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Increased prevalence of peripheral blood granulysin-producing cytotoxic T lymphocytes in preeclampsia

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ABSTRACT

Preeclampsia (PE) is a severe complication of pregnancy characterized by an excessive maternal systemic inflammatory response with activation of both the innate and adaptive arms of the immune system. Granulysin is a cytolytic and pro-inflammatory molecule expressed by activated human cytotoxic T lymphocytes and natural killer (NK) cells. Recent data show that serum granulysin levels are elevated in preeclampsia. The purpose of this study was to determine whether the proportion of peripheral blood cytotoxic T lymphocytes and NK cells that express intracellular granulysin is altered in PE. Twenty-two preeclamptic patients and 29 healthy pregnant women were involved in this case-control study. Intracellular granulysin expression of lymphocytes was determined with flow cytometric examination. In healthy pregnant women, the majority of NK cells and a small fraction of cytotoxic T cells expressed granulysin in their cytoplasma (median (25–75 percentile): 53.5 (45.6–68.0)% and 13.8 (8.5–23.1)%, respectively). In PE, the percentage of granulysin-positive cytotoxic T lymphocytes was markedly increased, while the proportion of granulysin-producing NK cells was unchanged as compared to healthy pregnant women (for cytotoxic T cells: 34.1 (19.3–45.6)%, $p < 0.001$; for NK cells: 57.2 (42.9–74.9)%, $p > 0.05$). Maternal age of healthy pregnant women showed a significant inverse correlation with the frequency of granulysin-expressing NK cells (Spearman $R = -0.44$, $p < 0.05$), while their BMI correlated positively with the proportions of granulysin-positive cytotoxic T cells and NK cells (Spearman $R = 0.43$, $p < 0.05$ for both). In conclusion, the majority of circulating NK cells but only a small population of cytotoxic T cells shows intracellular granulysin expression in normal pregnancy. In preeclampsia, the proportion of granulysin-producing cytotoxic T cells in the peripheral blood is markedly increased, which might contribute to the development of the pro-inflammatory Th1-type immune responses characteristics of the maternal syndrome of the disease.

1. Introduction

Preeclampsia, characterized by hypertension and proteinuria developing after the 20th week of gestation in a previously normotensive woman, is a severe complication of human pregnancy with a worldwide incidence of 2–10%. It is among the leading causes of maternal, as well as peri-

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natal morbidity and mortality, even in developed countries. Despite extensive research, the etiology and pathogenesis of preeclampsia are not completely understood. According to our knowledge, preeclampsia is a two-stage disorder. In the pre-clinical stage, insufficient trophoblast invasion in the first half of pregnancy results in poor placentation. Subsequent uteroplacental insufficiency leads to placental ischemia and oxidative stress. The ischemic and oxidatively stressed placenta releases pro-inflammatory (Th1-type) cytokines, lipid peroxidation products and trophoblast debris into the maternal circulation, which in turn trigger an exaggerated maternal systemic inflammatory response. The systemic inflammatory response with generalized endothelial dysfunction appears to be the cause of the maternal syndrome of preeclampsia in the second half of pregnancy (Redman et al., 1999; Redman and Sargent, 2005; Steegers et al., 2010). This study deals with the second (clinical) stage of preeclampsia.

Granulysin is a cytolytic and pro-inflammatory molecule expressed by activated human cytotoxic T lymphocytes and natural killer (NK) cells. It shares homology with other cytolytic molecules of the saposin-like protein (SAPLIP) family. Granulysin is localized to cytolytic granules of cytotoxic T lymphocytes and NK cells along with perforin and granzymes. This protein is broadly cytolytic against tumors and microbes, including Gram-positive and Gram-negative bacteria, fungi and parasites. In concert with perforin, it kills intracellular pathogens. Granulysin has been implicated in a myriad of diseases including infections, cancer, transplantation, autoimmunity and skin afflictions (Krensky and Clayberger, 2005, 2009). In the female reproductive system, granulysin has been found in endometrium, early pregnancy decidua and normal breast epithelium (Balogh et al., 2007; King et al., 2003; Mincheva-Nilsson et al., 2000). In addition, serum granulysin levels were strongly associated with the activities of cytotoxic T lymphocytes and NK cells and could be a useful novel serum marker to evaluate the overall status of host cellular immunity (Ogawa et al., 2003).

Recent data show that serum granulysin levels are elevated in preeclampsia and correlate with the clinical status such as mean blood pressure (Sakai et al., 2004). Furthermore, elevated granulysin concentrations were independently associated with an increased risk for preeclampsia (Qiu et al., 2006). The purpose of this study was to determine whether the proportion of peripheral blood cytotoxic T lymphocytes and NK cells that express intracellular granulysin is altered in preeclamptic patients as compared to healthy pregnant women. We also examined whether the clinical features of the study subjects were related to the proportions of granulysin-producing peripheral blood lymphocytes in our study groups.

2. Materials and methods

2.1. Study patients

Our study was designed using a case-controlled approach. Twenty-two preeclamptic patients and 29 healthy pregnant women with uncomplicated pregnancies were involved in the study. The study participants were enrolled in the First Department of Obstetrics and Gynecology, at the Semmelweis University, Budapest, Hungary between 2009 January and May. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The women were fasting, none of them were in active labour, and none had rupture of membranes.

Preeclampsia was defined by increased blood pressure ($\geq$140 mmHg systolic or $\geq$90 mmHg diastolic on $\geq$2 occasions at least 6 h apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria ($\geq$0.3 g/24 h or $\geq$1 + on dipstick in the absence of urinary tract infection). Blood pressure returned to normal by 12 weeks postpartum in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure $\geq$ 160 mmHg systolic or $\geq$110 mmHg diastolic, or proteinuria $\geq$5 g/24 h (or $\geq$3 + on dipstick). Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation (between 20 and 33 completed gestational weeks). Fetal growth restriction (IUGR) was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender, based on Hungarian birth weight percentiles (Joubert, 2000).

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University (IRB No. 188/2008), and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

2.2. Biological samples

Blood samples were obtained from an antecubital vein into heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by the standard Ficoll-Hypaque density-gradient centrifugation method. The aliquots of PBMC were stocked frozen in fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) at −80 °C until the measurements.

2.3. Flow cytometry

After thawing, isolated mononuclear cells were washed twice with phosphate-buffered saline (PBS) and their viability was assessed by trypan blue exclusion (consistently >90%). Non-specific binding sites were blocked by incubation with 10% mouse serum for 10 min at room temperature. Cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-human CD8 and phycoerythrin-cyanine 5 (PC5)-conjugated anti-human CD56 mouse monoclonal antibodies (BD Pharmingen, San Diego, California, USA) for 15 min at room temperature in dark and then washed with washing buffer. Red blood cells were lysed by incubation with 1.5 ml of 1× fluorescence-activated cell sorter (FACS) Lysing Solution (BD Biosciences, San Jose, California, USA) for 10 min at room tempera-

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ture in dark. Cells were centrifuged and the supernatant was removed. 500 μl of 1× FACS Permeabilizing Solution (BD Biosciences) was added and the mixture was incubated for 10 min at room temperature in the dark. After washing twice with washing buffer, the permeabilized cells were treated with biotinylated goat anti-human granulysin antibody (R&D Systems, Minneapolis, Minnesota, USA) for 30 min at room temperature in dark. Biotin-conjugated, isotype-matched goat immunoglobulin (Ig) G (R&D Systems) was used as a control for detecting non-specific binding. Following washing off the unbound biotinylated antibodies, the cells were stained with phycoerythrin (PE)-labelled streptavidin (Immunotech, Marseille, France) for 15 min at room temperature in the dark. After washing twice with washing buffer, the cells were resuspended in 1% paraformaldehyde in PBS. Flow cytometric analysis was performed on a FACSCalibur flow cytometer and data were processed using CellQuest Pro software (BD Biosciences). A real-time gate was set around the viable lymphocytes based on their forward scatter/side scatter profile. Contaminating monocytes and necrotic cells were excluded from the analysis.

2.4. Statistical analysis

The normality of continuous variables was assessed using Shapiro–Wilk's *W*-test. As the continuous variables were not normally distributed, nonparametric statistical methods were applied. To compare continuous variables between two groups, the Mann–Whitney *U*-test was used. The Fisher exact and Pearson χ^2 tests were performed to compare categorical variables between groups. The Spearman rank order correlation was applied to calculate correlation coefficients. As the proportions of granulysin-positive lymphocytes showed skewed distributions, we performed analysis of covariance (ANCOVA) with logarithmically transformed data.

Statistical analyses were carried out using the following software: STATISTICA (version 8.0; StatSoft, Inc., Tulsa, Oklahoma, USA) and Statistical Package for the Social Sciences (version 18.0 for Windows; SPSS, Inc., Chicago, Illinois, USA). For all statistical analyses, a two-tailed $p < 0.05$ was considered statistically significant.

In the article, data are reported as median (25–75 percentile) for continuous variables and as number (percentage) for categorical variables, if not otherwise specified.

3. Results

3.1. Patient characteristics

The clinical characteristics of the study participants are described in Table 1. There were no statistically significant differences in terms of age, gestational age at blood collection and the percentage of smokers between the two study groups. However, systolic and diastolic blood pressures, pre-pregnancy body mass index (BMI), BMI at blood sampling and the frequency of primiparas were significantly higher in preeclamptic patients than in healthy pregnant women. The gestational age at delivery and the fetal birth weight were significantly lower in the preeclamptic group compared with the group of healthy pregnant women. Fetal growth restriction was absent in healthy pregnant women, whereas the frequency of this condition was 45.5% in the preeclamptic group. Fourteen women had severe preeclampsia and 8 patients experienced early onset of the disease.

3.2. Intracellular granulysin expression of peripheral blood lymphocytes in healthy pregnant women and preeclamptic patients

Representative examples of intracellular granulysin expression by peripheral blood cytotoxic T ($CD8^+$) lymphocytes and NK ($CD56^+$) cells in a healthy pregnant woman and a preeclamptic patient are displayed in Fig. 1, as was determined by flow cytometric analysis. Table 2 and Fig. 2 reveal the proportion of granulysin-producing cells in relation to the parent populations in our study groups. In healthy pregnant women, the majority of NK cells and a small fraction of cytotoxic T cells expressed granulysin in their cytoplasma. In preeclamptic patients, the percentage of granulysin-positive peripheral blood cytotoxic T lymphocytes was markedly increased, while the proportion of granulysin-producing circulating NK cells was unchanged as compared to healthy pregnant women. Adjustment for age, primiparity, BMI and gestational age at blood draw in ANCOVA did not influence these results (Table 2).

Table 1
Clinical characteristics of healthy pregnant women and preeclamptic patients.

	Healthy pregnant women ($n = 29$)	Preeclamptic patients ($n = 22$)	Statistical significance (p value)
Age (years)	30 (26–32)	29.5 (27–38)	NS
Pre-pregnancy BMI (kg/m^2)	21.8 (20.3–23.0)	24.2 (21.5–26.7)	<0.05
BMI at blood collection (kg/m^2)	26.7 (24.8–29.8)	30.0 (27.2–33.1)	<0.05
Smokers	1 (3.4%)	1 (4.5%)	NS
Primiparas	11 (37.9%)	17 (77.3%)	<0.05
Systolic blood pressure (mmHg)	120 (115–127)	168 (150–180)	<0.001
Diastolic blood pressure (mmHg)	78 (70–80)	108 (100–110)	<0.001
Gestational age at blood collection (weeks)	37 (35–38)	35 (33–37)	NS
Gestational age at delivery (weeks)	39 (38–40)	35.5 (33–38)	<0.001
Fetal birth weight (grams)	3270 (3150–3740)	2295 (1740–2690)	<0.001
Fetal growth restriction	0 (0%)	10 (45.5%)	<0.001

Data are presented as median (25–75 percentile) for continuous variables and as number (percentage) for categorical variables.
BMI: body mass index; NS: not significant.

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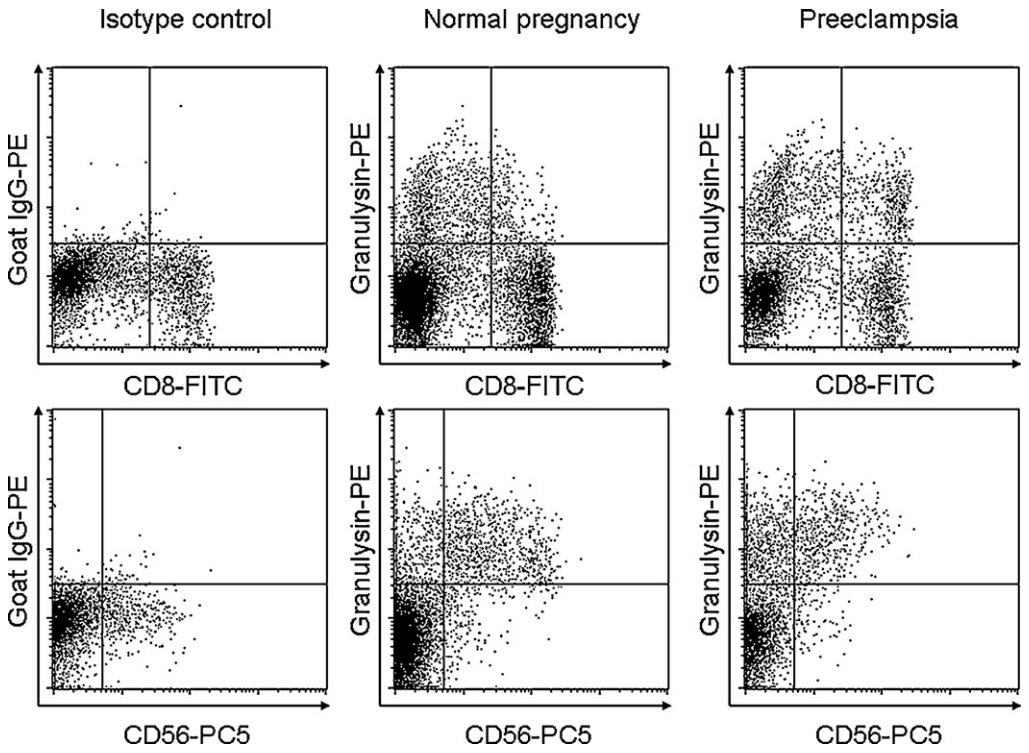

Fig. 1. Representative flow cytometric pattern demonstrating intracellular granulysin expression of peripheral blood $CD8^+$ and $CD56^+$ lymphocytes in a healthy pregnant woman and a preeclamptic patient. FITC: fluorescein isothiocyanate; PC-5: phycoerythrin-cyanine 5; PE: phycoerythrin.

In the group of preeclamptic patients, there were no significant differences in the frequency of granulysin-expressing lymphocyte populations between patients with mild and severe preeclampsia, between patients with late and early onset of the disease or between preeclamptic patients with and without fetal growth restriction (data not shown). Maternal age of healthy pregnant women showed a significant inverse correlation with the proportion of granulysin-positive NK cells (Spearman $R = -0.44$, $p < 0.05$; Fig. 3a). Furthermore, in the group of healthy pregnant women, significant positive correlations were observed between BMI at blood collection and the percentage of granulysin-producing cytotoxic T lymphocytes and NK cells (Spearman $R = 0.43$, $p < 0.05$ for both; Fig. 4a and b). However, these correlations did not reach statistical significance in the preeclamptic group (Figs. 3b, 4c and d). There was no other relationship between clinical features – including systolic and diastolic blood pressures – of the study subjects and the proportions of granulysin-expressing cells, either in healthy pregnant women or in preeclamptic patients.

4. Discussion

In the present study, we measured intracellular granulysin expression of peripheral blood cytotoxic T lymphocytes and NK cells in normal pregnancy and preeclampsia using flow cytometric examination. According to our findings, the majority of NK cells and a small fraction of cytotoxic T cells expressed granulysin in their cytoplasma in normal pregnancy. In preeclampsia, the proportion of granulysin-positive peripheral blood cytotoxic T lymphocytes was markedly increased, suggesting increased production of granulysin by circulating cytotoxic T cells in this pregnancy-specific disorder. However, the percentage of granulysin-producing peripheral blood NK cells was not altered in preeclamptic patients as compared

Table 2
Proportion of peripheral blood granulysin-positive lymphocyte populations in healthy pregnant women and preeclamptic patients.

Granulysin-positive cells/parent population	Healthy pregnant women ($n = 29$)	Preeclamptic patients ($n = 22$)	Statistical significance (p value)
$CD8^+Granulysin^+/CD8^+$ (%)	13.8 (8.5–23.1)	34.1 (19.3–45.6)	<0.001
Log ($CD8^+Granulysin^+/CD8^+$ (%)) (adjusted mean ± SE)	1.16 ± 0.06	1.42 ± 0.07	<0.05[a]
$CD56^+Granulysin^+/CD56^+$ (%)	53.5 (45.6–68.0)	57.2 (42.9–74.9)	NS
Log ($CD56^+Granulysin^+/CD56^+$ (%)) (adjusted mean ± SE)	1.75 ± 0.03	1.71 ± 0.03	NS[a]

Data are presented as median (25–75 percentile), if not otherwise specified.
SE: standard error; NS: not significant.
[a] Adjustment was carried out for age, primiparity, BMI and gestational age at blood draw in analysis of covariance (ANCOVA).

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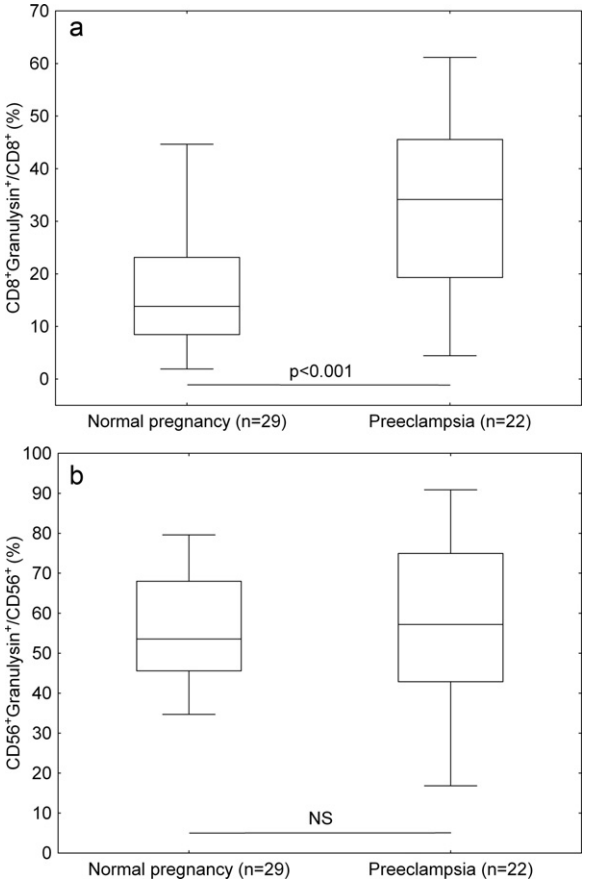

Fig. 2. The boxplot of granulysin-expressing $CD8^+$ (a) and $CD56^+$ (b) lymphocytes in proportion to the parent populations in healthy pregnant women and preeclamptic patients. Middle line: median; box: interquartile range (25–75 percentile); whisker: range (excluding outliers).

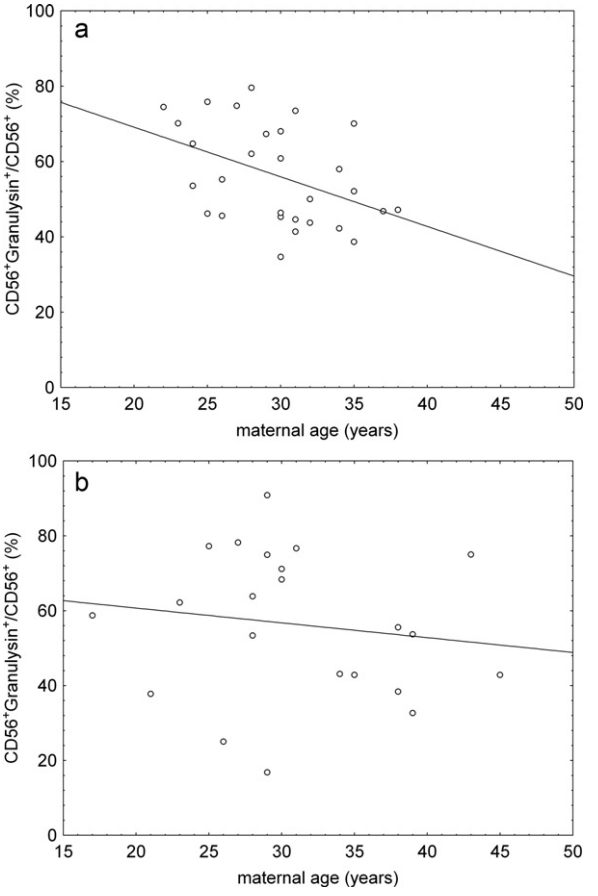

Fig. 3. Scatterplots and the regression line of the proportion of granulysin-expressing $CD56^+$ lymphocytes versus maternal age in healthy pregnant women (a) and preeclamptic patients (b).

to healthy pregnant women. Additionally, maternal age of healthy pregnant women showed a significant inverse correlation with the frequency of granulysin-positive NK cells, while their BMI at blood collection correlated positively with the proportions of granulysin-expressing cytotoxic T cells and NK cells.

Our findings that the majority of peripheral blood NK cells but only a small proportion of cytotoxic T cells showed intracellular granulysin expression in normal pregnancy are consistent with previous studies, which demonstrated NK cells to be the major granulysin source in normal PBMC (Obata-Onai et al., 2002; Ogawa et al., 2003). Granulysin production by circulating NK cells might provide healthy pregnant women with some degree of protection against infections and tumors. Granulysin can kill a variety of microorganisms and tumors by binding to the target cell surface based on charge and causing ion fluxes. An increase in intracellular calcium and efflux of intracellular potassium are associated with mitochondrial damage, the release of cytochrome C and apoptosis-inducing factor (AIF), and the blockade of electron transport with an increase of reactive oxygen species. Cytochrome C activates a caspase cascade, which together with AIF induces endonuclease activation and standard apoptosis (Kaspar et al., 2001; Okada et al., 2003). Granulysin can also induce a slower cytotoxicity pathway by activation of sphingomyelinase generating ceramide (Gamen et al., 1998). It might be plausible that the significant inverse correlation between maternal age and the frequency of granulysin-producing NK cells observed in our healthy pregnant group reflects impaired host defense mechanisms with advancing age.

The most remarkable observation of our study is that the proportion of granulysin-positive cytotoxic T cells in the peripheral blood is markedly increased in preeclampsia, which might be responsible – at least in part – for the elevated serum granulysin concentrations in this pregnancy-specific disorder reported earlier (Qiu et al., 2006; Sakai et al., 2004). There is an increasing body of evidence that an excessive maternal systemic inflammatory response to pregnancy plays a central role in the pathogenesis of the disease (Redman et al., 1999; Redman and Sargent, 2005). As part of the generalized intravascular inflammatory reaction, cytotoxic T lymphocytes are activated (Darmochwal-Kolarz et al., 2001; Saito et al., 1999), and cytotoxic T cell capacity to paternal antigens is increased in preeclampsia (de Groot et al., 2010), which might explain the increased prevalence of

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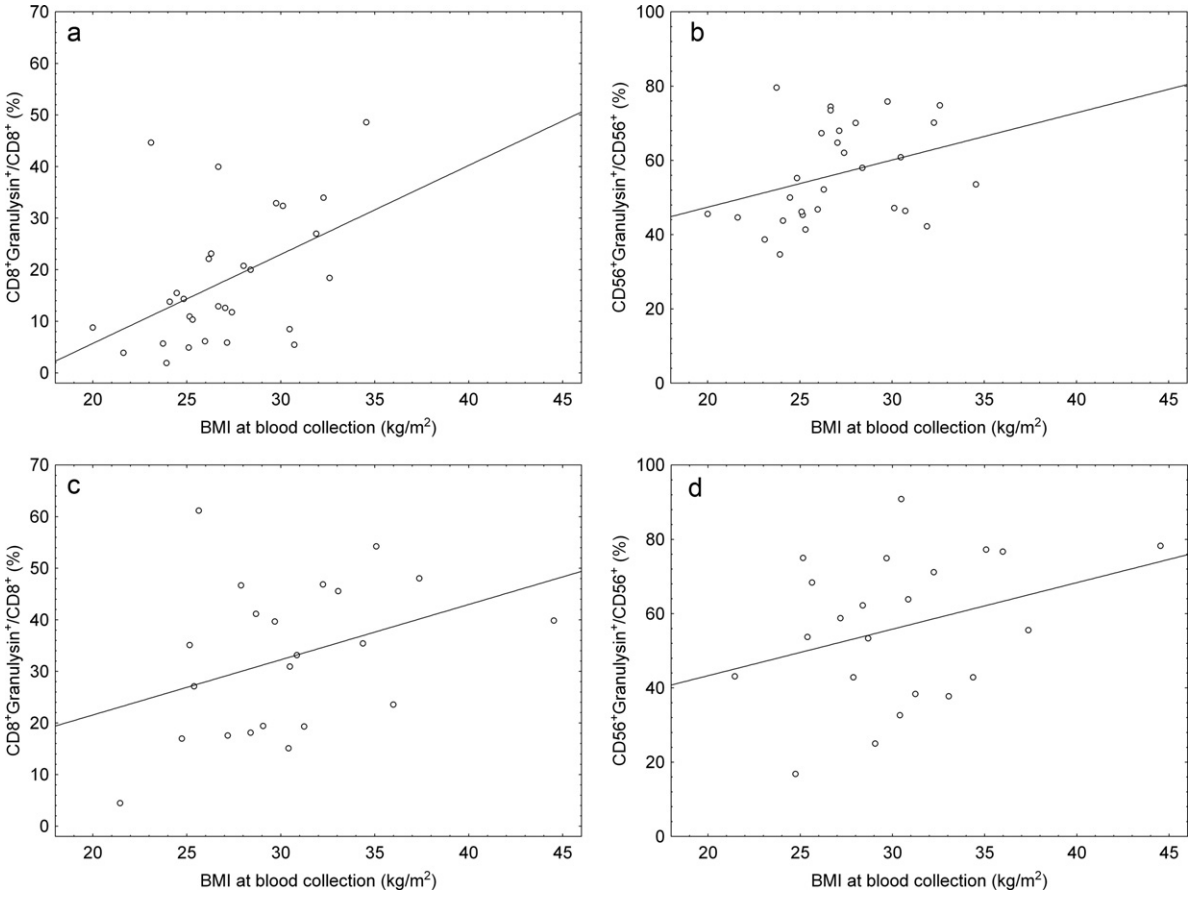

Fig. 4. Scatterplots and the regression line of the proportion of granulysin-expressing $CD8^+$ and $CD56^+$ lymphocytes versus BMI at blood collection in healthy pregnant women (a and b) and preeclamptic patients (c and d).

granulysin-producing cytotoxic T cells in this disease. Obesity is by itself an inflammatory condition (Vachharajani and Granger, 2009). Indeed, the proportion of granulysin-positive cytotoxic T cells and NK cells increased with BMI in our healthy pregnant group. Nevertheless, the difference in the frequency of granulysin-expressing cytotoxic T lymphocytes between our healthy pregnant women and preeclamptic patients remained significant even after adjustment for potential confounding variables, such as age, gestational age, BMI and primiparity. Preeclampsia is a multifactorial disorder with both genetic and environmental components (Roberts and Gammill, 2005). Thus, sequence variations in the gene encoding granulysin (Ericson et al., 2003) might also have an effect on granulysin expression of peripheral blood lymphocytes in preeclampsia. Further studies are required to explore the complex interaction between genetic and environmental factors in the regulation of granulysin expression in different tissues, ethnic populations and pathological processes.

Granulysin has been shown to act as a chemoattractant for monocytes, $CD4^+$ and $CD8^+$ memory (CD45RO) but not naïve (CD45RA) T cells, NK cells, and mature but not immature monocyte-derived dendritic cells. Furthermore, granulysin can activate monocytes to produce pro-inflammatory chemokines, including monocyte chemotactic protein (MCP)-1 and regulated upon activation normal T cell expressed and secreted (RANTES), and cytokines, such as tumor necrosis factor (TNF)-α (Deng et al., 2005). Therefore, it is tempting to speculate that increased production of granulysin by peripheral blood cytotoxic T cells suggested by our results might contribute to the development of the pro-inflammatory Th1-type immune responses, which are characteristic features of the maternal syndrome of preeclampsia (Saito and Sakai, 2003; Szarka et al., 2010). Indeed, we have previously reported that serum granulysin levels of preeclamptic patients correlate with the percentage of peripheral blood Th1 cells and Th1/Th2 ratios (Sakai et al., 2004). The fetus is a semi-allograft, and preeclampsia shares common pathogenetic mechanisms with a graft rejection (Labarrere, 1988). Interestingly, granulysin-expressing peripheral blood lymphocytes have also been implicated in mediating acute rejection of renal allografts (Sarwal et al., 2001).

Borzychowski et al. (2005) proposed a primary role of natural killer cells rather than T cells in the development of a predominant Th1-type immunological environment in preeclampsia. However, in the present study, no significant difference was observed in the prevalence of granulysin-positive peripheral blood NK cells between normal pregnant subjects and preeclamptic patients, sug-

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gesting that granulysin production of circulating NK cells is not substantially altered in preeclampsia. This is in line with recent findings that showed increased tumor rejection by $CD8^+$ T lymphocytes but no effect on NK cell-mediated tumor protection in mice transgenic for human granulysin (Huang et al., 2007). In addition, lysis of *Cryptococcus neoformans* by human $CD8^+$ T cells is granulysin dependent, but its NK cell-mediated killing is not (Ma et al., 2002, 2004). Interestingly, our research group revealed that granulysin produced by uterine natural killer cells induces apoptosis of extravillous trophoblasts in spontaneous abortion (Nakashima et al., 2008). Additional studies are needed to clarify the role of granulysin-producing decidual NK cells in the pathogenesis of preeclampsia.

In this study, the similar pattern of intracellular granulysin expression in peripheral blood cytotoxic T cells in preeclampsia regardless of the severity, the time of onset of the disease or the presence of fetal growth restriction might be explained by its multifactorial etiology. Several genetic, behavioural and environmental factors need to interact to produce the complete picture of this pregnancy-specific disorder. Our research group reported various genetic and soluble factors that were associated with the severity or complications of preeclampsia, including HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome and fetal growth restriction (Madach et al., 2008; Molvarec et al., 2008, 2009; Rosta et al., 2009; Varkonyi et al., 2010). However, it is also possible that the relatively small sample size of this study prevented to detect an effect in the subgroup analyses.

In conclusion, the majority of circulating NK cells but only a small population of cytotoxic T cells shows intracellular granulysin expression in normal pregnancy. In preeclampsia, the proportion of granulysin-producing cytotoxic T cells in the peripheral blood is markedly increased, which might contribute to the development of the pro-inflammatory Th1-type immune responses characteristics of the maternal syndrome of the disease. Nevertheless, a limitation of our study is its case-control design, which did not allow us to investigate the temporal relationship between the change in intracellular granulysin expression of circulating lymphocytes and the clinical stage of preeclampsia.

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