In conclusion, anti-β2GPI was found to represent a risk factor for developing PIH in this case-controlled cohort study, providing evidence to support the utility of anti-β2GPI determination as one of the laboratory criteria for APS classification. In the previous SAPPORO prospective study, aPLs were measured during pregnancy and women with a history of recurrent spontaneous abortion or thrombosis who tested positive for lupus anticoagulant or aCL underwent low dose aspirin therapy. The knowledge of the presence of these aPLs could potentially influence the physician in favour of an early pregnancy termination. These possible biases can be excluded in the present case-controlled study. However, the subject number in our study is relatively small and furthermore we did not measure anti-β2GPI repeatedly 12 weeks apart, as required by the updated SAPPORO criteria (Miyakis et al., 2006). It is well known that aPLs share antigen epitopes and presence of one aPL increases the chance of the presence of the other aPLs. In women positive for anti-β2GPI but negative for lupus anticoagulant, aCL, phosphatidylserine-dependent anti-prothrombin antibody, or aPE, the presence of anti-β2GPI was not a significant risk factor for development of PIH or preeclampsia. This may partly be due to small numbers but it is also possible that an anti-β2GPI is only a marker for the presence of other more important aPLs. Larger studies designed to include appropriate adjustments for the presence of several aPLs should be undertaken to clarify this.

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Correlation Between Natural Cytotoxicity Receptors and Intracellular Cytokine Expression of Peripheral Blood NK Cells in Women with Recurrent Pregnancy Losses and Implantation Failures

Atsushi Fukui[1], Evangelos Ntrivalas[2], Rie Fukuhara[1], Shunsaku Fujii[1], Hideki Mizunuma[1], Alice Gilman-Sachs[2], Kenneth Beaman[2], Joanne Kwak-Kim[2,3]

[1]Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, Japan;
[2]Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science/The Chicago Medical School, North Chicago, IL, USA;
[3]Department of Obstetrics and Gynecology, Rosalind Franklin University of Medicine and Science/The Chicago Medical School, Vernon Hills, IL, USA

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Correspondence
Atsushi Fukui, Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8562, Japan.
E-mail: a.fukuipon@mac.com

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Problem
Natural cytotoxicity receptors (NCRs) are unique markers, which regulate NK cell cytotoxicity and cytokine production. We investigated whether women with recurrent pregnancy losses (RPLs) and implantation failures have aberrant correlation between NCRs and intracellular cytokine expression of NK cells.

Method of study
Peripheral blood NK cells ($CD56^{dim}$ and $CD56^{bright}$) were analyzed for NCRs (NKp46, NKp44 and NKp30) and cytokine expression (TNF-α, IFN-γ, IL-4, IL-10) using flow cytometry in RPL ($n = 22$), implantation failures ($n = 23$) or controls ($n = 15$).

Results
In type 1 cytokine studies, $CD56^{bright}/NKp30^{+}$ cells in controls ($r = 0.696$, $P < 0.05$) were positively correlated with $CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$ cells. $CD56^{bright}/NKp46^{+}$ cells in implantation failures ($r = -0.76$, $P < 0.01$) were negatively correlated with $CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$ cells. RPL did not have any correlation. In type 2 cytokine studies, $CD56^{+}/NKp46^{+}$ cells ($r = 0.758$, $P < 0.01$) and $CD56^{+}/NKp30^{+}$ cells ($r = 0.637$, $P < 0.05$) were positively correlated with $CD56^{bright}/IL\text{-}4^{+}/IL\text{-}10^{+}$ cells in controls. $CD56^{+}/NKp30^{+}$ cells in implantation failures ($r = -0.778$, $P < 0.05$) were negatively correlated with $CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{+}$ cells. There were no correlations in RPL.

Conclusion
Recurrent pregnancy losses and implantation failures have lack of, or negative correlation between NCRs and intracellular cytokines expression. This observation suggests that excessive pro-inflammatory cytokine expression in NK cells in RPL and implantation failures may be exerted through the NCRs or interruption of signal transduction processes.

Introduction

Natural Killer (NK) cells are the potent cytolytic effector cells. The recent discovery of natural killer cytotoxicity receptors (NCRs), such as NKp30, NKp44 and NKp46 has demonstrated that NK cells have a unique series of triggering surface receptors responsible for the abolition of diverse tumor cells.[1] NCRs are involved in NK cytotoxicity and a direct correlation exists between the surface density of NCRs and the ability of NK cells to kill various tumors.[1]

NKp30 and NKp46 receptors are expressed on the surface of both activated and non-activated NK cells. However, NKp44 receptors are expressed on the surface of activated NK cells only. These receptors were reported to have various functions. NKp46 and NKp44, but not NKp30, recognize viral proteins such as hemagglutinin of influenza.[2,3] NKp30 and NKp46 receptors have a function in cytotoxic activity and immunoregulatory cytokine production of NK cells.[4] Recently, it has been reported that NKp30, NKp44 and NKp46 are responsible for NK cell apoptosis which is triggered by the engagement of NCR, which involves the production and release of FasL.[5]

NK cells play an important role in human pregnancy[6,7] and systemic regulation of peripheral blood NK cells contribute to reproductive success.[8,9] We have reported that $CD56^{+}/NKp46^{+}$ cells are markedly decreased in women with recurrent pregnancy losses (RPL) as compared with those of normal controls.[10] In addition, expression of NKp46, NKp44, NKp30, and a2V-ATPase on $CD56^{bright}$ NK cells was significantly up-regulated in women with RPL or implantation failures as compared with those of $CD56^{dim}$ NK cells.[10]

The cytokines produced by peripheral blood NK cells are mainly type 1 cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α. However, NK cells can produce type 2 cytokines, such as interleukin (IL)-4, 5, and IL-13 with stimulation. There are additional types of NK cells, which produce IL-10 or transforming growth factor (TGF)-β. It has been reported that cytokine production by NK cells facilitates decidualization, controls trophoblast invasion and promotes angiogenesis at the implantation site.[11,12] We have demonstrated that women with RPL or implantation failures have significant type-1 shift in cytokine producing $CD56^{bright}$ NK cells as compared with normal fertile population.[13]

The relationship between the expression of NCRs and the cytokines production by NK cells has not been clarified yet. In this study, we aimed to investigate the correlation between the expression of NCRs and intracellular cytokine expression of peripheral blood NK cells in women with RPL, implantation failures and normal fertile controls.

Materials and methods

Study Population

This is the sequential study of previously published studies of NCRs[10] and NK cell cytokine expression[13] in women with RPL and implantation failures. Twenty-two women with a history of two or more RPLs, 19 women with a history of two or more in vitro fertilization (IVF) failures in spite of total four or more embryos transferred and 13 age-matched normal fertile controls were included in the analysis. Others were enrolled for this study only: four women with two or more IVF failures and two more normal controls. Therefore, total 22 women with RPL, 23 women with implantation failures and 15 age-matched normal fertile controls were included in this study.

Study subjects were recruited at Reproductive Medicine Program. All study subjects had signed informed consents prior to entering the study, in accordance with the local institutional review board (IRB) protocol. Women with anatomic, infectious or genetic etiologies for RPL were excluded from the study. Women with evident endometriosis, anatomic or infectious etiologies of implantation failure were also excluded. Mean and standard deviation (S.D.) of ages are: RPL group, 34.8 ± 4.6 years (range, 25–45); implantation failures group, 33.3 ± 3.6 years (range, 28–38), and controls, 36.1 ± 4.8 years (range, 20–48). There are no differences in age distribution among the study and control groups. Mean and S.D. of spontaneous abortions of RPL group are 3.5 ± 1.0. Mean and S.D. of IVF-ET cycles in implantation failures are 2.4 ± 1.0. Mean and S.D. of live births in controls are 1.7 ± 0.7. Blood was drawn from study groups prior to any treatment.

Flow Cytometric Analysis of Peripheral Blood Lymphocytes

Dual-color flow cytometric analysis was performed to determine the co- expression of NCRs and intracellular cytokines in $CD56^{dim}$ and $CD56^{bright}$ natural

killer cells. For the expression of NCRs, peripheral blood leukocytes from each patient were reacted with a panel of monoclonal antibodies (mAbs) for 20 min at room temperature, then lysed, fixed and washed twice before analysis in a FACScalibur flow cytometer (Becton Dickinson; San Jose, CA, USA) as previously described.[10] The following mAbs were used to analyze the surface antigens of peripheral blood leukocytes: anti-CD56 fluorescein isothiocyanate (FITC) (BD Biosciences; San Jose, CA, USA), anti-CD335 (NKp46) phycoerythrin (PE) (BD Biosciences; San Jose, CA, USA), anti-CD336 (NKp44) PE (Beckman Coulter; Miami, FL, USA), and anti-CD337 (NKp30) PE (Beckman Coulter; Miami, FL, USA). Representative dot plots of the expression of NCRs (NKp46, NKp44 and NKp30) on $CD56^+$ NK cells and NK cell subsets in women with normal fertile control are shown in Fig. 1. Intracellular cytokine production by NK cells was measured using phorbol 12-myristate 13-acetate (PMA)-ionomycin-activated peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated by the Ficoll–Hypaque density centrifugation, then activated with PMA (25 ng/mL) and ionomycin (1μM) in the presence of brefeldin A (10 μg/mL) for 4 hr at 37°C in a 5% CO_2 humidified incubator. PBMCs were prepared, and immunofluorescence and triple-color flow cytometric analysis were performed to analyze intracellular cytokine expression (TNF-α, IFN-γ, IL-4, IL-10) of $CD56^{dim}$ and $CD56^{bright}$ NK cells with CD335, CD336 and CD337 natural cytotoxicity receptor co-expression as previously reported.[13] Representative dot plots of cytokine production by $CD56^+$ cells in women with normal fertile control are shown in Fig. 2. In brief, a gate was set on the lymphocyte population by characteristic forward- and side-scatter parameters. The lymphocytes were then analyzed in one parameter histograms for $CD56^{dim}$ and $CD56^{bright}$ cells and each of these populations were analyzed in two parameter histograms for co-expression of one of the natural cytotoxicity receptors and one of the intracellular cytokines. The percentages of these populations were measured and analyzed using BD cell QuestPro software (Becton Dickinson; San Jose, CA, USA) and FlowJo (Tree Star Inc., Ashland, OR, USA).

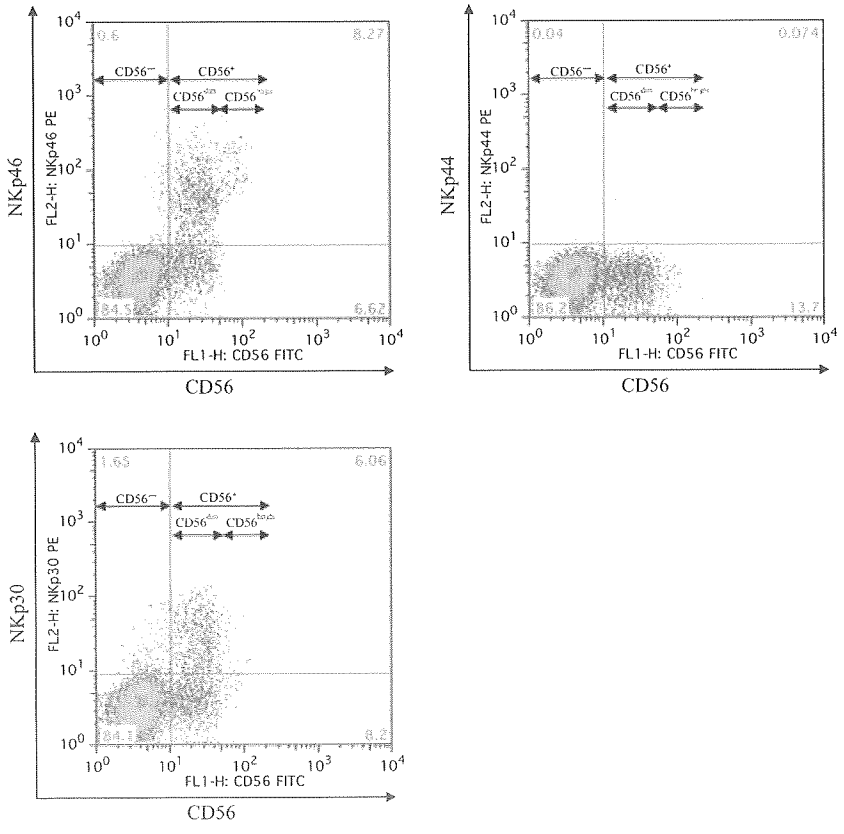

Fig. 1 Representative dot plots of the expression of natural cytotoxicity receptors (NCRs) on peripheral blood NK cells in normal fertile control. A gate is set on the lymphocytes. Lymphocytes are classified into $CD56^+$ and $CD56^-$ cells. $CD56^+$ cells are classified into $CD56^{bright}$ and $CD56^{dim}$ cells. Co-expression of CD56 with NCRs is evaluated.

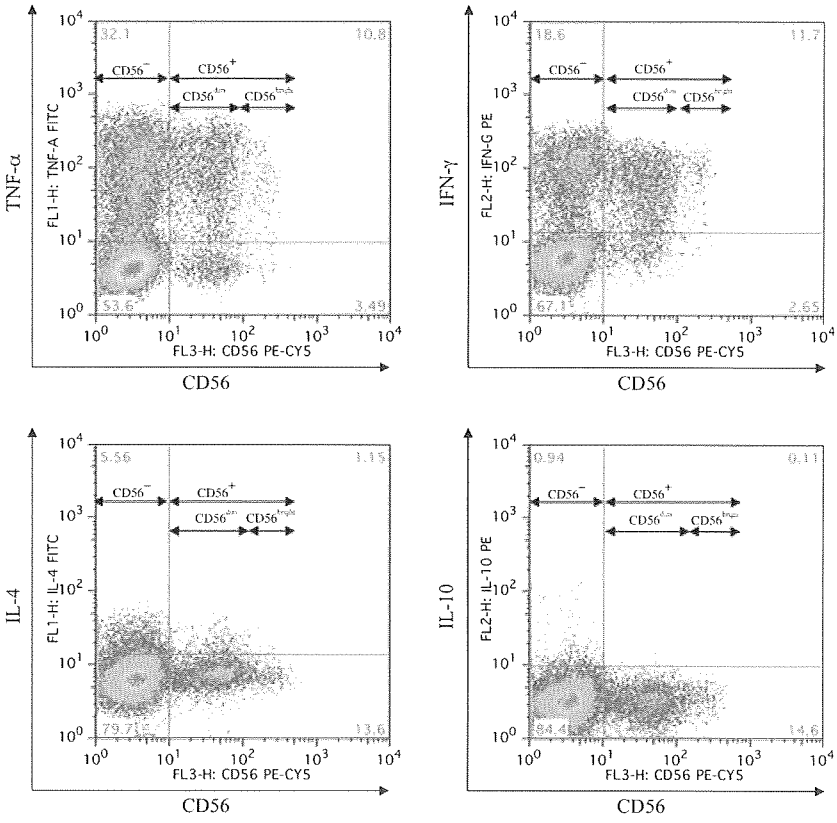

Fig. 2 Representative dot plots of cytokine production by peripheral blood NK cells in normal fertile control. A gate is set on the lymphocytes. Lymphocytes are classified into $CD56^+$ and $CD56^-$ cells. $CD56^+$ cells are classified into $CD56^{bright}$ and $CD56^{dim}$ cells. Cytokines production by CD56 cells is evaluated.

Statistical Analysis

All data are presented as mean ± S.D. The data were analyzed using StatView version 4.5 (Abacus concepts, inc., Japan). Age distribution of study groups and controls were analyzed by one-way ANOVA. Fisher's exact probability test of frequency distribution was applied for the comparison between two group differences. Correlations between the percentage of NCR-expressing NK cells and the percentage of cytokine-producing NK cells were analyzed by Pearson linear correlation. Correlations were considered significant if $r > 0.4$ and $P < 0.05$.

Results

Expression of NCRs and Cytokines Production by Peripheral Blood NK Cells

Expression of NCRs (NKp46, NKp44 and NKp30) on peripheral blood NK cell and cytokines production (IFN-γ, TNF-α, IL-10 and IL-4) by peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cell in women with RPL, implantation failures and normal fertile controls were shown in Table I. For the expression of NCRs by peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cell, there were no differences among the three groups. For the cytokines study, the percentage of $CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^-$ cells was significantly different among the three groups ($P < 0.05$). Furthermore, the percentage of $CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^-$ cells in the implantation failures group was significantly lower than that in controls ($P < 0.05$). The percentage of $CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$ cells was also significantly different among the three groups ($P < 0.05$). Furthermore, the percentages of $CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$ cells in the RPL ($P < 0.05$) and the implantation failures group ($P < 0.05$) were significantly higher than that in controls respectively. There were no differences among the three groups for the cytokines production by $CD56^{dim}$ NK cells.

Correlations Between NCRs and NK1 Cytokines

Correlations between NCRs-expressing NK cell ($CD56^{bright}$) levels and NK1 cytokine-expressing NK cell ($CD56^{bright}$) levels in women with RPL, implanta-

Table I Expression of NCRs (NKp46, NKp44 and NKp30) on Peripheral Blood NK Cell and Cytokines Production (IFN-γ, TNF-α, IL-10 and IL-4) by Peripheral Blood $CD56^{bright}$ NK Cell in Women with RPL, Implantation Failures and Normal Fertile Controls

	Controls (n = 15)	Implantation failures (n = 23)	RPL (n = 22)	P
$CD56^{dim}/NKp46^{+}$	68.7 ± 13.8	58.1 ± 23.2	62.0 ± 23.8	NS
$CD56^{dim}/NKp44^{+}$	1.6 ± 2.6	2.0 ± 2.1	1.1 ± 1.4	NS
$CD56^{dim}/NKp30^{+}$	51.2 ± 17.1	52.5 ± 46.1	46.1 ± 24.9	NS
$CD56^{bright}/NKp46^{+}$	98.1 ± 3.0	96.3 ± 6.9	94.0 ± 11.4	NS
$CD56^{bright}/NKp44^{+}$	22.4 ± 15.6	24.6 ± 24.3	12.5 ± 15.5	NS
$CD56^{bright}/NKp30^{+}$	66.3 ± 11.8	78.7 ± 22.7	80.3 ± 24.3	NS
$CD56^{dim}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	20.7 ± 7.5	15.8 ± 10.0	16.8 ± 12.0	NS
$CD56^{dim}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	45.2 ± 16.7	40.9 ± 19.2	40.3 ± 20.5	NS
$CD56^{dim}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	5.3 ± 3.5	6.8 ± 6.4	6.8 ± 6.0	NS
$CD56^{dim}/IL\text{-}10^{+}/IL\text{-}4^{-}$	0.6 ± 0.6	0.7 ± 0.5	0.8 ± 1.0	NS
$CD56^{dim}/IL\text{-}10^{+}/IL\text{-}4^{+}$	1.3 ± 2.5	1.3 ± 3.1	0.5 ± 0.7	NS
$CD56^{dim}/IL\text{-}10^{-}/IL\text{-}4^{+}$	3.2 ± 5.2	1.6 ± 3.7	1.1 ± 1.3	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	38.8 ± 8.4	28.2 ± 11.5	33.5 ± 14.8	<0.05
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	30.6 ± 9.9	41.3 ± 14.9	42.0 ± 15.6	<0.05
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	6.6 ± 4.0	7.7 ± 5.5	7.2 ± 7.2	NS
$CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{-}$	1.7 ± 3.0	4.9 ± 8.6	4.3 ± 10.2	NS
$CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{+}$	1.4 ± 1.8	2.2 ± 3.9	0.3 ± 1.3	NS
$CD56^{bright}/IL\text{-}10^{-}/IL\text{-}4^{+}$	2.5 ± 4.7	1.8 ± 4.9	0.9 ± 2.4	NS

**Data from Refs. 10 and 13.

tion failures and normal fertile controls were shown in Table II. In controls, the percentage of $CD56^{bright}/NKp30^{+}$ cells positively correlated with that of $CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$ cells ($r = 0.696$, $P < 0.05$). However, in implantation failure group, it did not correlate. Moreover, in implantation failure group, the percentage of $CD56^{bright}/NKp46^{+}$ cells negatively correlated with that of $CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$ cells ($r = -0.76$, $P < 0.01$) (Fig. 3b, red line). There were no correlations between any of the NCRs and type 1 cytokine-expressing NK cell levels (%) in women with RPL. For $CD56^{dim}$ cells, there were no correlations between any of the NCR and type 1 cytokine-expressing NK cell levels (%) in women with RPL, implantation failures and controls.

Correlations Between NCRs and NK2 Cytokines

Correlations between NCRs-expressing NK cell ($CD56^{+}$) levels and NK2 cytokine-expressing NK cell ($CD56^{bright}$) levels in women with RPL, implantation failures and normal fertile controls were shown in Table III. In controls, the percentage of $CD56^{+}/NKp46^{+}$ cells positively correlated with that of $CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{+}$ cells ($r = 0.758$, $P < 0.01$) and $CD56^{bright}/IL\text{-}10^{-}/IL\text{-}4^{+}$ cells ($r = 0.682$, $P < 0.05$) respectively (Fig. 4a green and blue lines). The percentage of $CD56^{+}/NKp30^{+}$ cells positively correlated with that of $CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{+}$ cells ($r = 0.637$, $P < 0.05$). In implantation failure group, the percentage of $CD56^{+}/NKp46^{+}$ cells did not have any correlations. However, the percentage of $CD56^{+}/NKp30^{+}$ cells negatively correlated with $CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{-}$ cells ($r = -0.778$, $P < 0.05$). Moreover, there were no correlations between any of the NCRs- and type 2 cytokine-expressing NK cell levels (%) in women with RPL. For $CD56^{dim}$ cells, there were no correlations between any of the NCR- and type 2 cytokine-expressing NK cell levels (%) in women with RPL, implantation failures and controls.

Discussion

Natural Killer cells can be subdivided by their surface marker expression such as $CD56^{bright}$ and $CD56^{dim}$. The major subset of uterine NK cell is $CD56^{bright}/CD16^{-}$ cell (approximately 80%), whereas that of peripheral NK cell is $CD56^{dim}/CD16^{+}$ cell (approximately 90%). It is reported that one of the roles of uterine NK cell is to control trophoblast invasion and uterine vascular remodeling during pregnancy.[14] A majority of circulating maternal NK

Table II Correlations Between NCRs and NK 1 Cytokine-Expressing NK Cell Levels in Women with RPL, Implantation Failures and Normal Fertile Controls

	r	*P*
Normal fertile controls		
$CD56^{bright}/NKp46^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.394	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.459	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.109	NS
$CD56^{bright}/NKp44^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.238	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.337	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	−0.032	NS
$CD56^{bright}/NKp30^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.049	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.696	<0.05
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.141	NS
Implantation failures		
$CD56^{bright}/NKp46^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.76	<0.01
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.447	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.273	NS
$CD56^{bright}/NKp44^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.137	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	−0.359	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	−0.615	NS
$CD56^{bright}/NKp30^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.319	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.39	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.003	NS
RPL		
$CD56^{bright}/NKp46^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.186	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	−0.214	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	−0.244	NS
$CD56^{bright}/NKp44^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.438	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.005	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.517	NS
$CD56^{bright}/NKp30^{+}$		
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$	−0.146	NS
$CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{+}$	0.033	NS
$CD56^{bright}/IFN\text{-}\gamma^{-}/TNF\text{-}\alpha^{+}$	0.23	NS

cells in blood is $CD56^{dim}$ cells and in intervillous space, trophoblasts are directly in contact with peripheral NK cells. Therefore, the manner in which the developing fetus evades NK cytotoxicity from these cells may determine the reproductive outcome. Consequently, regulation of peripheral NK cell cytotoxicity during pregnancy is very important.[15] $CD56^{bright}$ NK cells express low levels of perforin and CD16, but express high level of cytokines. These cells are the primary source of NK cell-derived cytokines. At the same time, $CD56^{dim}$ cells are cytotoxic. These cells produce low levels of cytokines, and are mediators of antibody-dependent cell-mediated cytotoxicity, lymphokine-activated killer activity and natural cytotoxicity.[16] Cytokines expression is mainly on $CD56^{bright}$ cell population. Hence, $CD56^{bright}$ and $CD56^{dim}$ cells are not only phenotypically diverse but also functionally different and it seems unnecessary that $CD56^{dim}$-producing cytokines are evaluated. In fact, type 1/2 cytokine expression was significantly higher in $CD56^{bright}$ cells as compared with those of $CD56^{dim}$ cells.[16]

In this study, the level of $CD56^{bright}/NKp30^{+}$ NK cells was positively correlated with that of type 1 cytokine-expressing $CD56^{bright}$ NK cells in normal controls, while negative correlation was noticed in women with implantation failures and no correlation in women with RPL. The function of NCRs during pregnancy is not well known. Our result provides the experimental evidence that NCRs and NK1 cytokines are co-regulated in normal fertile women. On the other hand, women with RPL or implantation failures have demonstrated lack of, or aberrant co-regulation.

Type 1 cytokines are important for trophoblast implantation and differentiation.[11,12,17,18] TNF-α has major role in trophoblast cell growth, cell differentiation, angiogenesis, and trophoblast apoptosis,[17] and IFN-γ promotes the vascular remodeling.[12] On the other hand, the large amount of TNF-α in pregnant mice leads to abortion.[19] In normal human pregnancy, IFN-γ production by peripheral blood NK cells is significantly decreased as compared with non-pregnant status.[20] Hence, the regulation of TNF-α and IFN-γ is the key for the reproductive success. It has been shown that one of the factors that regulate the cytokine expression in NK cells (towards the NK1 or NK2 subsets) is the expression of NCRs.[4,21] The lack of correlation between NCR and cytokine-producing NK cell levels that was identified in this study may be one of the mechanisms that lead to the NK1 cytokine predominance that is observed in women with RPL or implantation failures.

In this study, there were positive correlations between $CD56^{+}/NKp46^{+}$ and $CD56^{+}/NKp30^{+}$ cell levels, and type 2 cytokine-expressing $CD56^{bright}$ cell levels in normal fertile controls, while negative correlation was noticed in women with implantation failures and no correlation in women with RPL. In addition, the level of $CD56^{+}/NKp44^{+}$ cells were not

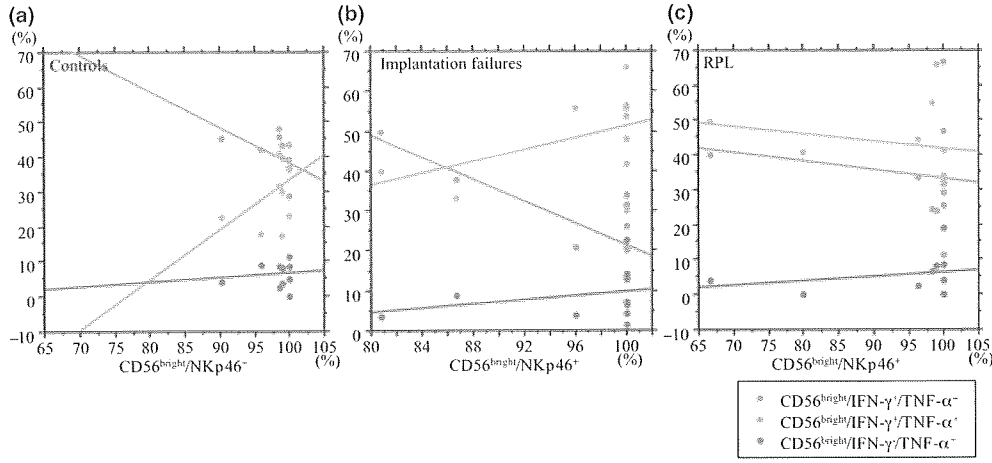

Fig. 3 Correlations between NCR ($CD56^{bright}/NKp46^{+}$) and type 1 cytokine-expressing cell levels (%) in women with RPL, implantation failures and normal fertile controls. The ordinate indicates the percentage of type 1 cytokines producing NK cells. The abscissa indicates the percentage of NCR positive NK cells ($CD56^{bright}/NKp46^{+}$ cells): (a) Correlation between $CD56^{bright}/NKp46^{+}$ and NK1 cytokine-expressing cell levels in normal fertile controls; (b) Correlation between $CD56^{bright}/NKp46^{+}$ and NK1 cytokine-expressing cell levels in women with implantation failures. The percentage of $CD56^{bright}/NKp46^{+}$ cells negatively correlated with that of $CD56^{bright}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$ cells ($r = -0.76$, $P < 0.01$) (red dots and line), and (c) Correlation between $CD56^{bright}/NKp46^{+}$ and NK1 cytokine-expressing cell levels in women with RPL.

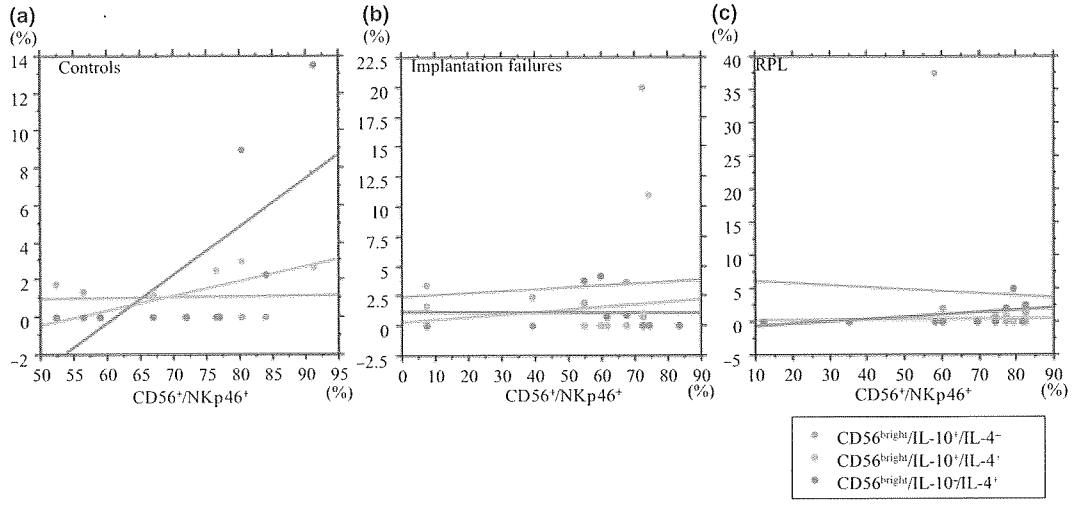

Fig. 4 Correlations between NCR ($CD56^{+}/NKp46^{+}$) and type 2 cytokine-expressing NK cell levels (%) in women with RPL, implantation failures and normal fertile controls. The ordinate indicates the percentage of type 2 cytokines producing NK cells. The abscissa indicated the percentage of $NKp46^{+}$ cells in total NK cells: (a) Correlation between $CD56^{+}/NKp46^{+}$ and NK2 cytokine-expressing cell levels in normal fertile controls. The percentage of $CD56^{+}/NKp46^{+}$ cells positively correlated with that of $CD56^{bright}/IL\text{-}10^{+}/IL\text{-}4^{+}$ cells ($r = 0.758$, $P < 0.01$) (green dots and line) and $CD56^{bright}/IL\text{-}10^{-}/IL\text{-}4^{+}$ cells ($r = 0.682$, $P < 0.05$) (blue dots and line), respectively; (b) Correlation between $CD56^{+}/NKp46^{+}$ and NK2 cytokine-expressing cell levels in women with implantation failures, and (c) Correlation between $CD56^{+}/NKp46^{+}$ and NK2 cytokine-expressing cell levels in women with RPL.

correlated with either type 1 or 2 cytokine-producing NK cell levels in normal fertile controls, RPL or implantation failures. This finding is consistent with a previous study, which indicated that NKp46 and NKp30 have a role in NK cytokines production, while the NKp44 does not play a similar role.[4]

Furthermore in this study experimental evidences are provided that NCRs and NK2 cytokines are co-

Table III Correlations Between NCRs and NK 2 Cytokine-Expressing NK Cell Levels in Women with RPL, Implantation Failures and Normal Fertile Controls

	r	*P*
Normal fertile controls		
$CD56^+/NKp46^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	0.028	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.758	<0.01
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	0.682	<0.05
$CD56^+/NKp44^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.135	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	−0.002	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	−0.153	NS
$CD56^+/NKp30^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.195	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.637	<0.05
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	0.392	NS
Implantation failures		
$CD56^+/NKp46^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	0.058	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.13	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	−0.02	NS
$CD56^+/NKp44^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	0.457	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	−0.147	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	−0.046	NS
$CD56^+/NKp30^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.778	<0.05
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.061	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	−0.074	NS
RPL		
$CD56^+/NKp46^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.062	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.215	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	0.45	NS
$CD56^+/NKp44^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.099	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	−0.183	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	−0.312	NS
$CD56^+/NKp30^+$		
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^-$	−0.157	NS
$CD56^{bright}/IL\text{-}10^+/IL\text{-}4^+$	0.291	NS
$CD56^{bright}/IL\text{-}10^-/IL\text{-}4^+$	0.555	NS

regulated in normal controls. There were correlations between IL-4 and/or IL-10 producing $CD56^{bright}$ cell levels and $NKp46^+$ and/or $NKp30^+$ NK cell levels. There was no correlation in $CD56^{dim}$ cells. This finding is in accordance with a previous study which showed that increased NK cell interaction via NKp30 could lead to increased production of IL-10, IFN-γ, and TNF-α.[22]

Recently, it has been reported that endometrial NK cells are negative for NKp30 and chemokine receptor expression. Therefore, pre-implantation NK cells are inactive and they are waiting for pregnancy.[23] For peripheral blood NK cells, we have shown a significant relationship between expression of NCRs and cytokines production by NK cells in normal fertile controls; however, this relation was not present in women with RPL and implantation failures even before pregnancy.

Often the interaction between peripheral blood NK cells and trophoblasts are not understood, and researchers are investigating various roles of peripheral and decidual NK cells.[24,25] NK cells preferentially kill targets with lower expression of MHC class I proteins, because fewer inhibitory receptors are engaging ligands. As a consequence, syncytiotrophoblasts are not free from peripheral blood NK cell cytotoxicity. Indeed, increased NK cell cytotoxicity has been reported to be associated with poor pregnancy outcome.[15,26]

It is reported that endometrial NK cells are different from peripheral blood NK cells for the expression of NCRs.[23] It is also reported that in decidua, not NKp46-, but NKp30-mediated cytokine secretion (IFN-γ and TNF-α) occurs in early pregnancy[21] and empowerment of uterine NK cells with angiogenic factors like VEGF C keeps them non-cytotoxic.[27] Interestingly, it has been reported that peripheral blood $CD56^{bright}$ cells can migrate and differentiate in the uterine lining. This study demonstrated that women with reproductive failures have dysregulated $CD56^{bright}$ NK cells in peripheral blood, which have a potential to migrate into the uterine lining.

In conclusion, clinical evidences for the correlations between NCRs and NK cell producing cytokines are provided. Correlations do not imply causality directly. However, there seems to be a close relationship between NCRs and cytokines production by NK cells in normal fertile population. Contrarily, in women with RPL or multiple implantation failures, lack of correlation or negative correlation between NCRs expression and cytokine production, were observed. We speculate that the presence of aberrant NCRs or interruption of signal transduction process after NCR activation may disintegrate co-regulation between NCRs and cytokine productions in NK cells of these women. In addition, these observations may suggest that excessive pro-inflammatory cytokine expression in NK cells in women with RPL and implantation failures may

be exerted through the NCRs or signal transduction processes may be disrupted. Further investigation of functional assays, testing the cytotoxic activity of the distinct NCR^+ NK cells subsets, and analyses of intracellular cytokines with regulatory potential such as VEGF in normal and abnormal pregnancies will contribute to the better understanding of the NK cell biology in women with RPL and implantation failures.

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H3N2 Influenza A Virus Replicates in Immortalized Human First Trimester Trophoblast Cell Lines and Induces Their Rapid Apoptosis

Quang Duy Trinh[1,2], Yasuyuki Izumi[1], Shihoko Komine-Aizawa[1], Toshikatsu Shibata[1], Yoshitaka Shimotai[1], Kazumichi Kuroda[1], Masashi Mizuguchi[2], Hiroshi Ushijima[3], Gil Mor[4], Satoshi Hayakawa[1]

[1]Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, Japan;
[2]Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan,
[3]Aino Health Science Center, Aino University, Tokyo, Japan,
[4]Department of Obstetrics, Gynecology and Reproductive Sciences, Reproductive Immunology Unit, Yale University School of Medicine, New Haven, CT, USA

Keywords
Apoptosis, first trimester trophoblast, influenza A virus

Correspondence
Satoshi Hayakawa, Nihon University School of Medicine, 30-1 Ohyaguchi Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: satoshih@med.nihon-u.ac.jp

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Problem
Epidemiological data suggested that pandemic influenza increased the risks of spontaneous abortion and premature labor, while seasonal influenza also increased the risk of schizophrenia in adolescence. However, their pathogenesis is so far unknown.

Method of study
The first trimester trophoblast cell lines, namely, Swan71 and HTR8 cells were challenged with A/Udorn/72 influenza virus (H3N2). At indicated time points, cells were examined for expression of influenza proteins. Viral replication in culture media, apoptosis and the expression of human leukocyte antigen (HLA)-G were also examined.

Results
Intracellular localization of viral proteins was observed. Twenty-four hours after inoculation, virus was detected in culture media while most cells fell into apoptosis. During apoptosis, expression of HLA-G was unchanged.

Conclusion
We revealed replication of low pathogenic influenza virus in the first trimester trophoblast cell lines. Placental damages are likely to be induced by direct cytopathic effects of influenza virus and subsequent apoptosis rather than down regulation of HLA-G expression and subsequent rejection by maternal immune system.

Introduction

Influenza is a highly contagious, acute viral febrile respiratory infection that results in global morbidity and mortality. During pregnancy poor outcome have been reported following the previous influenza pandemics.[1] During the influenza pandemic of 1918, remarkably high rates of spontaneous abortion and preterm birth were reported.[2,3] Asian influenza pandemic during 1957–58 also increased the rates of central nervous system defects and several other adverse outcomes, including birth defects, spontaneous pregnancy loss, fetal death, and preterm delivery,[4–6] while seasonal influenza did not increase the rates of pregnancy complications.[7] Recent studies have suggested the association of maternal influenza infection during pregnancy with three to seven-fold increase of schizophrenia in the offspring.[8–10] Brown et al. reported a

statistically significant association noted between elevated concentrations of cytokines or antibodies to influenza antigens in maternal serum and the incidence of schizophrenia in the offspring. They speculated that 4–21% of schizophrenia cases would have been prevented if the maternal infection had not occurred. Experimental rodent models have suggested that maternal influenza infection can influence the physiology, behavior, and neuropathology of adult offspring.[11] As viral RNA has not been detected in the fetal brain of influenza infected animals, these changes are speculated to be secondary to the maternal inflammatory responses, rather than results of direct viral effects.[12] To the best of our knowledge, the susceptibility and kinetics of influenza viruses in the early gestational trophoblasts have not been studied, while replication of influenza virus in freshly prepared cells from the term fetal membranes have been studied extensively by Uchide et al.[13]

In this study, we have hypothesized that invasive trophoblasts may be targets of influenza virus *in vivo* and *in vitro*. Taking into account the difficulty to obtain first-trimester human trophoblast primary cultures, we utilized the HTR8/SVneo and Swan71 (Sw.71) cell lines, both were derived from human first trimester extravillous trophoblast (EVT)[14] and have been employed as models of EVT cell proliferation, migration and invasiveness *in vitro*.[15–18] In this study, we observed that both cell lines were susceptible to non-pandemic influenza A (H3N2) virus and fell into apoptosis without remarkable reduction of human leukocyte antigen (HLA)-G expression.

Materials and methods

Cell Lines

The human first trimester trophoblast cell lines Swan71 (Sw.71, derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate described by Straszewski-Chavez)[19,20] and HTR8 (H8, originally were obtained from human first-trimester placenta and immortalized by transfection with a cDNA construct that encodes the simian virus 40 large T antigen)[14] were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sw.71) or RPMI 1640 (H8) normal growth medium (Gibco-Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 0.1 mM minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, 100 nM of penicillin and streptomycin (Gibco-Invitrogen). Cells were cultured in monolayer at 37°C and 5% CO_2.

Virus

The influenza virus A/Udorn/307/72 (H3N2) maintained in Nihon University School of Medicine was propagated either in 10-day-old embryonic chicken eggs or in Madin-Darby canine kidney (MDCK) cells. MDCK cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% FBS. Viral titers were checked with plaque formation assay before inoculation.

Viral Infections

Cells cultured in 6-well plates (2×10^5/well) were washed with phosphate-buffered saline (PBS) and infected with the influenza A/Udorn/72 at a multiplicity of infection of five (MOI 5) for 40 min at 37°C. After a 40-min adsorption, the inocula were removed, and cells were incubated with RPMI (for H8 cells) or DMEM (for Sw.71 cells) for the indicated times. Non-infectious influenza A/Udorn/72 was generated by incubating the virus at 56°C for 30 min, and inactivation was confirmed by the lack of cytopathic effect or replication on MDCK cell. For apoptosis examination, positive control was performed by incubating the cells with Actinomycin D (Nakarai Tesque, Inc., Kyoto, Japan), 1 μL of stock solution (1 mg/mL) per 10^5 cells.

Immunofluorescence Assay

H8 and Sw.71 cells grown on glass cover slips in 6-well plates were infected with the A/Udorn/72 at an MOI of 5. At the indicated time after infection, the cells were fixed with 4% paraformaldehyde solution for 10 min, washed with PBS, and incubated with rabbit anti-Udorn serum (1:1000 dilution), which had been prepared by immunizing rabbits with the purified virions as described previously,[21] for 1 hr at room temperature. Negative controls were placed by mock-virus treatment, heat-inactivated virus treatment as well as staining with rabbit sera without virus immunization. After washing with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG solution, prepared using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Invitrogen, Tokyo, Japan), for 40 min at room temperature. After washing, the cells were mounted with Fluoromount G (SounthernBiotech, Birmingham, AL, USA), and the

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fluorescent images were collected using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).[22] Each experiment was repeated at least three times, and Fig. 1 shows typical influenza viral protein staining.

Hemagglutination Assay

Serial twofold dilutions of specimens were made in 50 μL of PBS on 96-well U-bottom plates. To each well, 50 μL of 0.5% chicken erythrocytes in PBS was then added. The plates were kept at 4°C for 1 hr, then the hemagglutination (HA) patterns were read and HA titers were determined from the last dilution showing complete HA. Each experiment was repeated three times and the average values of the three independent measurements are shown.

RNA Isolation, cDNA Synthesis and Real Time Polymerase Chain Reaction

Cultured trophoblasts in 6-well plates and supernatant were solubilized with 1mL of TRIZOL® (Invitrogen). RNA was first extracted with chloroform, precipitated with absolute ethanol. Next, after washing with 75% ethanol, RNA was re-dissolved in RNAse-free water. The quantity and quality of the RNA were determined by ultraviolet absorbance at 260 nm. Then, DNase digestion step was performed using DNase I (TaKaRa Bio Inc., Shiga, Japan).

For cDNA synthesis, reverse transcription (RT) was performed using PrimeScript™RT Reagent Kit (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol, in which, the primer T7-Uni12 was used for influenza examination.

Real-time polymerase chain reaction (PCR) was conducted with the SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol with appropriate annealing temperature. For quantification of viral RNA copy numbers, dilutions of an external standard corresponding to 10^6 copies down to 1 copy of an influenza viral RNA solution were subjected to PCR in parallel using the primer pair T7 and vPB2qR3.[23] All

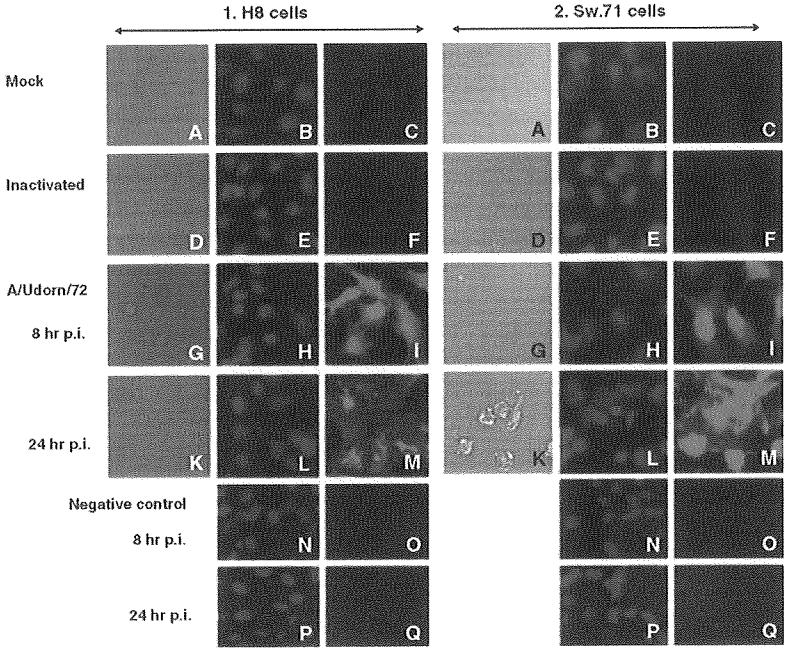

Fig. 1 Immunofluorescent images of H8 and Sw.71 cells infected with the A/Udorn/72 at an MOI of 5. At 8 h p.i. (panels 1G–I and 2G–I) and 24 h p.i. (panels 1K–M and 2K–M), the cells were fixed and stained for viral proteins using immunofluorescence with rabbit anti-Udorn serum. (1G, 1K, 2G, and 2K) differential interference contrast images; (1H, 1L, 2H, and 2L) the nuclei were counterstained with Hoesch stain (blue); (1I, 1M, 2I, and 2M) immunofluorescence staining of influenza viral proteins. Immunofluorescence (right) and corresponding brightfield (left) images are presented of the cells stained with antibodies for the viral proteins. Mock-infected cells and the cells incubated with the inactivated virus were also examined at 8 and 24 h p.i. (panels 1A–F and 2A–F). (Panels 1N–Q and 2N–Q), Udorn-infected H8 and Sw.71 cells stained with rabbit serum as negative controls.

reactions were performed on the ABI Prism™ 7500 sequence detection system (Perklin-Elmer Applied Biosystems, Tokyo, Japan). The primers were obtained from TaKaRa Co.Ltd (Tokyo, Japan). Oligonucleotide sequences of the primers used in this study are shown in Table I.

Detection of Apoptosis

The early apoptosis was measured using Apo-Strand™ELISA apoptosis detection kit (BIOMOL, Plymouth Meeting, PA, USA).This detection system employs monoclonal antibody to single-stranded DNA (ssDNA) which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis.[24] Tests were performed in cells grown in 96-well plates. For influenza virus inoculation Sw.71 and H8 cells were seeded (5000 cells/well) and cultured overnight before treatment. Cells were incubated with an MOI of 5 of influenza A/Udorn/72 and incubated for 18 hr. In brief, cells were fixed for 30 min with fixative indicated by the manufacturer and dried by incubating at 56°C for 20 min. Formamide was then added to the cells, and they were heated at 56°C for 30 min to denature DNA in apoptotic cells. Blocking solution was then added to all wells to block non-specific binding sites. Next, cells were incubated with antibody mixture for 30 min and rinsed with 1X wash buffer. After washing, cells were incubated with 100 μL of peroxidase substrate for 45 min, and absorbance was read using an ELISA plate reader at 405 nm. Reaction (color development) was stopped by the addition of 100 μL of 1% sodium dodecyl sulfate. Negative controls without viral inoculation, heat inactivated viruses and positive control with actinomycin D treatment were placed.

Flow Cytometric Analysis of HLA-G

For determination of the surface expression of HLA-G molecules, the cells (2×10^5) were washed twice with cold PBS containing 3% bovine serum albumin and then stained with FITC-conjugated mouse anti-human HLA-G monoclonal antibody (Clone MEM-G/11) (Exbio, Praha, Czech Republic) for 30 min at 4°C. After centrifugation, the cells were suspended in 0.5 mL of 1% paraformaldehyde and then subjected to flow cytometry analysis. Gated events were collected using the FACScalibur cytometer and analyzed with CellQuest software (Becton-Dickinson Biosciences, Tokyo, Japan).

Statistical Analysis

Analysis of variance was used for statistical analysis of the results. The resulting P-value <0.05 using Fisher's exact test was considered statistically significant.

Results

Imunofluorescence Detection of Influenza Virus Derived Antigens in H8 and Sw.71 Cells

H8 and Sw.71 cell lines were inoculated with non-pandemic influenza A Udorn/72 virus (H3N2). Intracellular localization of the virus in the infected cells was examined using immunofluorescence staining of the cells at indicated time points of 8 and 24 hr post infection (h p.i.) using rabbit anti-Udorn serum targeting with viral proteins of hemagglutinin protein (HA), nucleoprotein (NP)and matrix protein (M1). Non-immunofluorescence was detected in the mock-infected or heat-inactivated virus-treated cell (Fig. 1, panels 1A–F, and 2A–F) while strong intracellular localization of the virus was detected in the both cell lines at 8 and 24 h p.i. (Fig. 1, panels 1I, 1M for H8 cells and 2I, 2M for Sw.71 cells). Negative controls employing rabbit sera showed no immunofluorescence (Fig. 1, panels 1N–Q and 2N–Q). In addition, as seen in the Fig. 1, chromatin condensation and apoptotic nuclear fragmentation were observed by Hoechst staining (panels 1H and 2L) in influenza virus infected cells.

Table I Oligonucleotide sequences of the primers used in this study

Primer	Sequence 5′ to 3′	Sense
T7	TAA TAC GAC TCA CTA TAG G	+
T7-Uni12	TAA TAC GAC TCA CTA TAG GAG CAA AAG CAG G	+
vPB2qR3	TAG TAT CTC GCG AGT GCG AGA CT	–

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Apoptosis of the Cells Infected

To confirm the presence of apoptosis and to make quantitative analysis, we examined the amount of ssDNA using ELISA at 18 h p.i.. At the 18 h p.i., the absorbance of ssDNA at 405 nm was 2.1 ± 0.05 for H8 cells and 1.95 ± 0.15 for Sw.71 cells. For mock treated cells and inactivated virus-treated cells, the absorbances of ssDNA were 0.28 ± 0.08 and 0.29 ± 0.11, respectively for H8 cells; and in the case of Sw.71 cells, they were 0.30 ± 0.07 and 0.38 ± 0.13, respectively. The absorbance values of negative and positive controls were 0.81 ± 0.01 and 2.68 ± 0.16, respectively. The amount of ssDNA which suggests presence of apoptosis induced by influenza infection of the two cell lines were significantly higher than those of the corresponding mock-treated cells and inactivated-virus treated cells ($P < 0.0001$ for all cases) (Fig. 2).

Viral Replication in Infected Cells into Culture Media

By HA assay, we observed the evidence of exocytotic release of the viral progeny from both cell lines. Although at 8 h p.i., HA titers were under detectable levels in both cell lines, they were detected at 24 h p.i. (Fig. 3). We observed almost identical results with plaque formation assay (data not shown). Real time RT-PCR detected viral RNA at 8 h p.i. in the both cell lines and significantly increased at 24 h p.i. (Fig. 4).

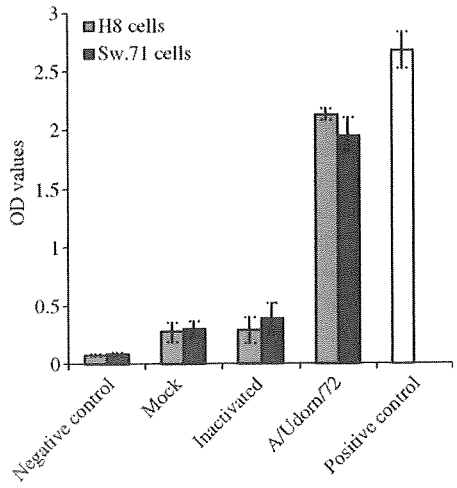

Fig. 2 ELISA detection of single-stranded DNA present in apoptotic cells. Each of the cell suspensions was prepared in a 96-well flat bottom plate as specified by the manufacturer. Then the cells were inoculated with the influenza A/Udorn/72 H3N2 at an MOI of 5. Single-stranded DNA provided in the ELISA kit was used as a control. Results are expressed as the means and standard deviations of three determinations

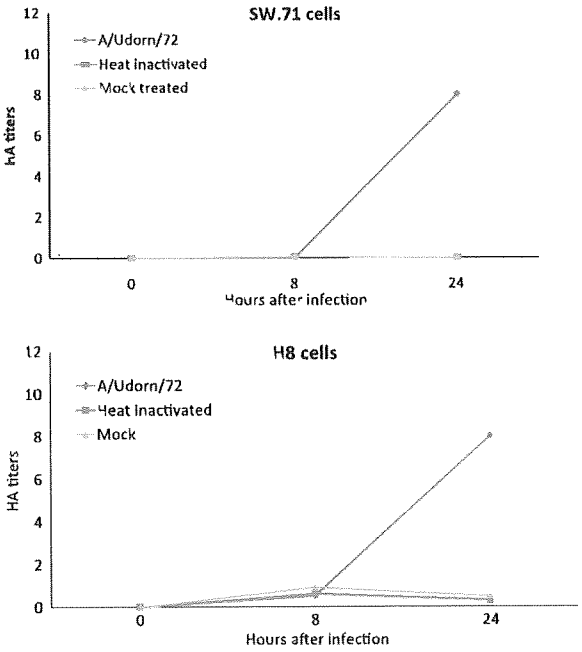

Fig. 3 Kinetics of influenza virus replication measured using the HA assay. H8 and Sw.71 cells were infected with the A/Udorn/72 at MOI 5. At the indicated time points post-infection (8 and 24 h p.i.), HA activity was measured in the culture supernatants.

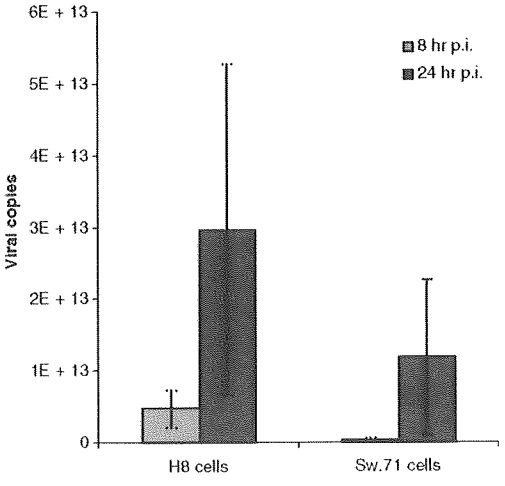

Fig. 4 Results of quantification of viral RNA copies in culture media using real-time PCR. *y*-axis: viral RNA copy numbers. The experiment was repeated three times. Each data point represents the mean ±S.D.

Expression of HLA-G

HLA-G is a non-classical major histocompatibility antigen with a restricted pattern of expression.[25] HLA-G is selectively expressed on cytotrophoblasts and invasive extravillous trophoblast at the feto-maternal interface where it may play a major role in maternal-fetal tolerance.[26] Its down-regulation caused by viral infections is considered to be a major cause of miscarriage.[27] We examined the expression of HLA-G using flow cytometry. Both in H8 and Sw.71 cells, we detected HLA-G in protein level. We observed no remarkable changes of HLA-G expression of the both cell lines at 8 and 24 h p.i. (Fig. 5). Forty-eight h p.i., we could detect no HLA-G protein because of cytopathic effects (data not shown).

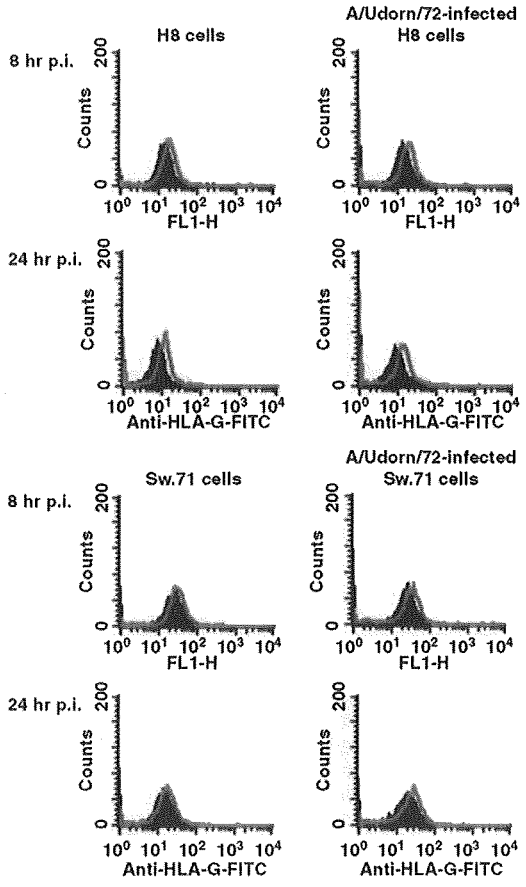

Fig. 5 Flow cytometry analysis of the non-classical class I MHC molecule HLA-G of H8 and Sw.71 cells by staining of HLA-G with FITC-conjugated mouse anti-human HLA-G monoclonal antibody. There was no shift in the histogram upon infection with the virus at 8 and 24 h p.i.

Discussion

In this study, we revealed that immortalized human trophoblast cell lines mimicking invasive trophoblasts are susceptible to H3N2 influenza virus. We consider that this finding is important because the human placenta is characterized by extensive invasion of cytotrophoblasts into the uterus wall, allowing their direct contact with the maternal blood, and by the extent of hormonal production.[28] In other words, trophoblastic invasion during the first trimester is a critical step to establish human pregnancy. Thus, insufficient invasion caused by various reasons including genetic abnormalities, disrupted maternal immune tolerance as well as viral infections results pregnancy failures. Productive infection and possible pathogenic roles of cytomegalovirus (CMV),[29] adenovirus,[30] adeno-associated virus-2 (AAV-2)[31] in early human trophoblasts have been reported while infection of influenza virus have not been studied despite its clinical importance. The most plausible explanation is that non-pandemic influenza is a relatively localized disorder in respiratory organs. Viremia is believed to occur infrequently in influenza.[32] A number of studies searching for influenza viremia after the onset of illness have failed to detect virus, supporting the notion that influenza viremia is at most a rare event in the post-symptomatic period and if it exists, it is not generally sustained for long periods. However, recent studies employing highly sensitive PCR suggested transient viremia before onset of respiratory is not rare.[33,34]

More importantly, viral RNA was detected from extrapulmonary sites including autopsy specimens of heart, kidney, brain, spinal cord, spleen, and liver of a pregnant 19-year-old woman who died as a result of A2/HongKong/8/68 infection.[35] Another case of a 24-year-old pregnant woman infected with influenza A/Bangkok (H3N2) showed positive results in fetal tissues and amniotic fluid.[36]

Taken together, these findings suggest fetoplacental tissues have a chance to be infected with influenza virus delivered by maternal systemic circulations. Limited viremia could be controlled with neutralizing antibodies evoked with the previous influenza infection or vaccinations.

However, in a case of pandemic, it is a completely different story. An influenza pandemic can develop with the emergence of a new virus with high transmission capability, and that harbors a novel HA that has not circulated for decades. In the 20th century, there were three overwhelming pandemics with

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influenza A, in 1918, 1957 and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu) and H3N2 (Hong Kong flu), respectively. During pandemics, pregnant subjects might have a higher risk of viremia and subsequent transplacental infection because of lack of previous immune exposures.

Gu et al. reported an autopsy case of pregnant woman who was killed by H5N1 influenza.[37] Employing in situ hybridization and immunohistochemical methods, they observed positive staining of influenza virus in placenta and fetal organs. They speculated that in addition to the lungs, H5N1 influenza virus disseminates to other organs including the brain and could also be transmitted from mother to fetus across the placenta.

Taken together, our findings suggest not only pandemic H5N1 influenza virus but also H3N2 and possibly other seasonal influenza viruses might replicate effectively in the invasive trophoblasts and subsequently induce placental apoptosis which might cause congenital anomalies as well as pregnancy failures. This cytopathic effect is independent from reduced HLA-G expression often observed in placental viral infections.

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Autoantibody associated disruption of kallikrein-kinin system in patients with recurrent pregnancy losses

Toshitaka SUGI

ABSTRACT

Factor XII, plasma prekallikrein and high molecular weight kininogen were first identified as coagulation proteins in the intrinsic pathway because patients deficient in these proteins had marked prolongation of in vitro surface-activated coagulation time. However, deficiencies of these proteins are not associated with clinical bleeding. Paradoxically, studies suggest that these proteins have anticoagulant and profibrinolytic activities. In fact, association between deficiencies of these proteins and thrombosis has been reported. Also, deficiencies of these proteins, autoantibodies to these proteins and anti-phospholipid antibodies are frequent haemostasis-related abnormalities found in unexplained recurrent aborters. Recently, evidence has accumulated for the presence of the kallikrein-kininogen-kinin system in the fetoplacental unit. Since contact proteins or kallikrein-kininogen-kinin system may play an important role in pregnancy especially in fetoplacental unit, autoantibodies to these proteins may be associated with pregnancy losses. These possibilities will be reviewed, the functions of the individual components will be summarized, and their role in blood coagulation and pregnancy discussed.

Key words; factor XII, antiphosphatidylethanolamine antibody, kininogen

Department of Obstetrics and Gynecology, Specialized Clinical Science, Tokai University School of Medicine, Kanagawa, Japan
Address correspondence to Toshitaka Sugi, Department of Obstetrics and Gynecology, Specialized Clinical Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa, 259-1193, Japan. (Tel: +81-463-93-1121; Fax: +81-463-91-4343; E-mail: sugi@is.icc.u-tokai.ac.jp).