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# INTERLEUKIN-8 PRODUCTION BY CD16$^-$CD56$^{bright}$ NATURAL KILLER CELLS IN THE HUMAN EARLY PREGNANCY DECIDUA

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SUMMARY Decidual CD16$^-$CD56$^{bright}$ natural killer (NK) cells were sorted from the decidual mononuclear cells (MNC) at the early pregnancy using a fluorescence activated cell sortor. The CD16$^-$CD56$^{bright}$ NK cell population occupies a major population in the decidual MNC, in contrast to a very small population (<1%) in the peripheral blood MNC. These decidual CD16$^-$CD56$^{bright}$ NK cells produced a large amount of IL-8, i. e., mean of 96.7$\pm$19.8 ng/ml in the 24 hr-cultured supernatants without any stimulant, which was comparable to the IL-8 production by LPS-stimulated peripheral blood MNC. Most of the IL-8 was ascribable to the production from decidual CD16$^-$CD56$^{bright}$ NK cells. Intracytoplasmic IL-8 in the decidual CD56$^{bright}$ NK cells was also detected by flow cytometry. RT-PCR methods confirmed IL-8 mRNA expression in this population, while no or very scarce expression of IL-1 $\alpha$ and IL-1 $\beta$ mRNA was observed. The present study is a first observation revealing that decidual CD16$^-$CD56$^{bright}$ NK cells express IL-8 mRNA and produce IL-8. 

It has recently been revealed that NK cells can be divided into CD16$^+$CD56$^{dim}$ NK cells and CD16$^-$CD56$^{bright}$ NK cells (1,2). The CD16$^-$CD56$^{bright}$ NK cells occupy only a very small percentage (<1%) of lymphocytes in peripheral blood, and, for this reason, studies on this type of NK cells have not been initiated until very recently (2). We and other groups have demonstrated that CD16$^-$CD56$^{bright}$ NK cells express both IL-2 receptor alpha-chain (IL-2R $\alpha$) and IL-2R beta-chain (IL-2R $\beta$) (3-5) and are abundant in fetal livers (6,7) and in human decidua (5,8-10). Since this type of NK cells have been found also in the liver of 6-week old fetuses and because they possess CD3 $\varepsilon$ and CD3 $\delta$ chains in their cytoplasm (7), they are thought to represent undifferentiated NK cells. We have already demonstrated

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that $CD16^-CD56^{bright}$ NK cells secrete various cytokines such as granulocyte colony-stimulating factor(G-CSF), granulocyte macrophage CSF(GM-CSF), macrophage CSF(M-CSF, CSF-1), leukemia inhibitory factor (LIF), tumor necrosis factor alpha (TNF- $\alpha$) and interferon-gamma(IFN- $\gamma$) (2,11,12). It is also known that large amounts of IL-8 are secreted into the decidua (13-15) and that IL-8 is secreted from $CD3^-$ large glanular lymphocytes ($CD3^-$ LGL) (16). With this in mind, we investigated the IL-8 mRNA expression in $CD16^-CD56^{bright}$ NK cells using reverse transcriptase polymerase chain reaction (RT-PCR) method and IL-8 production by this type of NK cells.

## MATERIALS AND METHODS

**Separation of decidual tissues and enrichment of $CD16^-CD56^{bright}$ NK cells:** Decidual tissues were sampled from women during artificial abortion in weeks 6-10 of pregnancy. Informed consent was obtained from all women prior to decidual tissue sampling. Each sample was cut into small pieces and shaken for one minute, followed by filtration through a Nylon mesh (pore size:30 $\mu$ m) and subsequent Ficoll-Hypaque density gradient centrifugation. Decidual mononuclear cells thus obtained were subjected to sorting by two color cytometry, using two antibodies, i. e., an FITC-labeled Leu 11a antibody (CD16:Becton Dickinson, USA) and a PE-labeled Leu 19 antibody (CD56, Becton Dickinson, USA). The sorting of $CD16^-CD56^{bright}$ NK cells was performed using an FACStar (Becton Dickinson, USA) as described elsewhere (5, 11) and purity of these cells were over 98%.

**Amplification of RNA using RT-PCR method:** From the $CD16^-CD56^{bright}$ NK cells, total RNA was extracted using a RNA Zol B (CINNA/BIOTEX, USA). For the preparation of cDNA, 4 $\mu$ g of RNA were incubated at 40℃ for 1 hr together with a 1xPCR buffer (Stratagene, USA), 1mM dNTP, 50U RNAse inhibitor (Takara Shuzo, Japan), 20U reverse transcriptase (Takara Shuzo, Japan) and 1nM random hexamer (Takara Shuzo, Japan), for a total volume of 20 $\mu$ l as described elsewhere (11,12). A 50 $\mu$ l solution, containing 1 $\mu$ l cDNA solution, 200 pM dNTP, 0.5 $\mu$ M primer and 1.25 U Taq polymerase (Toyobo, Japan), was subjected to 30 cycles of PCR, with each cycle consisiting of 50 sec at 94℃, one min at 55℃ and one min at 72℃. Part (10 $\mu$ l) of this solution was subsequently electrophoresed on 6% polyacrylamide gel and stained with ethidium bromide. Oligonucleotide primers of sense (5'-TGACGGGGTCACCCAC-ACTGTGCCCATCTA-3') and antisense (5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3') for $\beta$-actin, sense (5'-ATGGCCAAAGTTCCAGACATGTTTG-3') and antisense (5-'GGTTT-TCCAGTATCTGAAAGTCAGT-3) for IL-1$\alpha$, sense (5'-CTTCATCTTTGAAGAAGAACCTATCTTC-TT-3) and antisense (5'-AATTTTTGGGATCTACACTCTCCAGCTGTA-3') for IL-1$\beta$ and sense (5'-TCTGCAGCTCTGTGTGAAGGT-3') and antisense (5'-TGAATTCTCAGCCCTCTT-CAA-3') for IL-8 were synthesized. The size of amplfied fragement for $\beta$-action, IL-1$\alpha$, IL-1$\beta$ and IL-8 were 661bp, 816bp, 331bp and 252bp, respectively.

**Measurement of IL-1$\alpha$, IL-1$\beta$ and IL-8 :** Before and after sorting, cells were suspended in RPMI1640 medium containing 10% FCS at a concentration of $1\times10^6$ cells/ml in a 96-well flat culture plate, and incubated at 37℃ for 24 hr. Cytokine concentration in the cultured supernatants were determined as described(11), using ELISA assay kits for IL-1$\alpha$ and IL-1$\beta$ (Otsuka Pharmaceutical Co., Tokushima, Japan). IL-8 was determined by ELISA as described previously (17,18).

**Intracytoplasmic IL-8 in decidual $CD56^{bright}$ NK cells :** Decidual mononuclear cells were stained with FITC labeled anti-CD56 (Leu 19). These cells were washed with balanced salt solution (BSS), and fixed with cold 4% paraformaldehyde for 10 min. These cells were then washed with BSS containing 0.005% saponin, and were inculbated with biotinylated anti-human IL-8 monoclonal antibody, WS-4 ($IgG_1$, 17). After washing these cells with BSS-saponin, the samples were incubated with streptavidine conjugated

phycoerythrin for 30 min at room temperature. For every sample tested, a biotinylated mouse IgG1 was used to look at nonspecific binding. The intracytoplasmic IL-8 on decidual mononucler cells was analyzed on FACScan (Becton-Dickinson).

## RESULTS

**Cytokine production by decidual mononuclear cells and $CD16^-$ $CD56^{bright}$ NK cells:** We already found that human placental and decidual tissues contained a large amount of cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, TNF-$\alpha$ IFN-$\gamma$ as well as G-CSF, GM-CSF, or M-CSF (11,12). Here, we investigated whether they also produce IL-8, a chemotactic factor for neutrophil, T cells and basophils, since significant levels of IL-8 as well as IL-6 and G-CSF were detected in the amniotic fluids from preterm and term parturition (15). As shown in Table 1, MNC obtained from the decidua

Table. 1 IL-8, IL-1$\alpha$ and IL-1$\beta$ secretion by decidual mononuclear cells and decidual $CD16^-CD56^{bright}$ NK cells[1)]

| | | IL-8(ng/ml) | IL-1$\alpha$ (pg/ml) | IL-1$\beta$ (pg/ml) |
|---|---|---|---|---|
| 1. | 6ws[2)]MNC[3)] | 112.8 | 60 | 300 |
| | $CD16^-CD56^{bright}$ NK[4)] | 22.6 | <10 | <20 |
| 2. | 6wsMNC | 115.5 | 55 | 240 |
| | $CD16^-CD56^{bright}$ NK | 21.6 | <10 | <20 |
| 3. | 7wsMNC | 98.6 | 35 | 150 |
| | $CD16^-CD56^{bright}$ NK | 60.9 | <10 | <20 |
| 4. | 7wsMNC | 116.4 | 45 | 290 |
| | $CD16^-CD56^{bright}$ NK | 64.8 | <10 | <20 |
| 5. | 8wsMNC | 91.5 | 40 | 185 |
| | $CD16^-CD56^{bright}$ NK | 18.6 | <10 | <20 |
| 6. | 9wsMNC | 8.8 | 40 | 150 |
| | $CD16^-CD56^{bright}$ NK | 53.9 | <10 | <20 |
| 7. | 10wsMNC | 73.5 | 20 | 75 |
| | $CD16^-CD56^{bright}$ NK | 75.5 | <10 | <20 |

1) Decidual mononuclear cells and isolated $CD16^-CD56^{bright}$ NK cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum at the concentration of 1 $\times 10^6$ cells/ml in a 96-well flat microtiter plate. IL-1$\alpha$ , IL-1$\beta$ and IL-8 levels in the 24 hr cultured supernatants were determined by ELISA.

2) Weeks of gestation.

3) Decidual mononuclear cells before sorting.

4) Sorted decidual $CD16^-CD56^{bright}$ NK cells.

at 6 to 10 weeks of gestation contained high levels of IL-8, ranging 68.8 to 116.4 ng/ml (mean±SD=96.7 ± 19.8 ng/ml), compared to the minimal levels of IL-1 α (42.1±13.2 pg/ml) and IL-1 β (198.6±82.2 p/ml). The amount of IL-8 produced by isolated $CD16^-$ $CD56^{bright}$ NK cells were 51.1±30.5% (n=7) of the total amounts of IL-8 produced from MNC before sorting. No significant levels of IL-1 α (<10 pg/ml) and IL-1 β (<20 pg/ml) were produced by this subset. It should be noted that these cytokines were produced spontaneously in the culture without any stimulation. These spontaneous production is comparable to the IL-8 level produced by LPS-stimulated normal peripheral blood MNC, which was estimated to be approximately 50 to 100 ng/ml of IL-8 (16).

Dectection of cytokine mRNA expression by RT-PCR:We attempted to confirm these cytokine mRNA expression in the $CD16^-CD56^{bright}$ NK cells by the RT-PCR method. We found that decidual MNC contained 252 bp band corresponding to IL-8 mRNA , 816 bp band for IL-1 α and 331 bp band for IL-1 β , respectively (Fig.1 ; lane 1 and 3). As shown in Fig.1, decidual $CD16^-CD56^{bright}$ NK cells separated from two decidual MNC contained IL-8 mRNA, while the expression of IL-1 α and IL-1 β mRNA was absent or very scarce (Fig.1; lane 2 and 4).

Detection of intracytoplasmic IL-8 in decidual $CD56^{bright}$ NK cells by flow cytometry : As shown in Fig.2, intracytoplasmic IL-8 was clearly detected in decidual $CD56^{bright}$ NK cells. Mean fluorescence intensity stained with monoclonal anti-IL-8 antibody, WS-4 (40. 3) was two-fold stronger than that stained with a control antibody (19.2)

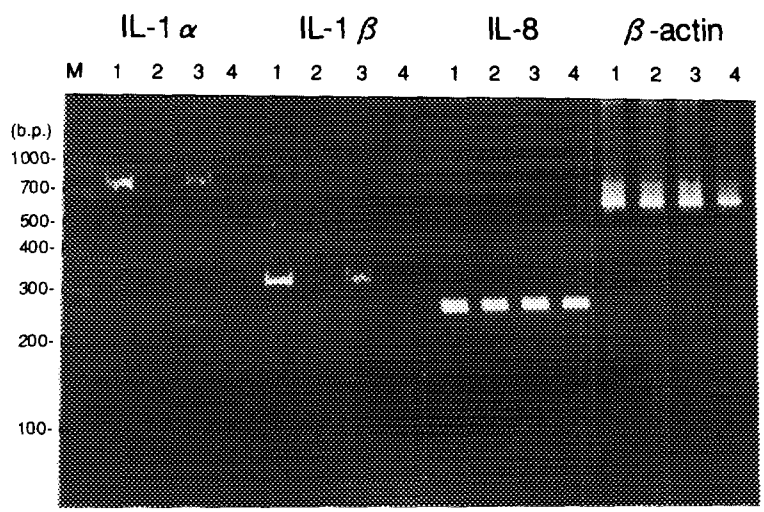


Fig.1. IL-1 α , IL-1 β and IL-8 mRNA expression in decidual mononucler cells (lane 1 and 3) and sorted decidual $CD16^-CD56^{bright}$ NK cells (lanes 2 and 4) using RT-PCR method. We performed the RT-PCR method in 2 cases (case 1 and case 2 ; case 1 at 7ws and case 2 at 8ws). Lanes 1 and 2 were derived from case 1 and lanes 3 and 4 were from case 2. The size of amplified fragments for IL-1 α , IL-1 β IL-8 and β -actin were 816bp, 331bp, 252bp and 661, respectively.

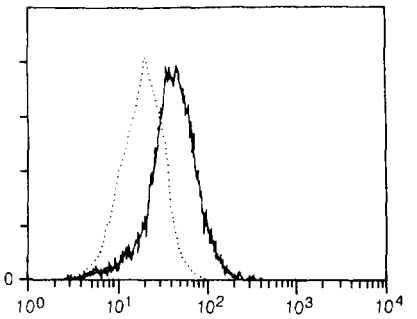


Fig.2. Expression of intracytoplasmic IL-8 in decidual $CD56^{bright}$ NK cells by flow cytometry. Biotinylated control IgG(----) and biotinylated anti - human IL-8 monoclonal antibody , WS - 4(——). Samples were analysed by FACScan displayed as histogram.

## DISCUSSION

Decidual mononuclear cells were composed of macrophages (20-30%), $CD16^-CD56^{bright}$ NK cells (60-70%), T cells (10%), $CD16^+CD56^{dim}$ NK cells (1-2%) and B cells (less than 1%) (5,8,9). The IL-8 detected in the supernatant of cultured decidual mononuclear cells can be interpreted as representing the total of IL-8 secreted from macrophages, $CD4^+$T cells, $CD16^-CD56^{bright}$ NK cells and $CD16^+CD56^{dim}$ NK cells. By the present study $CD16^-CD56^{bright}$ NK cells were the major population to secrete IL-8. It has been reported that IL-8 production by $CD3^-$LGL is induced by stimulation with PMA (16). It is interesting that CD69 (an early activation antigen) is expressed on decidual $CD16^-CD56^{bright}$ NK cells (5, 19). Because CD69 is expressed on the cell surface soon after stimulation with protein kinase C (PKC) activator (20), it seems likely that the $CD16^-CD56^{bright}$ NK cells detected in the decidua have already been stimulated with some PKC activators to promote IL-8 production. Further detailed examination of $CD16^-CD56^{bright}$ NK cells with PMA, ionomycin, may be required. IL-8 serves as a chemotactic factor mainly for neutrophils (21,22). Although the decidua contains high levels of IL-8 (12-14), the role of IL-8 remains to be obscure in that no marked infiltration by neutrophils, basophils or T cells inside the decidua is evident. Therefore, there may be some mechanism which inhibits the action of IL-8 in the decidua. In addition, a recent report indicates that progesterone suppresses the secretion of IL-8 from placental and decidual cells (14). It is therefore conceivable that IL-8 secretion from decidual cells may be suppressed *in vivo*. Once infection has occurred, however, decidual cells appear to immediately secrete IL-8 as a host defense mechanism against infection at the feto-maternal interface.

It is known that $CD16^-CD56^{bright}$ NK cells possess a high-affinity IL-2R and that their NK activity is reinforced even by small amounts of IL-2 (3-5). It has been previously reported that $CD16^-CD56^{bright}$ NK cells secrete M-CSF, GM-CSF G-CSF, LIF and TNF- $\alpha$ (11). The present study revealed that $CD16^-CD56^{bright}$ NK cells also secrete IL-8. These findings from previous and present studies suggest that $CD16^-CD56^{bright}$ NK cells are involved in the

early phase of host defenses and have a pivotal role against infection with viruses or bacteria.

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