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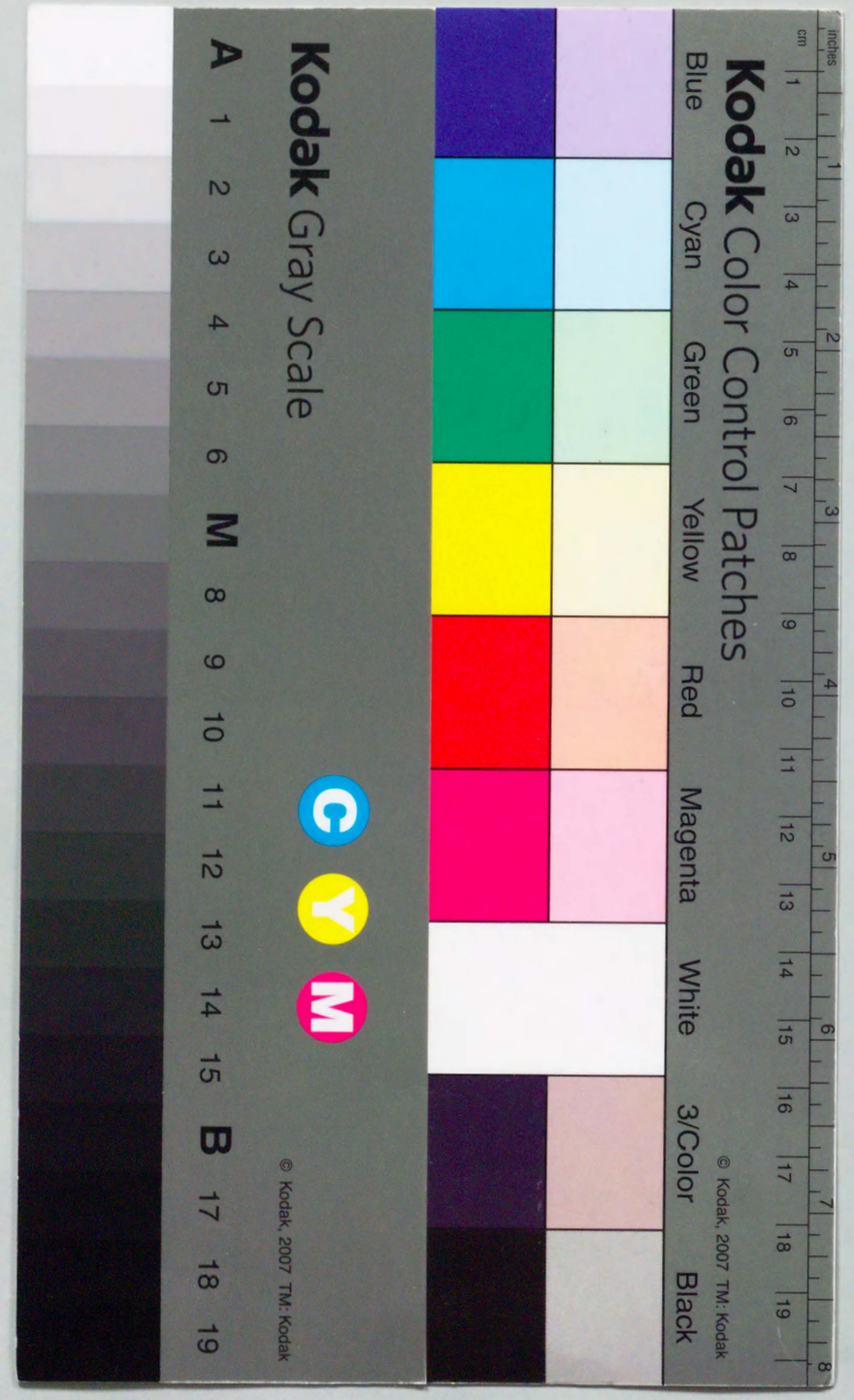

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ABSTRACT

Objective: Our purpose was to characterize neutrophil degranulation activity related to the manifestation of preeclampsia.

Materials and Methods: Studied were nine nonpregnant healthy women, nine normal pregnant women, and six cases with preeclampsia in the third trimester. Neutrophil preparations obtained for each case were divided into one nonstimulation and two stimulation groups using 10 and 100 nmol/L of N-formyl-methionyl-leucyl-phenylalanine (FMLP).

Main Outcome Measure: β-glucuronidase activity measured by fluorimetrical enzyme release assay was used for evaluating degranulation function.

Results: (1) The total β-glucuronidase activity in preeclampsia was significantly lower than in the other groups, whereas no difference was noted between the nonpregnant and the normal pregnant groups. (2) In the nonstimulation group, the degranulation proportional release in preeclampsia was significantly higher than in the other two subject groups, although there was no difference between the nonpregnant and normal pregnant subjects. (3) In the 10 nmol/L-FMLP stimulation group, preeclamptic women showed significantly higher values than those in the nonpregnant and normal pregnant groups. (4) In

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the 100 nmol/L-FMLP stimulation group, there was no significant difference in values among nonpregnant, normal pregnant, and preeclamptic women.

Conclusions: These findings indicate that neutrophil degranulation is already activated in preeclampsia, with a decrease in the total granule content retained in neutrophil. This suggests that in preeclampsia, a possible modification mechanism exists, specific in neutrophil degranulation and different from the FMLP-induced O_2^- producing system.

Key Words: Neutrophil; Degranulation; Preeclampsia; β-glucuronidase.

INTRODUCTION

Preeclampsia has been hypothesized to be a pregnancy-induced disease involving cell-to-cell interaction failure, particularly occurring between the endothelium, platelet, and white blood cell, to maintain homeostasis of microcirculation system function during pregnancy, leading from generalized vasoconstriction through to the manifestation of clinical signs such as hypertension, proteinuria, and edema (1–3).

Recently, from the standpoint that both neutrophil-derived oxidants and proteolytic enzymes act together synergistically to damage endothelial cells in tissue injury mechanism (4), authors have taken a strong interest in finding what contributes to the triggering of pathological change in the cells involved in preeclampsia (5,6).

In 1989, Greer et al. first demonstrated a positive association between the neutrophil-producing plasma elastase concentration and the pathogenesis of preeclampsia (7). β-glucuronidase is also known to be an enzyme of azurophil granule components derived from neutrophil, feasible for accurate measurement when using the fluorimetrical enzyme release assay method (8,9).

Thus, by assessing β-glucuronidase activity in the neutrophil in situ, we herein attempted to clarify whether neutrophil degranulation function plays upon cell level pathology in relation to the manifestation of preeclampsia.

MATERIALS AND METHODS

Study Population

Included were a total of 24 subjects, divided into three groups: nine nonpregnant healthy women, nine women with normal pregnancy, and six pregnant women complicated with preeclampsia in the third trimester (Table 1). Mothers were cared for in the Maternity and Perinatal Care Unit of Kyushu University Hospital, and the nonpregnant subjects were volunteers who all gave informed consent to participate in this study.

The subjects were diagnosed as having preeclampsia according to the definition by the American College of Obstetrics and Gynecology (10), when indicating

Table 1. Clinical Profile of Population Studied

VARIABLES	NORMAL NONPREGNANT (n = 9)	NORMAL PREGNANCY (n = 9)	PREECLAMPSIA (n = 6)	STATISTICAL SIGNIFICANCE
Age (yr)	27.0 (23–29)	27.0 (25–30)	27.5 (24–33)	ns
Gravidity	1.0 (0–2)	1.0 (1–3)	1.5 (1–3)	ns
Parity	0.0 (0–2)	0.0 (0–2)	0.5 (0–2)	ns
Gestation at time of sample (wks)	—	36.5 (35–38)	36.0 (34–38)	ns
Mean arterial pressure (mmHg)	86.7 (76.7–90.0)	83.3 (75.3–90.0)	121.7 (113.3–133.3)	$p < 0.005$
Proteinuria (g/24h)	Undetectable	Undetectable	2.9 (2.4–3.2)	—

Note. n: number of cases, values: median (range), yr: years old, wks: gestational weeks, *ns*: not significant.

proteinuria (> 0.3 g/24 h) and showing a rise in blood pressure to over 140/90 mmHg after 20 weeks' gestation. All cases were nonsmokers with neither alcohol abuse nor clinical or laboratory evidence of infection causing an increase in neutrophil activity, with a white cell count of less than 12,000/mm^3. There was also no indication of complications such as renal disease, cardiovascular disease, diabetes mellitus, or collagen disease, or any incidence of drug administration up to the examination of blood sampling. Age, gravidity, and parity were comparable between the three groups, as were the gestational ages of the two pregnant groups at the time of blood sampling (Table 1).

Three nonpregnant women and three cases with normal pregnancy were excluded due to a lack of sufficient neutrophil count needed to examine degranulation-dependent β-glucuronidase activity (described below).

Chemicals

Purchased were N-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochalasin B (Sigma Chem. Co., St. Louis, MO), and 4-methyl-umbelliferyl-β-D-glucuronide (4MU) compounds (WAKO Pure Chem. CO., Osaka, Japan).

Preparation and Incubation of Neutrophils

A 45–50 ml heparinized blood sample was obtained once from each subject. Neutrophils were then isolated according to the following procedure: (a) Dextran sedimentation, (b) hypotonic lysis of erythrocytes, and (c) Conray-Ficoll method (11). With 98% of the cells confirmed to be viable by the trypan blue dye–exclusion test, we obtained the final preparation containing more than 98% neutrophils and less than 2% lymphocytes, monocytes or eosinophils. This sample was then washed and suspended in HANKS saline buffer (140 mmol/L NaCl, 4.8 mmol/L KCl, 1.26 mmol/L $CaCl_2$, 0.81 mmol/L $MgSO_4$, 0.34 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2PO_4, 4.16 mmol/L $NaHCO_3$, 4 mmol/L glucose, pH 7.4) in order to

yield a concentration at 5×10^6 cells/mL, processed for each case, and divided into nonstimulation and stimulation groups. The former was incubated for 15 min at 37°C, while the latter was incubated first for 5 min at 37°C with cytochalasin B (5 μg/mL), and then separately with a 10-min additional stimulation using two different concentrations of FMLP, at 10 nmol/L and 100 nmol/L. After the indicated period of incubation, the reaction was stopped by immediate cooling in an ice bath. The reaction mixture was centrifuged at 1500 × g for 10 min at 4°C in an Eppendorf microfuge (12). The supernatant (termed "first supernatant") was decanted and stored at 4°C until experimental use. The pellets were, on the other hand, resuspended in HANKS saline with 0.2% (w/v) Triton X-100, which were then ultrasonicated in an ice bath at three 20-s bursts (50% duty cycle) using an ultrasonic disrupter (Model UD-201, TOMY Co., Tokyo, Japan) with an output of 2.0 (20 watts). This homogenate was further ultracentrifuged at 105,000 × g for 30 min, and the soluble fraction (termed "second supernatant") was also decanted for subsequent analysis.

Determination of β-glucuronidase Activity

The β-glucuronidase included in the two kinds of samples: the first and second supernatants were assayed with 4 MU compounds as substrates, where enzyme activity was determined by incubating 100 μl of the sample with 100 μl substrate (10 mmol/L 4-methylumbelliferyl-β-D-glucuronide dissolved in 0.1 mol/L sodium acetate pH 4.0, containing 0.1% Triton X-100) for 15 min at 37°C. The 4-methylumbelliferone formed was measured fluorometrically (excitation: 365 nm, emission: 460 nm) in a spetrofluorometer (Model 650-40, Hitachi, Ltd., Tokyo, Japan). β-glucuronidase activity was calculated and represented in 4-methlumberiferone nmol/min per 5×10^6 cells (8,9). From our preliminary study, the intraassay variation was found to have a mean value of 7.6% (n = 10) (data not shown).

For both the first and second supernatants, the mean β-glucuronidase activity obtained from three independent measurements for each subject was used as a measurement.

Variable Definition

Using β-glucuronidase as an indicator, the extent to which neutrophil could release granules was measured in two ways: (1) total β-glucuronidase activity = total of both the first and second β-glucuronidase activity; (2) degranulation proportional release (%) = (first supernatant β-glucuronidase activity)/(total of both the first and second β-glucuronidase activity) × 100.

Statistical Analysis

Differences in variables: age, gravidity, parity, mean arterial pressure, total β-glucuronidase activity, and degranulation proportional release between the three groups (nonpregnant, normal pregnant, and preeclamptic women) were first assessed using the Kruskal Wallis *H*-test for multigroup comparisons. When found to be significantly different, the data were further analyzed using the Mann-Whitney *U*-test to compare any pair of groups (13). Multiple comparisons were performed using the Bonferroni method to examine the differences in distribution between normal nonpregnant, normal pregnancy and preeclampsia. Thus, the reported P values are corrected according to Bonferroni (13,14). Comparison of the gestational week between the 2 pregnant groups was also made using the Mann-Whitney *U*-test. Differences were considered significant when $p < 0.05$.

RESULTS

Total β-glucuronidase Activities

In nonpregnant, normal pregnant and preeclamptic women, total β-glucuronidase activity was 5.1 (4.3–5.8) (median (range)), 5.2 (4.2–6.2) and 3.5 (2.7–4.3) nmol/min/5 × 10^6 cells, respectively (Fig. 1). The value in preeclampsia was significantly lower than in the other two subject groups, whereas no difference was noted between the nonpregnant and the normal pregnant groups.

Neutrophilic Degranulation Abilities

Non-Stimulation Group

In nonpregnant, normal pregnant, and preeclamptic women, degranulation proportional release was 5.4 (4.1–6.2) (median (range)), 5.3 (5.0–5.6) and 10.4

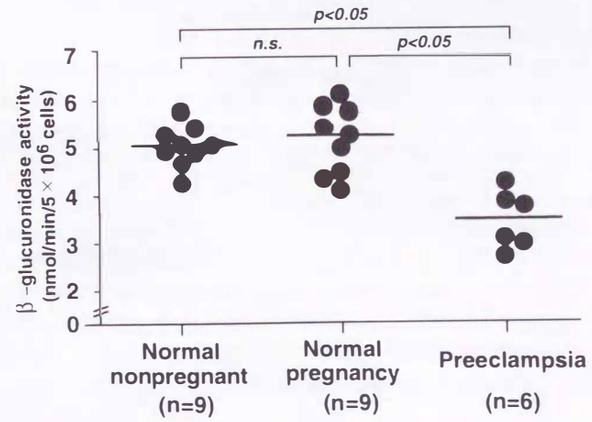

Figure 1. Total neutrophil β-glucuronidase activities in nonpregnant women, normal third-trimester pregnancies, and pregnancies with preeclampsia. *Horizontal bars* = median values. n.s. = not significant. $p < 0.05$ based on the corrected *p*-values for multiple comparisons.

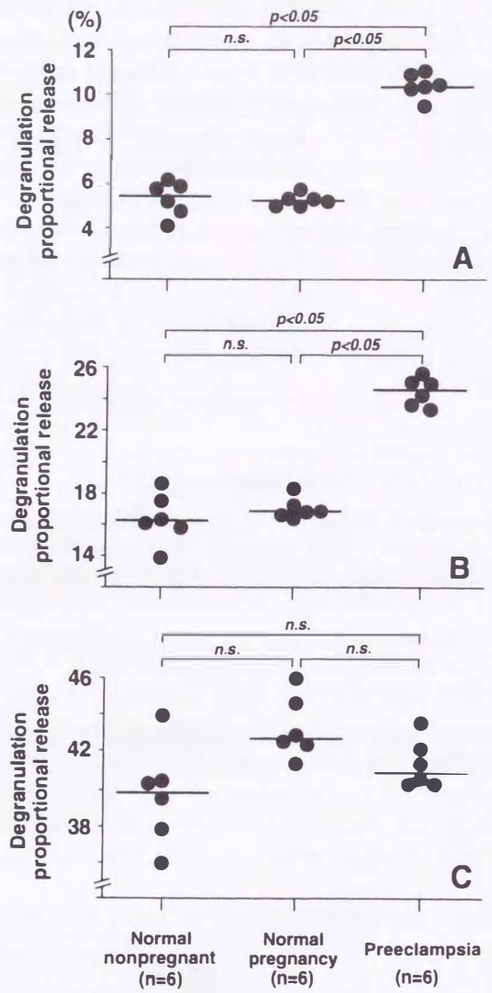

Figure 2. The degranulation proportional release (%) of neutrophil β-glucuronidase in nonpregnant women, normal third-trimester pregnancies, and pregnancies complicated by preeclampsia, with (A) nonstimulation, (B) 10 nmol/L-FMLP stimulation, and (C) 100 nmol/L-FMLP stimulation. *Horizontal bars* = median values. n.s. = not significant. $p < 0.05$ based on the corrected *p*-values for multiple comparisons.

(9.6–11.0)%, respectively (Fig. 2A). The degranulation proportional release in preeclampsia was significantly higher than in the other two subject groups, although there was no difference between the nonpregnant and the normal pregnant subject.

10 nmol/L-FMLP Stimulation Group

In nonpregnant, normal pregnant, and preeclamptic women, degranulation proportional release was 16.2 (13.9–18.6), 16.9 (16.4–18.3) and 24.6 (23.3–25.3)%, respectively (Fig. 2B). Preeclamptic women showed significantly higher values than those in the other two groups.

100 nmol/L-FMLP Stimulation Group

In nonpregnant, normal pregnant, and preeclamptic women degranulation proportional release was 39.9 (35.8–43.9), 42.7 (41.3–45.9) and 40.8 (40.1–43.5)%, respectively (Fig. 2C), without statistical significance.

DISCUSSION

In this study, considering β-glucuronidase measurement in the supernatant first obtained as a variable indicating extracellular activity and that obtained in the second supernatant as a variable implying intracellular activity, we examined both total β-glucuronidase activity and the degranulation proportional release of neutrophils, in nonpregnant, normal pregnant and preeclamptic women.

In contrast to the indirect measurement of plasma elastase concentration, derived from neutrophil using radioimmunoassay (7), our experimental system directly measures neutrophil degranulation activity, i.e., with β-glucuronidase, using fluorimetrical enzyme release assay on granulocyte suspension.

In nonpregnant and normal pregnant women, both total β-glucuronidase activity and the spontaneous degranulation proportional release of neutrophils are consistent with previous reports (total β-glucuronidase activity: around 1.0 nmol/min/10^6 cells (9,12) and spontaneous degranulation proportional release: 3–6%) (9,15,16). In preeclampsia, however, the total β-glucuronidase activity was significantly lower, and the spontaneous degranulation proportional release was significantly higher, compared with the other two subject groups studied. These findings demonstrate that neutrophil degranulation is already activated in this disorder, with a decrease in the total granule content retained in the neutrophil.

FMLP is well known to stimulate the degranulation function of neutrophils under pretreatment with cytochalasin B, in a dose-dependent manner, such that the neutrophil produces O_2^-.

In preeclampsia, when neutrophils are stimulated with 10 nmol/L FMLP, higher degranulation values are found, whereas no significant difference is noted

with 100 nmol/L FMLP stimulation. Using 1 μmol/L FMLP, no significant difference was found in degranulation proportional release between nonpregnant and preeclamptic neutrophils (42.5% versus 43.5%). Moreover, during 10 nmol/L-FMLP stimulation, differences between preeclampsia and nonpregnancy, and between preeclampsia and normal pregnancy are almost equal in value to those in spontaneously occurring reactions. These findings suggest that a possible modification mechanism exists, specific to neutrophil degranulation in preeclampsia, different from the O_2^- producing system, since enhanced FMLP-induced O_2^- production was seen only in the neutrophils obtained from preeclamptic cases (17).

In our preliminary study, without FMLP, the superoxide dismutase inhibitable reduction of ferricytochrome *c* was undetectable in preeclamptic neutrophils (17). In addition, sera from preeclampsia did not have the potential of direct activation on neutrophil O_2^- production. These observations indicate that NADPH oxidase, which catalyzes O_2^-, remains inactive in preeclampsia, until the neutrophilic cells become activated by some stimulants, in contrast with the degranulation reaction.

Various factors such as granulocyte and granulocyte-macrophage colony-stimulating factor, tumor necrosis factor, interferon-γ, interleukin-1, interleukin-8, and endothelin-1, have been reported to influence neutrophil degranulation reaction. When comparing preeclamptics and normal pregnant women, however, we found no significant differences in the serum concentrations of the above factors (17). It remains unclear whether or not cytokine cascades were activated.

Proteases included in the primary (azurophil) granules, such as elastase, chymotripsin, and cathepsin G, have an enhanced effect on the O_2^- production of neutrophils (18). Elastase and cathepsin G have been reported to generate the potent vasoconstrictor angiotensin II (19,20). These proteases also initiate platelet aggregation (21) and participate in adhesion molecule expression (22).

Referring to these reports, we deduce that substances released by the degranulation process may cause a cell-to-cell interaction activating O_2^- production. Subsequently, the combined action of proteases and oxidants seems to be necessary in the pathogenic manifestation of preeclampsia.

Our results may therefore lead to a better understanding of the activation of neutrophil degranulation function when attempting to study the triggering system involved in vascular complications of preeclampsia.

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