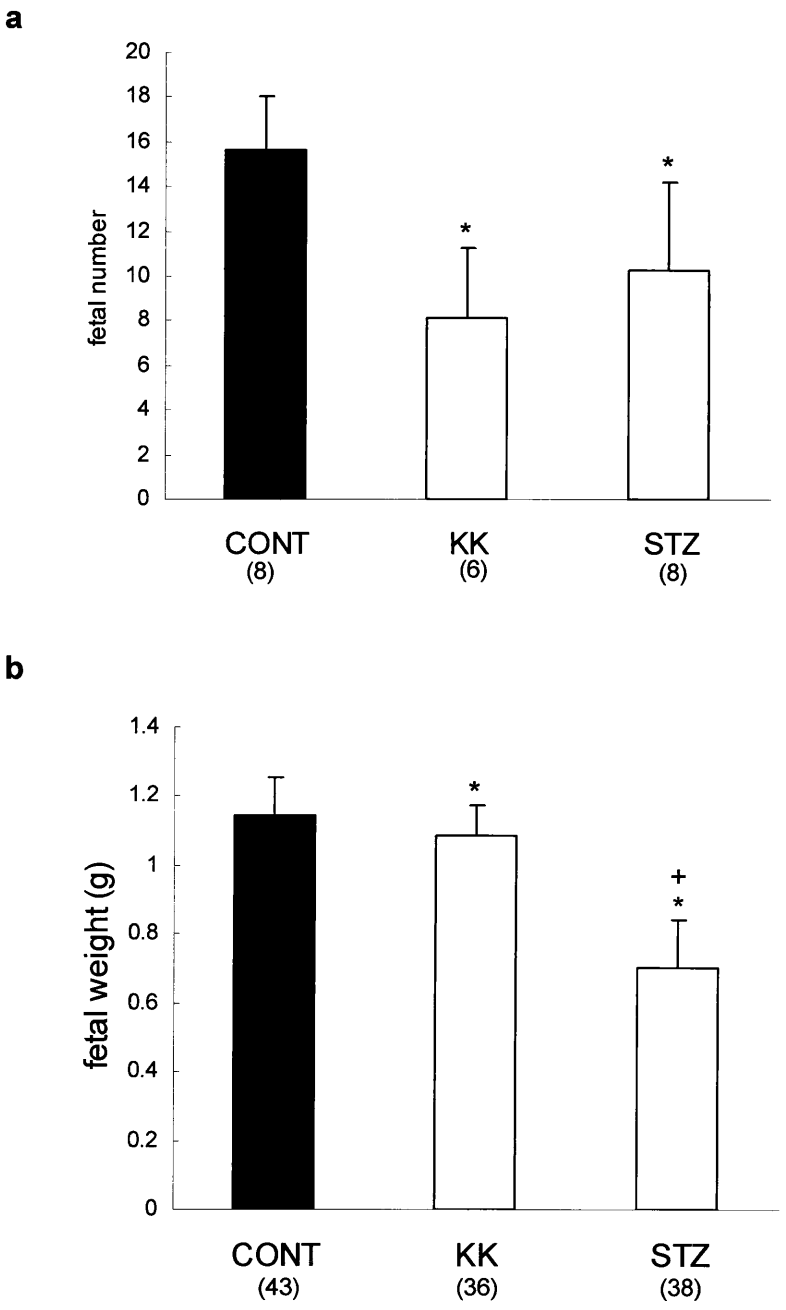


Figure. 2.2 The number of fetuses (a) and the fetal weight (b) on Day 18 of pregnancy in control, KK and STZ mice. Each column represents the mean ± S.D. Parentheses in (a)= number of mothers, parentheses in (b)= number of fetuses. *P<0.01 vs control, $^{+}$P<0.01 vs KK.

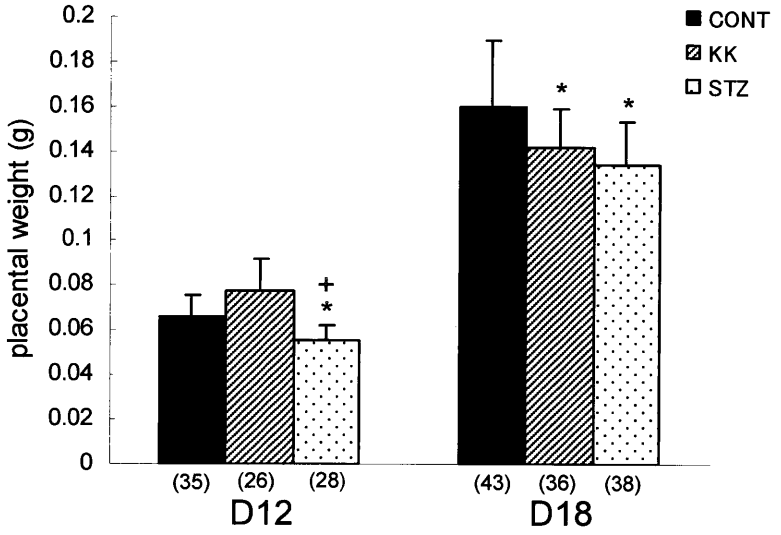


Figure 2.3 The placental weight on Day 12 and Day 18 of pregnancy. Each column represents the mean ± S.D. Parentheses=number of placentas. *P<0.01 vs control, +P<0.01 vs KK.

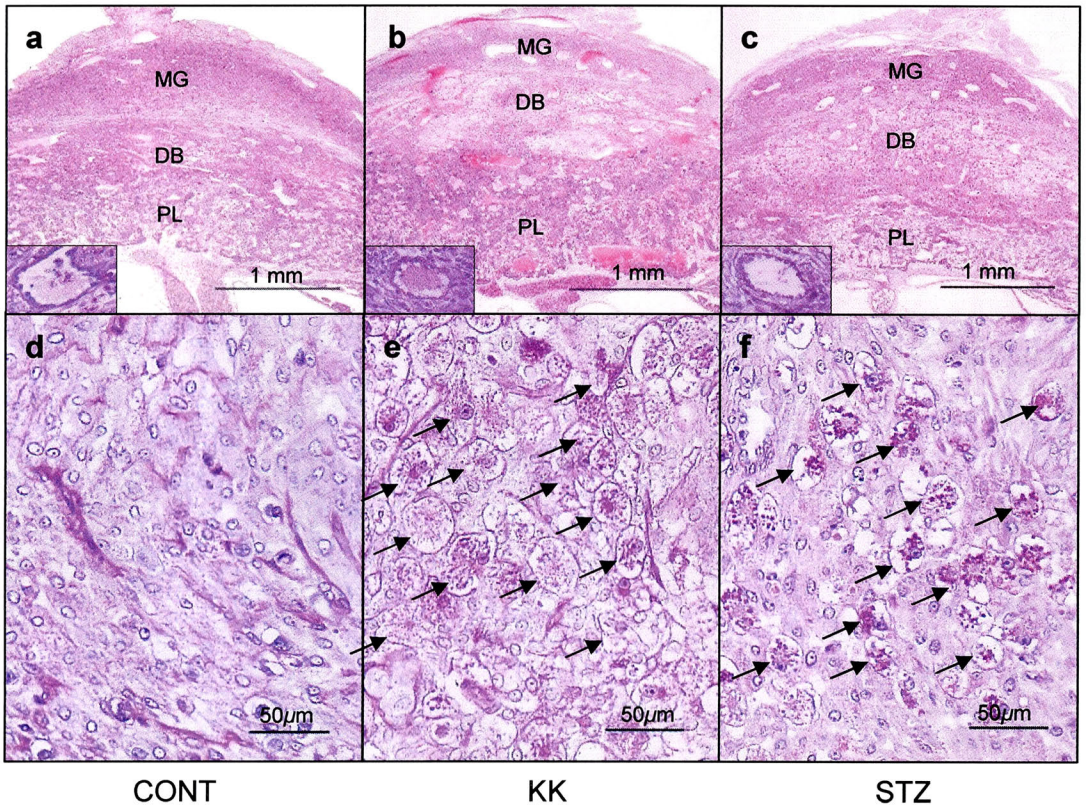
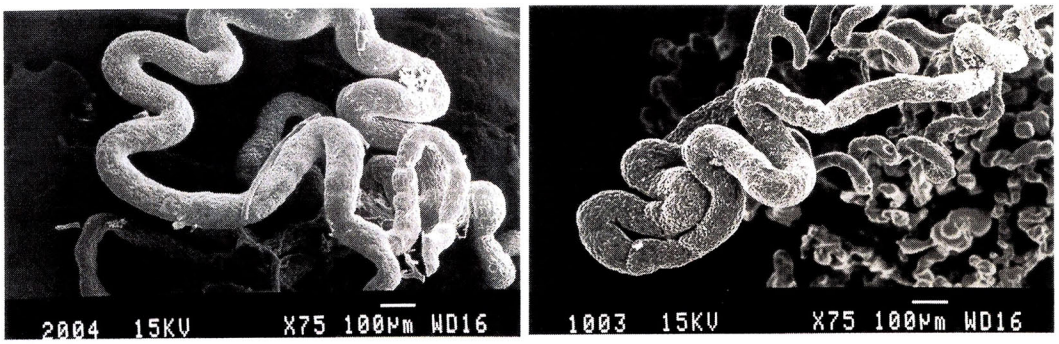


Figure 2.4 Placentas in each group (a-c) on Day 12 of pregnancy. Inset: the spiral artery of Day 12 of pregnancy, showing the same structure in 3 groups. uNK cells (arrows) in each group on Day 18 of pregnancy (d-f), showing that uNK cells still remain in the KK (e) and STZ mice (f) in the metrial gland. DB= decidua basalis, MG= metrial gland, PL= placental labyrinth. PAS stain.



CONT STZ

Figure. 2.5 Scanning electron microscopy of corrosion casting specimens in the maternal placenta on Day 12 of pregnancy. The spiral artery in STZ mice is the same structure to the control mice.

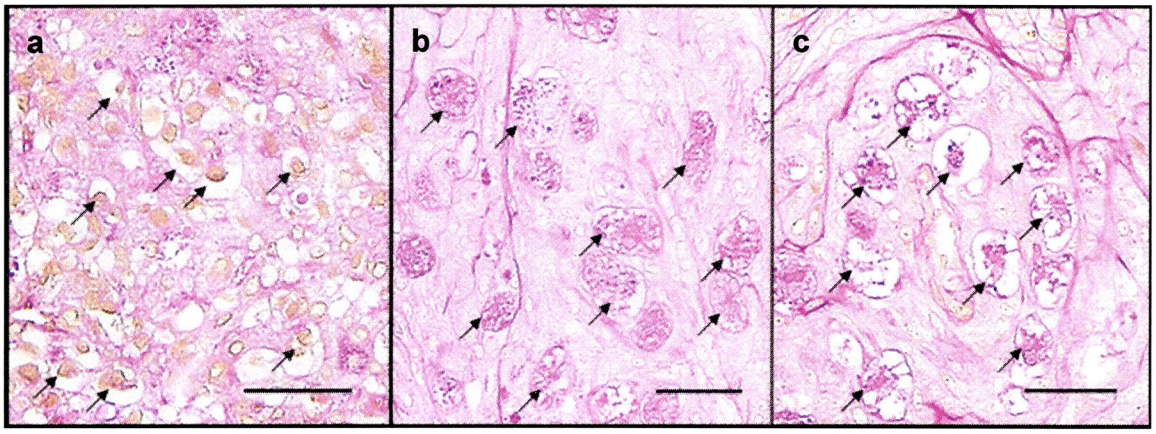
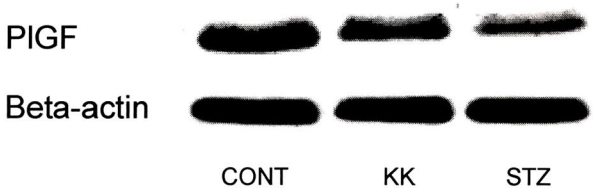


Figure 2.6 Immunohistochemical detection of apoptosis cells by the anti-ssDAN antibody on Day 18 of pregnancy. uNK cells in the control (a) are possitive for the anti-ssDNA antibody, while those in both diabetes mice (b, c) negative for the anti-ssDNA antibody. arrows= uNK cell, Bar= 50 μm.

a



b

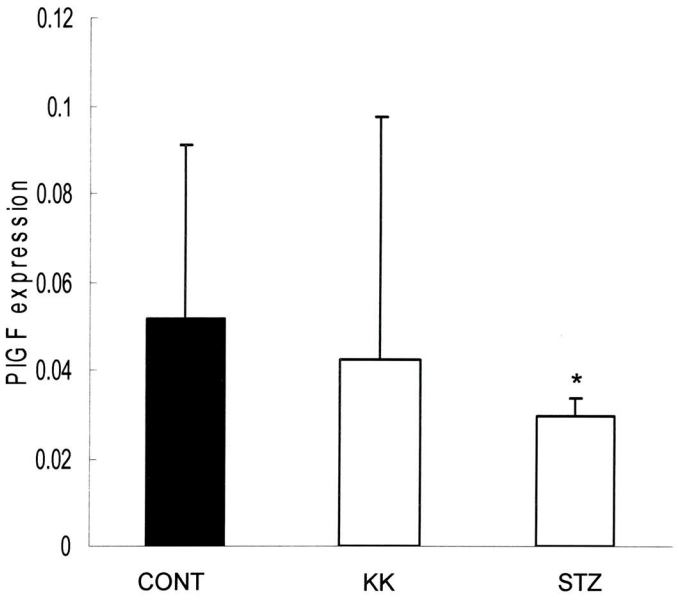


Figure 2.7 Western blot analysis (a) and real-time PCR analysis (b) in placental tissues of control, KK and STZ mice on Day 12 of pregnancy. PIGF protein does not change, but its expression in STZ nice is significantly decreased when compared to control mice. The number of placentas tested is 6 in each. *P<0.01 vs control.

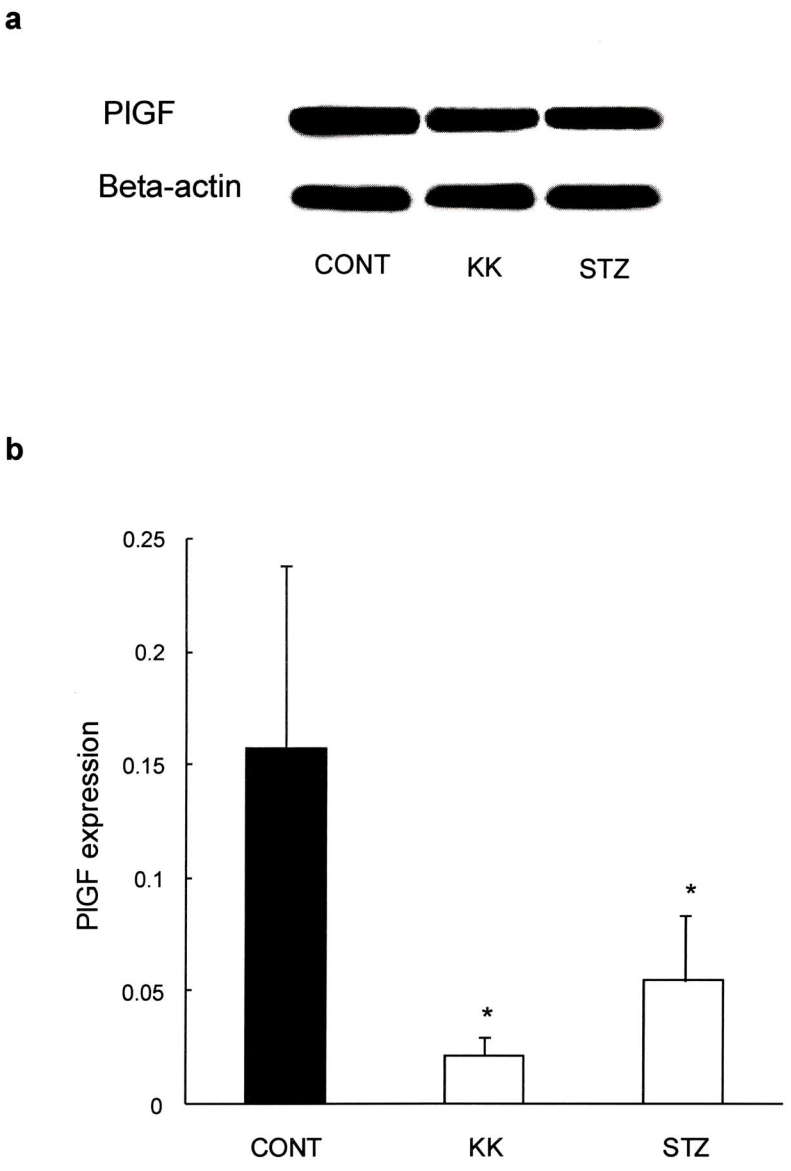


Figure 2.8 Western blot analysis (a) and real-time PCR analysis (b) in placental tissues of control, KK and STZ mice on Day 18 of pregnancy. PIGF protein does not change, but its expression is significantly decreased when compared to control mice. The number of placentas tested is 6 in each. *P<0.01 vs control.

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SUMMARY

Studies on differentiation of uterine natural killer cells at the feto-maternal interface in perforin-deficient, β_2-microglobulin-deficient and diabetes mice

Natural killer (NK) cells are a part of the innate immune system and defend against allogenic cells under stress such as virally infected or tumor cells. NK cells are present in the uterus during pregnancy, called uterine NK (uNK) cells. They are increased in number, reach at the maximum level until Day 12 of pregnancy, and finally disappear from the uterus in the near term due to apoptosis. The uNK cells acquire cytoplasmic granules that contain perforin, serine proteases and phosphatases, but also secrete various cytokines and growth factors, e.g., IL-1, LIF, CSF-1, EGF and PlGF, which are useful for pregnancy success. uNK cells are considered to play an important role in placental growth, angiogenesis and immune responses at the feto-maternal interface.

The first chapter is on uNK cells in perforin and β_2-microglobulin deficient mice. The uNK cells partly share common functions with peripheral NK cells, which are not restricted by major histocompatibility complex (MHC) class I antigen, and have cytotoxic activity dependent on perforin. Our result showed that the cell population of uNK cells in $P^{-/-}$ mice was tended to be higher than B6 mice on Day 10 and Day 12 of pregnancy. The cell population of uNK cells in the decidua basalis of $\beta_2m^{-/-}$ mice was significant increased when compared to B6 mice on Day 10 of pregnancy. The cell population of uNK cells in the metrial gland of $\beta_2m^{-/-}$

mice was significantly increased on Day 12 of pregnancy comparing to B6 and $P^{-/-}$ mice. On the other hand, the cell population of uNK cells in the decidua basalis of $\beta_2m^{-/-}$ mice was tended to be lower than B6 and $P^{-/-}$ mice. These results indicate that β_2m may be involved in proliferation of uNK cells in the metrial gland, and that β_2m may affect the maturation of uNK cells in the decidua basalis.

The second chapter is on reproductive performance in diabetes mice with a special reference to uNK cells and placental growth factor (PlGF). Diabetes is a syndrome characterized by disordered metabolism and hyperglycemia, causing abnormalities of intrauterine metabolic and hormonal milieu during pregnancy, but there is little information about the influences of diabetes on reproductive performance, placental morphology and immune responses at the feto-maternal interface. Thus, we studied here the influences of diabetes on reproductive performance by using two kinds of diabetes mice, i.e., KK/TaJcl (KK) mice with Type-II diabetes and streptozotocin-induced diabetes (STZ) mice with Type-I diabetes. The number of fetuses, the fetal weight and placental weight in both diabetes mice were significantly decreased when compared to control mice. Surprisingly, uNK cells in both diabetes mice persisted in the metrial gland even at the near term of pregnancy, while those in controls were not observed. The cell density (cells/mm^2) of uNK cells in the metrial gland of KK and STZ mice was significantly increased on Day 18 when compared to control mice. In addition, the immunohistochemical detection revealed that the uNK cell nuclei in KK and STZ mice were negative for anti-ssDNA antibody. Persistence of uNK cells in both diabetes mice during late pregnancy may be due to inhibition of apoptosis by Fas/Fas-L system. Although PlGF expression in both diabetes mice was

significantly decreased, PlGF protein did not change. These results show that diabetes condition affected reproductive performance, particularly uNK cell behavior, but not PlGF production.

Further studies on the molecular basis are needed to determine the differentiation and function of uNK cells and the relation between uNK cells and cytokine networks.

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LIST OF PUBLICATIONS

1. **Thanmaporn Phichitrasilp**, Eiichi Hondo, Ken Kusakabe, Yumiko Nakagawa, Kaori Ihara, Shoichi Wakitani, Yasuo Kiso; Uterine natural killer cells in perforin and β_2-microglobulin deficient mice.

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(Chapter 1)

2. **Thanmaporn Phichitrasilp**, Eiichi Hondo, Worawut Rerkamnuaychoke, Shoichi WAKITANI, Makoto Sugiyama, Jumpei Terakawa, Yasuo Kiso; Reproductive performance in diabetes mice with a special reference to uterine natural killer cells and placental growth factor.

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(Chapter 2)