

Research Article

The Role of HLA-C and NK Cells in Fetal Growth Restriction***Peran HLA-C dan Sel NK pada Pertumbuhan Janin Terhambat***

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Abstract

Objective: To determine the role of HLA-C and NK cell expressions in fetal growth restriction (FGR).

Methods: A cross sectional study design was used. This study was conducted at the Obstetrics and Gynecology Department of Dr. Moewardi General Hospital, Surakarta, its affiliated hospitals, and at the Pathological Anatomy Laboratory of the Faculty of Medicine, University of Sebelas Maret Surakarta. A total of 40 samples were included in this study. The samples consisted of 20 normal pregnancies and 20 pregnancies with FGR. HLA-C expression in the trophoblast and NK cells in decidua of the subjects who met the inclusion and exclusion criteria were examined using immunohistochemical method and statistical analysis with T test.

Results: The mean expression of HLA-C in the trophoblast in the pregnant group with FGR was 9.02 ± 1.30 , normal pregnancy was 7.96 ± 0.97 , $p=0.01$ ($p<0.05$). The mean expression of NK cells in decidua of pregnancy with FGR was 10.59 ± 2.11 , normal pregnancy was 0.91 ± 8.18 , with $p=0.00$ ($p<0.05$).

Conclusion: The expressions of HLA-C in trophoblast and NK cells in decidua of pregnancy with FGR were higher compared with those of normal pregnancy.

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Keywords: fetal growth restriction, HLA-C, NK cells

Abstrak

Tujuan: Untuk menentukan peran ekspresi HLA-C dan sel NK pada pertumbuhan janin terhambat (PJT).

Metode: Penelitian ini menggunakan desain studi potong lintang. Penelitian dilakukan di bagian Obstetri dan Ginekologi RS Dr. Moewardi Surakarta dan rumah sakit-rumah sakit jearingnya, dan Laboratorium Patologi Anatomi Fakultas Kedokteran Universitas Sebelas Maret Surakarta. Besar sampel adalah 40, terdiri dari 20 kehamilan normal dan 20 kehamilan dengan PJT. Subjek yang memenuhi kriteria inklusi dan eksklusi dilakukan pemeriksaan ekspresi HLA-C di trofoblas dan sel NK di desidua dengan metode imunohistokimia dan dilakukan analisis statistik dengan uji t.

Hasil: Rerata ekspresi HLA-C di trofoblas pada kelompok hamil dengan PJT $9,02 \pm 1,30$, kehamilan normal $7,96 \pm 0,97$, dengan $p=0,01$ ($p<0,05$). Rerata ekspresi sel NK di desidua pada hamil dengan PJT $10,59 \pm 2,11$, kehamilan normal $0,91 \pm 8,18$, dengan $p=0,00$ ($p<0,05$).

Kesimpulan: Ekspresi HLA-C di trofoblas dan Sel NK di desidua hamil dengan PJT lebih tinggi bila dibandingkan dengan kehamilan normal. [Maj Obstet Ginekol Indones 2017; 5-3: 142-148]

Kata kunci: HLA-C, pertumbuhan janin terhambat, sel NK

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INTRODUCTION

Fetal Growth Restriction (FGR) is still a major health burden as it is associated with increased neonatal morbidity and mortality.¹ It occurs in 10 - 15% of pregnant women and is usually diagnosed during pregnancy, while some fetuses were diagnosed after birth.² FGR incidence varies from country to country. In developing countries, its incidence is 25% higher compared to developed countries, which is amounted to 4-8%, where the highest prevalence is in Asia (75%), especially in Southeast Asia and followed by Africa (20%), whereas in Latin America is 5%.^{2,3} The prevalence

of FGR in 4 fetomaternal centers in Indonesia in 2004-2005 was 4.4 %, with the highest prevalence (6.44%) were reported in dr. Sardjito Central General Hospital Yogyakarta. The perinatal mortality rate were 7-8 times higher than normal babies. Approximately 26% or more stillbirth events were associated with FGR.⁴

FGR is defined as a condition where the fetal weight is below 10 percentile of gestational age or less than 2 standard deviations below the mean of gestational age.³ It can also be diagnosed when abdominal circumference is less than or equal to 5 percentile or femur length (FL) / abdominal circumference (AC) >24 .⁵

The identification of FGR is important. It is essential to know the risk factors in FGR, which include maternal cause and fetal cause, and placental factor. Intrauterine growth determines the perinatal, postnatal, and adulthood development. FGR is often associated with an increased risk that develops to metabolic diseases in the adulthood including hypertension, diabetes mellitus, obesity, dyslipidemia, and metabolic syndrome.²

The success of pregnancy process depends on many factors, one of which is immune adaptation of the pregnancy where fetal maternal immune tolerance is very important. Fetus as semi-allogeneic can cause immune maladaptation that will produce poor perinatal outcomes such as FGR to mother.⁶

The cause of FGR among other is the disturbance of uteroplacental blood flow that is now often associated with a condition where intolerance interruption on maternal immune system occurs and results in disruption of trophoblast invasion into decidua during placentation process, in the form of inadequate invasion that results in disruption invasion of placenta which will lead to poor uteroplacental perfusion resulting in the occurrence of pregnancy complications such as FGR.⁷ Plasma cells' infiltration in the decidua will cause decidua vasculopathy, where macrophage infiltration participate in extravillous interstitial trophoblast cell death by apoptosis which causes leukocytes to surround or attack the placental villi. This portrait was found in FGR placenta.⁸

Histocompatibility antigen is a gene that encodes a protein that affects the tissue transplant compatibility. The antigen is divided into two systems, namely minor histocompatibility antigen and major histocompatibility complex (MHC). MHC gene is known as Human Leucocyte Antigen (HLA) in humans. MHC is divided into regions that encode three different classes of HLA molecules, namely HLA-I, II, and III which are different structurally and functionally. Classic HLA molecule is encoded by three loci, namely HLA-A, HLA-B, and HLA-C which are very polymorphic.⁹

The expression of class I non-classic HLA molecules is limited to specific certain type of cell, but is generally expressed in placental trophoblast cells where HLA-G has the strongest expression while HLA-E has medium expression. Both class II HLA molecules and class I classic HLA molecules are not expressed in the placenta, with the excep-

tion of the existence of a weak expression of classic HLA-C molecule. This makes the function of non-classical HLA molecules and classical HLA-C molecules interesting, in line with mother's acceptance to semi-allogeneic fetus during pregnancy. High expression of HLA-G and HLA-E will bind to Natural Killer cells (NK cell) receptor inhibitor that will causes prevention on NK cells to secrete pro-inflammatory cytokines. Whereas, if high levels of HLA-C inhibit NK cell function that causes the secretion of angiogenic and mitogenic endothelial ineffective, placentation and vascular remodeling will be interfered.¹⁰

Human Leucocyte Antigen-C molecule is a heterotrimer composed of glycoproteinsized 45 kD, a 12 kD β 2m invariant, and a binding peptide. Cell surface of proteins is highly polymorphic, with 1016 known all that encode 750 proteins. HLA-C has expression level on low cell surface (i.e. 10% of the HLA-A or B). The main role of the HLA-C is a ligand for KIR. NK cell receptor for HLA-C is a member of a multigene Killer Inhibitor Receptor (KIR) family. Locus of HLA-C is dimorphic and both groups of HLA-C interact with different KIR. NK cell receptors tend to aim to HLA-C introduction in the uterus. All women express KIR in both HLA-C all groups, and because HLA-C is polymorphic, the interaction between non-self paternal fetus HLA-C and maternal decidua NK cell KIR occurs during pregnancy progresses. Every pregnancy will involve different combinations of HLA-C paternal fetus and maternal KIR, therefore it is possible that some combinations are less than optimal for implantation and ultimately lead to failure of pregnancy. It becomes the basis of pathogenesis of important diseases in pregnancy, such as the fetal growth retardation. Trophoblast cells that invade the maternal uterine tissue express HLA-C at high levels in a stable conformation of β 2. HLA-C with trophoblast cells perform direct contact with maternal NK cells that express KIR at the site where placentation take place.^{6,7}

Decidua NK cells affect trophoblast ability to invade and alter spiral arteries and regulates blood flow into the intervillous chamber by regulating the transformation of trophoblast arteries. NK cells are a type of lymphocyte with a large size that contains many granules in the cytoplasm (LGL). The cells function to kill cells, produce cytokines, and produce growth factors such as VEGF and PLGF.⁶ Decidua NK cells express specific KIR receptors that bind HLA-C. The binding of HLA-C on KIR

inhibitory signals as well as activating signals of NK cells, depending on the type of the KIR. Decidua NK cells express specific KIR that binds HLA-C on a higher level than peripheral NK cells. Some ligands of the receptor are class I HLA molecules and these interactions can activate KIR/KIR2DS1 or inhibit KIR/KIR2DL1.^{11,12} NK cells express receptors with different complement that have a chain either activating or inhibiting signal. CD 56 Bright uterine NK cells express diverse receptors including immunoglobulin receptor killer which is known to recognize HLA-C.⁶

All HLA-C all types can be grouped into two main KIR epitopes, C1 and C2. C1 group is ligand for receptor of KIR2DS2 NK cell activator that is associated with increased birth weight, and C2 group is ligands for receptor of KIR2DL1 NK cell inhibitor that is related with pregnancy disorders as abnormal placentation.¹¹ Fetus vascular supply control relies on the interaction between maternal KIR on decidua NK cells and paternal HLA-C expressed by trophoblast. HLA-C has a relationship with the development of fetal growth retardation. The combination of maternal decidua NK cells, namely maternal KIR with HLA-C2 fetal genotype (HLA-C receptor inhibitor), can increase the risk of fetal growth retardation. When maternal AA genotype coincided with HLA-C2 genotype heterozygous or homozygous fetus, decidua NK cells expressing KIR genotype AA will be inhibited, because haplotype A has KIR receptor inhibitor (KIR2DL1). This receptor can inhibit cytokines secretion from NK cells that are believed to facilitate the normal trophoblast invasion. If NK cells are not activated, these cells potentially cause inadequate trophoblast invasion, which resulted in the failure of placentation and lead to fetal growth retardation.⁷ C1 does not cause the same inhibition on KIR haplotype A that is caused by weak binding of HLA-C1 on the corresponding inhibitor KIR2DL2/3 compared to HLA-C2 binding on KIR2DL1. Therefore, C1 does not inhibit NK cell on the same level with the C2. When C2 coincided with B KIR haplotype, then activated KIR haplotypes will neutralize the inhibition signals.¹¹

This study is aimed to analyze the expression of HLA-C and NK cells in FGR, and is expected to be a predictor of FGR occurrence so as to prepare FGR therapy.

METHODS

A cross sectional study design was used. This study was conducted at the Obstetrics and Gynecology department of Dr. Moewardi General Hospital Surakarta and its affiliated hospitals. HLA-C and NK cells expression was assessed using immunohistochemistry method performed in the Pathological Anatomy Laboratory, Faculty of Medicine, Universitas Sebelas Maret Surakarta.

The study was conducted on pregnant women with FGR (fetal weight $\leq 10^{\text{th}}$ percentile or fetal abdominal circumference ≤ 5 percentile or the ratio of femur length/abdominal circumference (FL/AC) >24) on ultrasound examination and normal pregnant women to analyze HLA-C expression in trophoblast and NK cells in the decidua. The sample size is determined based on Multi formula that for each group contains 20 people.¹³ The inclusion criteria were mothers aged 20 - 35 years, 34 - 40 weeks of gestational age in pregnancy with FGR cases, and 37 - 40 weeks of gestational age in normal pregnancy, single fetus, remind alive, and have willingness to participate in the research. Exclusion criteria were mothers with diabetes mellitus, kidney disease, heart disease, liver disease, chronic hypertension, infectious disease and smoking, fetal death in utero, fetus with major congenital abnormalities. Both groups underwent examination on the expression of HLA-C and NK cells in extravillous trophoblast which expressed HLA-C and decidua NK cells accumulated in the surrounding area.

Immunohistochemistry Examination

Tissue slide preparation was done in several steps, and was initiated by taking cotyledon tissue sample from the placenta (trophoblast). Trophoblast was fixed using buffered formalin solution for 8 hours or up to 48 hours, and then inserted into tissue cassette and soaked in 50%, 70%, 80%, and 95% alcohol solution, followed by cleaning using xylol 3 times for 60 minutes or each preparation. During the embedding process, each preparation was immersed in liquid paraffin with a melting point of 58°C at a temperature of 45°C in an incubator for 18 hours, then paraffin blocks were made and affixed to the holder and were cut 4 - 5 microns thick with a rotary microtome. The tissues were laid on poly L-lysine slides. Glass objects resulted from paraffin blocks were immersed in xylol 4 times for 5 minutes each. Next, rehydration

was applied using series alcohol (absolute, 95 70%) and then rinsed with the distilled water (H₂O) for 5 minutes. The slides were washed with PBS pH 7.4, twice for 5 minutes and was dripped with endogenous peroxidase methanol H₂O₂ 0.3% for 15 minutes then rinsed with running water for 5 minutes and rinsed again with distilled water for 5 minutes. Next repeated rinse was applied using PBS 2 x 5 minutes, and followed with retrieval using Tris EDTA pH buffer 9 in microwave or decloing chamber and then washed with PBS 2 x 5 minutes and dripped with snifer background when it was already cool. It must be drained before dripping with prepared monoclonal antibodies HLA-C and incubated at 4°C for 18 hours. Subsequently, it was washed again with PBS 2 x 5 minutes, and afterwards, dripped with secondary antibody (universal trekkielibk) for 15 minutes and washed with PBS 2 x 5 minutes, then followed with dripping streptavidin for 10 minutes, washed with PBS 2 x 5 minutes after administration of peroxidase substrate diethyl amino benzene for 10 minutes. Another wash was given with water for 5 minutes, and then dripped with hematoxylin for 2 minutes and rinsed with running water for 5 minutes and mounted using entelan and covered with glass cover. Finally, light microscope was used to observe. The expression of HLA-C and NK cells were shown with a bluish tint for weak positive, golden yellow for moderate positive, and brown for strong positive on the trophoblast. Observations were made as much as 9 field of view. Trophoblast cell number was calculated based on the expression intensity, and the percentage was made from whole number of cells. The percentage obtained was converted into numbers and calculated according to histological score formula. Scoring of HLA-C and NK cell image was conveyed as Histology Score (HS) which was done based on the formula: HS = (PK x IK) + (PS x IS) + (PL x IL) + (PN x IN)

Formula explanation:

P = Percentage N = Negative L = Weak
K = Strong I = Intensity S = Medium

The HS of HLA-C and NK cells was classified as follows. The percentage/percentage of number of cells: 0-25% = negative; 26 - 50% = weak positive; 51-75% = moderate positive; 76-100% = strong positive; for the qualitative meaning: 0.00 to 3.75 = negative; 3.76 to 7.50 = weak positive; 7.51 - 11.25 = moderate positive; 11.26 to 15.00 = strong positive.^{14,15}

Data were analyzed using T test. Reagent used for the expression of HLA-C was HLA-C (H-5) antibody reagent sc-166 134 Santa Cruz Biotechnology, Inc. Meanwhile, the reagent used for the expression of NK cell was rabbit polyclonal antibody Anti KIR2DL1 bs-2419R BIOSs, Inc., and KIR was used to see the expression of NK cell receptors. Observation on intensity of the color was done by using Olympus CX-21 series light microscope, 400x magnification on 9 field of view. The number of HLA-C and NK cells on trophoblast was calculated based on the intensity of the reddish-brown color and the percentage. The higher the histological score, the stronger the expression.¹⁵

Ethical Clearance

Ethical Clearance was obtained from EIK Health Research Commission Dr. Moewardi General Hospital - Faculty of Medicine, University of Sebelas Maret Surakarta Number: 461/VI/HREC/2015 dated June 25, 2015.

RESULTS

Table 1. Characteristics of Research Subjects

Variable	N	Min	Max	Mean	SD
Maternal age (years)	40	20.00	35.00	29.50	4.06
Gestational age (weeks)	40	37.00	40.00	38.87	0.88
Birth weight (grams)	40	1800.00	3100.00	2421.25	504.32
Body mass index	40	17.31	24.15	19.89	1.83

Characteristics of the research subjects showed the average age of pregnant women was 29.50±4.06 years, gestational age was 38.87±0.88 weeks, birth weight was 2421.25±504.32 g, BMI was 19.89±1.83.

The mean expression of HLA-C in pregnancies with FGR was higher (9.02±1.30), compared with normal pregnancy (7.96±0.97), p=12.01 (<0.05). Average NK cells (KIRDL1) in pregnancy with FGR were higher (10.59±2.11), compared to normal pregnancies (8.18±0.91) with p=0.00 (<0.05).

HLA-C expressed in trophoblast in brown color on the core which extends into the cytoplasm of trophoblast extravillous. From the immunohistochemical method reading, HLA-C mean score on

trophoblast tissue in FGR group was higher when compared with normal pregnancy group. HLA-C expression on trophoblast was assessed using Olympus CX-21 series light microscope with a magnification of 400 times.

The color of expressed decidual NK cells in cytoplasm was red. The mean score of NK cell expression on FGR group tissues was higher was compared to the normal pregnancy group.

Expression of NK cells in the decidua by Immunohistochemistry method using Olympus CX-21 series light microscope with magnification of 400 times. Figure 2A: NK cell expression in normal pregnancy. Figure 2B: NK cells expression in FGR. NK cells positive if the color is red in the cytoplasm.

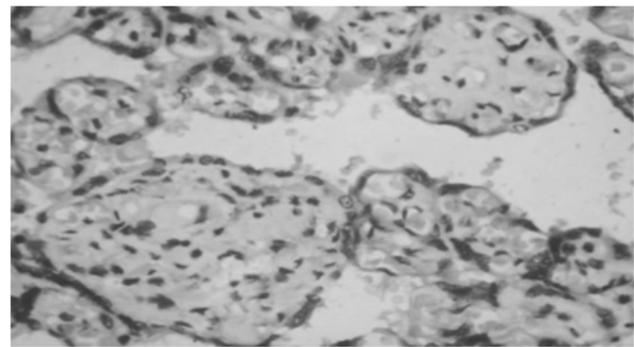
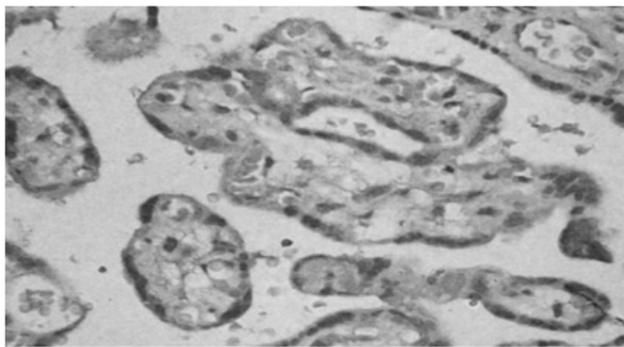


Figure 1. HLA-C Expression on FGR and Normal Pregnancy.

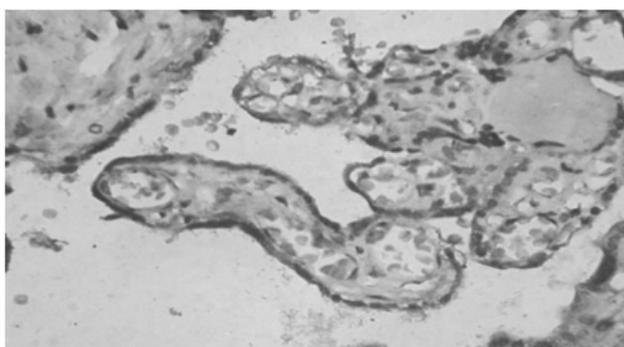


Figure 2. NK Cell Expression in FGR and Normal Pregnancy.

Table 2. Mean Expression of HLA-C and NK Cells in Pregnancy with FGR and Normal Pregnancy

Variable	Pregnancy with FGR (N=20)	Normal Pregnancy (N=20)	p
HLA-C (% cells / field of view)	9.02±1.30	7.96±0.97	0.01*
NK cells (% cells / field of view)	10.59±2.11	8.18±0.91	0.00*

Description *p significant <0.05

DISCUSSION

The success of pregnancy depends on many factors, one of which is immune adaptation of the pregnancy in which fetal maternal immune tolerance is very important. Immune maladaptation can cause miscarriage, preeclampsia, fetal growth restriction, preterm, or gestational diabetes mellitus associated with excessive activation of immunology and dysregulation.⁶ Fetus is the result of an interaction of placenta with maternal immune system that has direct contact with semi-allogeneic fetus during pregnancy. A special type of cell derived from the fetus, called trophoblast, invades the uterus and contact directly with the maternal immune system.¹⁰ Extravillous trophoblast cells produce histocompatibility polymorphic antigen known as human leukocyte antigens-C (HLA-C) that will interact with killer immunoglobulin receptors (KIR) on maternal decidua NK cells that is suspected to have a role in regulating physiological functions related to placenta development.¹⁶ Therefore, it is said that HLA-C expression plays a role in the success of pregnancy.⁷

In this study, the mean of HLA-C expression in pregnancy with fetal growth retardation was higher (9.02 ± 1.30), compared to normal pregnancy (7.96 ± 0.97), with $p=0.006$ ($p < 0.05$). The mean of NK cells (KIR2DL1) expression in the group with fetal growth retardation was higher (10.59 ± 2.11) compared to normal pregnancy (8.18 ± 0.91), with $p=0.00$ (< 0.05).

The results were consistent with a study conducted by Hviid (2006) where HLA-C expression on cases of intrauterine growth was higher than normal. High HLA-C would inhibit NK cell function so that the secretion of angiogenic and mitogenic endothelial was ineffective that would interfere placentation and vascular remodeling. The balance between non-classical HLA molecules and the classical HLA-C was required for the success of normal pregnancy.¹⁰ A study conducted by Haumonte (2014) was also consistent with this research that HLA-C, which is part of the MHC Class I, was high in cases of intrauterine fetal death, fetal growth retardation, and the highest frequency in preeclampsia.¹⁷

HLA-C has a relationship with intrauterine growth due to the success of pregnancy depends on the expression of HLA-C.⁷ Vascular supply control of the fetus depends on the interaction between maternal KIR on decidua NK cells and

paternal HLA-C expressed by trophoblast. The combination of maternal decidua NK cells, namely maternal KIR with fetus HLA-C2 genotype (ligand for receptor of KIR2DL1 NK cell inhibitor) can increase the risk of fetal growth retardation. When the AA genotype maternal concurred with genotype HLA-C2 heterozygous or homozygous fetus, decidua NK cells expressing KIR genotype AA is to be inhibited, because haplotype A KIR receptor inhibitor (KIR2DL1). These receptors can inhibit the secretion of cytokines from NK cells are believed to facilitate the normal trophoblast invasion. If NK cells are not activated, these cells potentially cause inadequate trophoblast invasion, which resulted in the failure of placentation leading to fetal growth retardation. On FGR expression of HLA-C gene C2 is higher in trophoblast.^{7,11}

To reinforce that HLA-C2 type with KIR2DL1 inhibitor as a cause of FGR which is C1 (ligands for receptors of NK KIR2DS2 cell activator) do not cause same inhibition on A KIR haplotype that is caused by weak binding of HLA-C1 to the correspondent inhibitor KIR2DL2/3 compared to HLA-C2 binding on KIR2DL1. Therefore, C1 cannot inhibit NK cell on the same level with the C2. When C2 coincides with the KIR B haplotype, the activated KIR haplotype will neutralize inhibition signals.⁷

Farrel (2014) found no relationship between KIR and HLA-C which were seen in bad placenta and demonstrated the importance of maternal-fetal immune interactions to determine the outcome of the pregnancy. Maternal KIR inhibition combined with HLA-C2 fetal is associated with low fetal weight, while the activation of fetal maternal KIR with HLA-C2 is associated with increasing fetal weight.¹⁸ In this study, the high expression of KIR2DL1, an inhibitor of maternal KIR, is associated with fetal growth restriction.

Beaman (2014) showed a little difference but was still relevant to our study that the combination of KIR receptor activation that interacted with HLA-C1 haplotype would produce GM-CSF that was useful in placentation. If trophoblast expresses HLA-C2 inhibitor that interacts with receptors on NK cells, so that the cells are inactive and will result in preeclampsia, FGR, and recurrent miscarriage. A suitable combination will cause activations of NK cells and angiogenesis during placental growth and cause a good pregnancy, if the combination does not proper, it brings difficulty during pregnancy period.¹⁹

According to a theory by Hanna (2006), the risk of fetal growth restriction would increase if the mother had KIR haplotype inhibitor and the fetus had HLA-C2 gene which would lead to inhibition of NK cell activity which is not expected to occur during pregnancy.²⁰

Sharkey (2008) found that KIR/HLA-C interaction was biologically important in regulating placentation. KIR that binds HLA-C is expressed more strongly in decidua NK cells than peripheral NK cells derived from the same woman at the same time. The strongest expression of KIR is in early gestation (6 weeks), and it decreases slowly after the first trimester.²¹

Decidual NK cells produce several chemokines and angiogenic cytokines. It shows that NK cells play a role by releasing its cytokines in controlling pregnancy, besides functioning through cytotoxic mechanism. Activated KIR may not only activate the function of cytotoxic killer, but also activates the production of certain cytokines that can induce trophoblast invasion. This can explain the reason that NK cells have activating receptors for class I HLA molecule. Decidua NK clones that express activated KIR2DS4 receptors secrete large amounts of growth factors (VEGF, PLGF, IL-8 and IP-10), when incubated with cells that have experienced transfection with HLA-C2 group. Decidua NK cells that express KIR2DL1 receptor inhibitor secrete fewer growth factors when transfected with HLA-C2.²⁰

CONCLUSION

The expression of HLA-C and NK cells are higher in fetal growth restriction compared to normal pregnancy.

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