An evaluation of the activity of histidine-rich glycoprotein on differentiated neutrophil-like cells from human cell lines

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differentiated neutrophil-like HL-60, dHL-60
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human serum albumin, HSA
Microchannel array flow analyzer, MC-FAN
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Nickel-nitrilotriacetic acid, Ni-NTA
N-formyl-L-methionyl-L-leucyl-phenylalanine, fMLP
nitroblue tetrazolium, NBT
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Abstract

Background: Histidine-rich glycoprotein (HRG) treatment ameliorated the survival rate of septic mice by suppressing excess immunothrombus formation. Although such findings suggested that HRG may be one of the most useful drugs for sepsis, obtaining a stable experimental system to standardize the HRG drug product is difficult to achieve using neutrophils isolated from volunteers. This is due to the short survival time and individual differences of human neutrophils. In the present study, we determined whether the differentiated neutrophil-like cell lines exhibited similar responses to HRG compared to human purified neutrophils. Method: All-trans retinoic acid (ATRA) was employed to induce the differentiation of the human myeloid leukemia cell lines, HL-60 and NB-4. Thereafter, the cells were treated with Hank's balanced salt solution, human serum albumin, or HRG. The effects of HRG on these cells were evaluated according to cell shape, microcapillary passage, ROS production, neutrophil extracellular traps (NETs) formation, the expression of activated CD11b, and cell viability. Result: HRG maintained the round shape of differentiated neutrophil-like cells, decreased the time required by cells to pass through the microcapillaries, and inhibited ROS production, NETs formation, and the expression of activated CD11b on the cell surface. Moreover, the cells could survive longer in the presence of HRG than the control. Conclusion: The ATRA-induced differentiated cell lines could be used as alternatives to neutrophils to investigate the effects of HRG on neutrophils. This method can thus be used as
an essential standardization test in pharmaceutical development.

**Significance Statement**

Human neutrophils exhibit varying responses to histidine-rich glycoprotein (HRG); however, all-trans retinoic acid-induced differentiated neutrophil-like cell (NLC) lines can be used as reliably proxies to investigate the effects of HRG on neutrophils. Additionally, these cell lines can be employed in the development of therapies for the treatment of sepsis.
Introduction

HRG is a 75-kDa glycoprotein characterized by many histidine residues and GHHPH repeat sequences, synthesized in and secreted from the liver (Borza, Tatum, & Morgan, 1996; Koide, Foster, Yoshitake, & Davie, 1986; Poon, Patel, Davis, Parish, & Hulett, 2011). It exists in healthy human plasma at 60-100 µg/mL (approximately 1 µM) (Poon et al., 2011) and is reported to possess a variety of physiological activities, including the suppression of erythrocyte aggregation (Zhong et al., 2018), vascular endothelium protection (Gao et al., 2019; Gao et al., 2020), and regulation of blood cell adhesion (Wake et al., 2016). In the animal experiments, we reported that serum HRG levels in septic mice with cecal ligation and puncture (CLP) markedly decreased while their survival rate increased after HRG supplementation, thus HRG was identified as an effective therapeutic strategy for sepsis (Wake et al., 2016). In the clinical studies, it was suggested that plasma HRG level may be not only an excellent biomarker for septic patients but also a prediction factor for the prognosis of septic patients in an intensive care unit (Kuroda et al., 2018; Nishibori, Wake, & Morimatsu, 2018).

In 2016, a major revision to the definition of sepsis was performed. As a result, sepsis is now defined as a “life-threatening organ damage due to uncontrolled host reactions to infections” (Singer et al., 2016). The global burden of sepsis has been increasing, which require progressive and strategic treatment for patients with the life-threatening condition
(Angus & Poll, 2013; Fleischmann et al., 2016; Rhee et al., 2017). However, there is no specific treatment for sepsis, thereby contributing to its high mortality (Reinhart et al., 2017). Therefore, discovering an alternative biomarker and a therapeutic target for sepsis is urgently required (Opal, Dellinger, Vincent, Masur, & Angus, 2014; Reinhart, Bauer, Riedemann, & Hartog, 2012). We have already started the project to develop human plasma-derived HRG preparation for the treatment of sepsis. Thus, to standardize purified HRG as a biological material, it is imperative to establish the convenient and reliable assay system for evaluating each lot of HRG prepared from pooled plasma which would be used as the treatment for septic patients.

In vitro experiments using human purified neutrophils, in particular, showed that the passage of neutrophils through a microvascular model was remarkably improved by HRG supplementation as it maintained the spherical neutrophil shape with smooth surface without microvilli and modified their adhesiveness without attachment to the vascular wall model (Wake et al., 2016). HRG also inhibited reactive oxygen species (ROS) production as well as neutrophil extracellular traps (NET) formation from neutrophils, and cell death (Wake et al., 2016). Collectively, HRG could maintain the quiescent state of neutrophils. The rapid decrease in plasma levels of HRG in septic mice might result in a loss of its regulatory effects on circulating neutrophils, leading to the attachment of neutrophils to vascular endothelial walls and the intravascular formation of immunothrombus.
Because the response of neutrophils to severe infections is closely related to the pathology of sepsis, identifying the effects of HRG on neutrophils is crucial. However, owing to the difficulty associated with the use of human neutrophils in experiments because of their short lifespan, the individual differences in neutrophils among donors, and the challenges to establish a stable experimental system, the establishment of standardized cells to estimate the effects of human plasma-derived HRG is desirable. Therefore, we examined the effects of HRG on the all-trans retinoic acid (ATRA)-induced differentiated neutrophil-like cells (NLCs) from HL-60 and NB-4 (Barber, Belov, & Christopherson, 2008; Gupta, Shah, Malu, Berliner, & Gaines, 2014). Herein, we aimed to determine whether the NLCs could be a reliable proxy for neutrophils in HRG- and sepsis-related researches.
Materials and Methods

Reagents

Nickel-nitrilotriacetic acid (Ni-NTA) agarose gel was obtained from Qiagen (Hilden, Germany). N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) was obtained from Peptide Institute, Inc. (Minoh, Japan). ATRA, nitroblue tetrazolium (NBT), isoluminol, horseradish peroxidase type IV and zymosan A from Saccharomyces cerevisiae, and Phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). Hoechst33342, calcein-AM, and SYTOX orange were obtained from Life Technologies (Carlsbad, CA). Anti-histone H3 Ab (citrulline R17 +R2 + R8) (cit-histone H3) and Alexa Fluor 488-labeled anti-rabbit IgG Ab were obtained from Abcam (Cambridge, UK). FITC-labeled anti-human activated CD11b Ab (clone: CBRM1/5) was obtained from eBioscience (San Diego, CA). Blocking One was obtained from Nacalai Tesque (Kyoto, Japan). Anti-human HRG monoclonal antibody (Rat) and anti-human HRG polyclonal antibody (Rabbit) were synthesized in our laboratory.

Isolation of human neutrophils from human peripheral blood

Peripheral blood polymorphonuclear cells were purified from healthy donors using Polymorphprep density gradient media (Alere Technologies AS, Oslo, Norway). In line with the ethical guidelines of Okayama University, written informed consents were obtained from
healthy volunteers (n = 5), and we drew blood from the cubital vein. In brief, heparinized peripheral blood were layered over Polymorphprep and centrifuged at 500 × g for 35 minutes. Polymorphonuclear cells were then harvested and diluted with PBS. After centrifugation at 400 × g for 10 minutes, the purity of neutrophils was confirmed to be more than 95% under microscope. Subsequently, neutrophils were used at appropriate concentrations for each experiment.

**HRG purification from human plasma**

HRG was purified from human plasma (obtained from the Japanese Red Cross Society) by affinity chromatographic methods using Ni-NTA, as previously described (Mori, Takahashi, Yamaoka, Okamoto, & Nishibori, 2003). Briefly, protease inhibitors were added to human plasma. Thereafter, the mixture was centrifuged twice at 10,000 rpm at 4 °C for 15 minutes. The supernatant was incubated with Ni-NTA agarose gel, which was pre-equilibrated with 10 mM Tris-buffered saline (TBS, pH 8.0) and suspended in the same buffer. After gently mixing for 2 h at 4 °C, the gel suspension was transferred into a column, washed with 50 mM imidazole in 10 mM TBS (pH 8.0), 2 M NaCl in 10 mM TBS (pH 8.0), and 100 mM imidazole in 10 mM TBS (pH 8.0) to enable the passage of all unbound proteins through the column. Finally, HRG was eluted with 10 mM TBS (pH 8.0) containing 500 mM imidazole. After exchanging the buffer by dialysis, the eluate was loaded onto a Mono Q column (GE
Healthcare, Little Chalfont, United Kingdom) and separated by an NaCl gradient. The purified HRG was identified and collected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a western blot with an anti-human HRG polyclonal antibody. The obtained human HRG was stored at −20 °C until use.

**Differentiation of HL-60 and NB-4 by ATRA**

The human acute myelogenous leukemia (AML) cell lines, HL-60 (at the myeloblast stage of development, AML-M2) and NB-4 cells (at the promyelocyte stage of development, AML-M3), were obtained from RIKEN Cell Bank (RCB0041) and CLS Cell Lines Service (GmbH). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific, Waltham, MA), L-glutamine (Sigma-Aldrich), and penicillin Streptomycin (Thermo Fisher Scientific), and maintained in a humidified 5% CO₂ environment at 37 °C. The media were routinely passaged and diluted every 3-4 days to maintain exponential growth. All experiments were performed with cells from passages between 10 and 25.

ATRA (Sigma-Aldrich) was dissolved in 99.5% ethanol as a 10 mM stock solution. The solution was stored at -80 °C until use. The final concentration of ethanol in the culture was maintained at less than 0.1% v/v. HL-60 or NB-4 cells were diluted to 2.5 × 10⁵ cells/ml in RPMI-1640 culture medium supplemented with 10% FBS. ATRA was added to the HL-60
and NB-4 cell culture to yield the final concentrations of 10 mM and 1 mM, respectively.

HL-60 and NB-4 were passaged two or three days after the addition of ATRA. Live cells were isolated with Dead Cell Removal Kit per the manufacturer’s protocol (Miltenyi Biotec Inc., Auburn CA).

The NBT reduction test is one of the neutrophil function tests that examines the phagocytic activity and function of neutrophil enzyme systems (Blair, Carbone, & Sartorelli, 1985). This method was used as a functional measure of the maturation of HL-60 and NB-4 cells. A total of $1.0 \times 10^6$ cells was collected by centrifugation and resuspended in 0.25 ml of RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific). Subsequently, 0.25 ml of 0.1% NBT with 5 µl PMA was added to each sample, and the cell suspensions were incubated at 37 °C for 30 min. Samples were then fixed by the addition of an equal volume of 2% paraformaldehyde in PBS. Cells were observed with a BZ-8000 microscope (Keyence Corporation, Osaka, Japan). If the cells were differentiated into neutrophils, the yellow NBT dye was internalized, thereby resulting in the formation of black formazan due to reduction. The stained and non-stained cells were counted manually under the microscope. Both differentiated neutrophil-like HL-60 cells (dHL-60) and differentiated neutrophil-like NB-4 cells (dNB-4) showed stable differentiation rates (Figure 1A, B, C, and D).
Observation of the morphological changes in differentiated cells

To clarify the effects of HRG on the morphology of dHL-60 and dNB-4 cells, cell shape was observed by calcein-AM (for cytosol) and Hoechst33342 (for nuclei) staining as described previously (Wake et al., 2016). Thereafter, 100 µl (5×10^4 cells/well) of the two cell lines and human neutrophils (as a positive control) were aliquoted into each polystyrene well. The cells were incubated with different concentrations of HRG (0.01 to 1 µM) in the presence of control IgG or anti-HRG Ab, human serum albumin (HSA; 1 µM), BSA (1 µM), or fMLP (1 µM) at 37 °C for 1 h. The In Cell Analyzer 2000 System (GE Healthcare, Little Chalfont, UK) was used for morphological observation. The data were analyzed with the In Cell Investigator Version 1.62 (GE Healthcare, Little Chalfont, UK). Additionally, the morphological characteristics of cells were assessed by cell area (µm^2/cell); the inclusion area per cell in the extended cytoplasm area was automatically determined using the In Cell Analyzer 2000 system.

Microchannel array flow analyzer (MC-FAN)

The cell samples were prepared as described above. Briefly, the cells were incubated with either of the following reagents (HRG, HSA, BSA, fMLP: each at a final concentration 1 µM) at 37 °C for 1 hour. Thereafter, the passage of cells through the microcapillary slits (7.0 µm width, 4.5 µm depth) was evaluated by MC-FAN (MC Lab, Japan). Briefly, the cells
flowed through the silicon-based artificial microchannels under a constant suction of –20 cm H₂O. Thereafter, the passage time of the 100-μl sample through the microcapillaries was determined. The passage time are dependent on both the cell size and the cell adhesiveness, and the cells adhere to the silicon-coated microchannels based on their adhesiveness.

**Expression analysis of activated CD11b on the cell surface by flow cytometry**

Both dHL-60 and dNB-4 samples were stimulated with 100 ng/ml of PMA in the presence of one of the reagents (Hank's balanced salt solution (HBSS), HSA, or HRG, each at a concentration of 1 μM) for 1 h at 37 °C in a 5% CO₂ atmosphere. Thereafter, the cells were stained with FITC-labeled anti-human-activated CD11b antibody for 25 min at 4 °C and fixed with 0.5% PFA in PBS for 30 min at 4 °C. The extent of activated CD11b expression in cells was analyzed using MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).

**Determination of extracellular ROS production**

Because of the short half-life and reactivity of ROS, direct observation of the amount of ROS production is challenging. Under the presence of ROS, horseradish peroxidase type IV oxidize isoluminol and induce luminescence. Our method harnessed the reaction and observed the amount of ROS production indirectly. Briefly, isoluminol (final concentration,
50 µM) and horseradish peroxidase type IV (final concentration, 4 U/ml) were added to the
dHL-60 and dNB-4 cell suspensions. Thereafter, the suspensions (100 µl; 5 × 10⁵ cells) were
aliquoted into a 96-well plate (BD Biosciences). Subsequently, HRG, HSA (each at a final
concentration of 1 µM), or HBSS was added to each well. In addition, the zymosan
suspension (final concentration of 100 ng/ml) was simultaneously added to each well.
Extracellular ROS production was measured ten times (every 5 min interval) from the
beginning of incubation at 37 °C by detecting chemiluminescence intensity using Flexstation
3 (Molecular Devices, Sunnyvale, CA).

Observation of NET formation

Citrullination of histone H3 and H4 by peptidylarginine deaminase 4 is required for
chromatin de-condensation, which is an essential step for NET formation. We observed NET
formation using citrullinated histone H3 (cit-histone H3) with immunocytochemical analysis.
The dHL-60 and dNB-4 cells were prepared, as mentioned above. For dead cell imaging, the
cells were treated, in advance, with a cell-impermeable DNA fluorescent dye (5 µM SYTOX
orange). PMA 100 ng/ml and one of the reagents (HBSS, HSA, HRG, each at concentration
of 1 µM) were added to the cell suspensions. Thereafter, the cell suspensions (100 µl; 5 × 10⁵
cells) were aliquoted into a 96-well plate (BD Biosciences). The cells were incubated for 2h
(neutrophils) or 4 h (dHL-60 and dNB-4 cells) at 37 °C in 5% CO₂ to stimulate neutrophil
extracellular traps (NETs) formation. Subsequently, the supernatant was gently removed, and
the cells were fixed with 4% of PFA for 20 min at 4 °C. To evaluate NET formation by
immunocytochemical analysis, the samples were permeabilized with 0.1% Triton X-100 in
TBS (T-TBS) for 1 min and blocked with Blocking One for 10 min at room temperature. After
the addition of the primary antibody (anti-cit-histone H3 Ab at 10 μg/ml) to the samples, the
samples were incubated for 2 h at room temperature. After 3 rounds of washing with TBS, the
secondary antibody (Alexa Fluor 488-labeled anti-rabbit IgG at 2 μg/ml) was added to the
samples followed by incubation for 1 h at room temperature. Dead cells (SYTOX orange
positive site) and NET formation (cit-Histon H3 positive site) were observed using the In Cell
Analyzer 2000 system.

**Cell viability**

Purified human neutrophils, dHL-60, and dNB-4 cells were prepared, as mentioned
above. To evaluate cell viability, live cells were stained by calcein-AM (for cytosol) for 15
min at 37 °C in 5% CO₂. PMA 100 ng/ml was added to the cell suspensions in the presence
of one of the reagents (HBSS, HSA, HRG, each at a concentration of 1 μM). Each suspension
(100 μl; 5 × 10⁵ cells) was aliquoted into a 96-well plate (BD Biosciences) and incubated for
8 h at 37 °C in 5% CO₂. Cell viability was defined as the percentage of live cells at 8 h
post-incubation. The number of live cells was calculated using the In Cell Analyzer 2000 system based on the presence of calcein-AM staining.

Statistical Analysis

Statistical significance was evaluated by Student’s t-test for comparisons between two groups or Tukey’s test for multiple comparison. All data are presented as the mean value ± SD. The $P$-values less than 0.05 was considered as to be statistically significant. The analyses were conducted with SPSS software version 24 (SPSS, Chicago, IL, USA).
Result

Effects of HRG on the morphology of dHL-60 and dNB-4 cells

The dHL-60 or dNB-4 cells pre-stained with calcein-AM (for cytosol) and Hoechst33342 (for nuclei) were observed using the In Cell Analyzer 2000 System. The morphological characteristics of cells were assessed according to the cell area. HRG reduced the cell volume of dHL-60 and dNB-4 and the purified human neutrophils in a dose-dependent manner, and exhibited a significant effect at concentrations ≥ 0.03 μM (Fig. 2A, B). When 1 μM of HRG was assumed to cause the maximal effects, the EC50 of HRG that caused the less-surface-area effect on dHL-60, dNB-4, and neutrophils was 0.046 μM, 0.060 μM, and 0.056 μM respectively. These effects of HRG were not observed with human serum albumin (HSA), bovine serum albumin (BSA), and fMLP (Fig. 2C, D). Additionally, HRG significantly reduced the cell surface-area in both cell lines and human neutrophils(Fig. 2C, D). The effect of HRG on dNB-4 was inhibited by the addition of three types of anti-HRG antibodies and human neutrophils. However, in dHL-60, there was a significant reversal in the effect of HRG by the addition of the mouse monoclonal anti-HRG antibody (IgG2b) (Figure 2E, F, G, and H).

HRG decreased the adhesion property of dHL-60 and dNB-4 cells
To examine the effects of HRG on the adhesion property of dHL-60 and dNB-4, the passage of cells through the microcapillary was observed using MC-FAN. Owing to the abundant cytoplasm and high adhesiveness, cells without HRG treatment did not smoothly pass through the slits. Moreover, the flow path was often completely blocked around the slits (red arrowhead) (Figure 3A). In the group treated with HRG (1 μM), the cells could pass through the microcapillaries at a significantly faster rate than the other groups (Figure 3B). Subsequently, we determined whether HRG (1 μM) inhibited the activated-CD11b expression on the surface of cells by flow cytometry. The extent of activated-CD11b expression on cells was analyzed using the MACSQuant Analyzer. HRG significantly decreased the cell surface activated-CD11b expression on dHL-60 (Figure 4A-B). Additionally, HRG significantly reduced the activated-CD11b expression on dNB-4 compared to the HBSS control. However, the effect was not statistically significant compared to the HSA group (p= 0.076).

**HRG inhibited ROS production and NET formation in dHL-60 and dNB-4**

To determine the production of ROS by the two differentiated cell lines, we measured the chemiluminescence produced by the oxidation of isoluminol. Because the differentiated NLCs cannot produce ROS without additional stimulation, zymosan was employed to induce ROS production. Thus, 10 mg/ml of the zymosan suspension was added in the presence of HRG (1 μM), HSA (1 μM), or HBSS to both dHL-60 and dNB-4. After 20 min of incubation
at 37 °C, extracellular ROS production was evaluated. Figure 5 demonstrates the significant inhibition of zymosan-induced ROS production in dHL-60 and dNB-4 caused by HRG compared to that observed in the other groups. To assess the effect of HRG on NET formation, PMA, a chemical inducer of NET formation, was added to the dHL-60 and dNB-4 cells pre-stained with SYTOX orange (cell-impermeable DNA fluorescent dye), in the presence of one of the reagents (HBSS, HSA, or HRG, each at a concentration of 1 μM). Thereafter, the cells were incubated either for 2h (neutrophils) or for 4 h (dHL-60 and dNB-4 cells) at 37 °C in 5% CO₂ on a plastic 96-well plate. NET formation was evaluated as a cit-histone H3 positive site by immunocytochemical analysis on the In Cell Analyzer 2000 system with the cell area. Compared to the HBSS and HSA groups, HRG significantly inhibited NET formation in both dHL-60 and dNB-4 cells (Figure 6A, B, and C).

dHL-60 and dNB-4 survived longer than purified human neutrophils and HRG extended their survival

After the dHL-60 or dNB-4 cells were pre-stained with calcein-AM (for cytosol) and Hoechst33342 (for nuclei), the number of live cells was counted using the In Cell Analyzer 2000 System. Neutrophils viability was significantly lower than that of dHL-60 and dNB-4 at 8 h after incubation (Figure 7A).
PMA was added in the presence of the reagents (HBSS, HSA, HRG, each at a concentration of 1 μM) to cells for an 8-h incubation at 37 °C in 5% CO₂. PMA stimulation markedly reduced the viability of all three cells; however, the addition of HRG significantly prolonged the survival time of every cell type under PMA stimulation (Figure 7B, C, and D).

Discussion

The promyelocytic leukemia cell lines, HL-60 and NB-4, are known to differentiate into neutrophil- or monocyte-like cells after induction by chemical stimuli (Gupta et al., 2014; Idres, Benoît, Flexor, Lanotte, & Chabot, 2001; Jasek, Mirecka, & Litwin, 2008). ATRA has been used to induce the differentiation of these cell lines into NLCs, which are often characterized by the NBT reduction test (Blair et al., 1985). In the present study, we obtained more than 80% NBT-positive cells from both HL-60 and NB-4 when the cells were exposed to 10 μM and 1 μM of ATRA for five days. Thus, the procedure was simple and reliable. Additionally, it repeatedly yielded a considerable number of NLCs. In previous studies (Gao et al., 2019; Wake et al., 2016), HRG was observed to induce the round shape of neutrophils, their smooth surface, and few microvilli. These neutrophils could easily pass through the microchannels and cause the lowest levels of spontaneous ROS production and less adhesion to the vascular endothelial cells and the artificial polystyrene wear. Therefore, we determined whether NLCs differentiated from HL-60 and NB-4 by ATRA exhibited similar responses to
HRG to evaluate the usefulness of the differentiated cells in the standardized regulation tests for the purified HRG materials from plasma.

One of the major effects of HRG on neutrophils involves the induction of a round shape with a smaller diameter and a smooth cellular surface as revealed by scanning electron microscopy. Under septic conditions, neutrophils are exposed to various immunochemical stimuli, which leads to cellular enlargement. Neutrophil enlargement is a result of over-activation, where they are unable to function properly. These over-activated cells have excessive cell adhesiveness to microcapillaries, leading to immunothrombus formation and subsequent worse clinical outcomes in septic patients (Wake et al., 2016). In the present study, we observed similar concentration-dependent morphological effects of HRG on the differentiated NLCs from HL-60 and NB-4. However, these effects were inhibited by the addition of the antibody against HRG, thereby indicating the specificity of the effect of HRG. The morphological effects of HRG on neutrophils and NLCs were comparable according to the potencies of HRG on each cell as determined with the concentration-effect curves. The EC50 values for neutrophils, HL-60, and NB-4-derived cells were 0.056 μM, 0.046 μM, and 0.060 μM, respectively, suggesting a similar mechanism of action of HRG on human neutrophil and NLCs. The most distinctive physiological effects of HRG is considered as the spherical shape-inducing effect, and in our laboratory, we assess the quality of purified HRG with the extent of spherical shape-inducing effects on neutrophils as well as a western blotting.
Notably, the effective concentrations of HRG on human vascular endothelial cells were within the same range (Gao et al., 2019). HRG inhibited the PMA-induced upregulation of the expression of the activation epitope on CD11b on NLCs as observed in neutrophils (Wake et al., 2016).

Because the activation of CD11b is related to the functional control of cellular adhesion through LFA-1 on neutrophils, NLCs might receive functional regulation by HRG. Although NLCs have a larger size than human neutrophils, measuring the time taken by NLCs to pass through the microchannels of MC-FAN revealed that treatment with HRG reduced the time required for the passage of 100 µl of cell suspension compared to those treated with other proteins. Further, HRG significantly prolonged the survival time of NLCs and neutrophils under the stimulation of PMA. Excessive immunothrombus formation secondary to neutrophil intravascular cell adhesion detonates multiorgan dysfunction in septic patients, leading to high mortality. Previous studies suggested that HRG reduced the immunothrombus formation (Wake et al., 2016) and reduced HRG concentration in severe septic patients (Kuroda et al., 2018). Altogether, analyzing neutrophil flow provides insight into the neutrophil function in sepsis, and these results suggest that NLCs responded to HRG in a very similar manner to human neutrophils.

Recent studies suggest that the pathophysiological cascade in septic conditions may include excessive ROS production in neutrophils, which is associated with NETosis, leading
to the formation of immunothrombi. Although NETosis was initially characterized as a barrier system to prevent the diffusion of invasive bacteria, more global pathophysiological roles have been suggested (Brinkmann et al., 2004; Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010; Phillipson & Kubes, 2011; Remijsen et al., 2010). The intravascular events that simultaneously damage the vascular endothelial cells should exacerbate microcirculation in the tissues and quickly produce multiple organ failure (MOF) and disseminated intravascular coagulation (DIC). Herein, we used NLCs to demonstrate the NETosis inhibiting effects of HRG. Thus, NLCs could be used to understand the mechanisms of NETosis and the effects of HRG on this process.

This study had some limitations. First, although standardized NLCs were produced herein, the reproducibility of the experiment has not been confirmed in other laboratories. Second, the responsiveness of the NLCs to HRG may not be identical to that of neutrophils as NLCs are derived from the differentiation of the human myeloid leukemia cell lines. Third, the reactivity of NLCs might be subject to cultural and differentiation-inducing conditions. Despite the limitations with NLCs, several issues arise when the mechanism used by HRG to modulate the shape or function of human neutrophils is investigated. First, human neutrophils are very short-lived and have a circulating half-life of <8 h (Summers et al., 2010). As shown in Fig. 7D, isolated neutrophils also have a very short life span in the culture media. Second, there might be individual variations in survival time and responsiveness after isolation. Thus,
it is impractical to conduct regulatory tests with isolated human neutrophils to evaluate the activity of purified HRG as a candidate drug for the treatment of sepsis (Kuroda et al., 2018; Nishibori, Wake, & Morimatsu, 2018; Wake et al., 2016). However, NLCs from HL-60 and NB-4 may be competent replacements for this purpose. Additionally, differences in the control test response and responsiveness to HRG were identified between NLCs derived from HL-60 and NB-4.

In the present study, ATRA was employed to induce the differentiation of the human myeloid leukemia cell lines, HL-60 and NB-4, into NLCs. Based on our findings, the differentiated NLCs had very similar reactivity to HRG relative to human neutrophils. Additionally, a stable experimental system could be easily obtained. Altogether, the methods developed herein to acquire the differentiated NLCs could be an essential tool not only for researchers but also for pharmaceutical developers to drive the sustainable drug-discovery efforts for sepsis treatment.

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Authorship Contributions

Participated in research design: Yukinori Yoshii, Hidenori Wake, Kiyoshi Teshigawara, Dengli Wang and Masahiro Nishibori

Conducted the experiments: Yukinori Yoshii

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Figure Legends

**Figure 1. Differentiation of the human myeloid cell lines to neutrophil-like cells**

HL-60 and NB-4 are human AML cell lines that can be induced by ATRA to form mature neutrophil-like differentiated cells. (A, B) The NBT-stained dHL-60 and dNB-4 cells were induced for 5 days in 10 μM and 1 μM of ATRA. (C) The ATRA-induced cell differentiation rates were determined by manually counting the NBT positive cells at 10x original magnification. The results are presented as the average of five different visual fields. 80 to 90% of HL-60 and NB-4 cells were differentiated to neutrophil-like cells (dHL-60 and dNB-4 cells, respectively) by ATRA. (D) HL-60 or NB-4 cells were diluted to 2.5 × 10⁵ cells/ml (total 2.5 × 10⁶ cells), and ATRA was added to the HL-60 and NB-4 cell culture. After the induction of cell differentiation, the absolute number of cells was counted. The results are the mean ± SD of 5 experiments. *P < 0.05.

**Figure 2. The effect of HRG on neutrophil-like cell surface area**

The shape of the differentiated cells and purified human neutrophils were analyzed after staining of the cell nuclei and cytosol with Hoechst33342 and Calcein-AM, respectively, for 15 min. Changes in cell morphology were observed with the In Cell Analyzer 2000 System. The data were analyzed with In Cell Investigator Version 1.62. (A) The cells were incubated with 0.01 to 1 μM of HRG. Scale bar, 20 μm. (B) Cell Area (μm²/cell) represents the inclusion
area per cell in the extended cytoplasm area. HRG caused a change in shape to spherical, and
inhibited the irregularity and expanding of the cell shape of dHL-60 and dNB-4 cells and the
human neutrophils according to the concentration. The results are the mean ± SD of 12
experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs HBSS. (C) The cells were incubated
with one of the reagents at the same concentration of 1 μM for comparison. (D) The spherical
shape-inducing effects of HRG were evident. The results are the mean ± SD of 12
experiments. ***P < 0.001 vs HBSS, †††P < 0.001 vs HSA. Scale bar, 20 μm. (E) The cells
were incubated with HSA (1 μM), HRG (1 μM), HRG, and control IgG or anti-HRG Ab (10
μg/mL) for 1 h. The anti-HRG mono/polyclonal Ab could significantly inhibit the spherical
shape-inducing effects of HRG. (F, G, and H) In the NB4 cell line, the change to a spherical
shape was reversed by all antibodies and neutrophils. The results are the mean ± SD of 12
experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar, 20 μm.

Figure 3. HRG ameliorated microcapillary passage of neutrophil-like cells

Cell incubation was initiated with one of the reagents (HRG, HSA, BSA, fMLP: each at a
final concentration of 1 μM) at 37 °C for 60 min. Thereafter, the cells were applied to a
MC-FAN and allowed to flow through silicon-based artificial microchannels under a constant
suction of −20 cm H₂O. (A) Red arrowheads indicate the adhesion of cells to the
microcapillary during the experiment. (B) The passage time of the 100-μl samples through
microcapillaries was determined. The results are the mean ± SD of 6 experiments. \(*\star\star\star P < 0.001 \text{ vs HBSS, } \dagger\dagger\dagger P < 0.001 \text{ vs HSA.}

**Figure 4. The expression level of CD11b on the surface of neutrophil-like cells decreased after HRG treatment**

The dHL-60 or dNB-4 cells were stimulated with 100 ng/ml of PMA for 1 hour at 37 °C in a 5% CO₂ atmosphere. After the cells were stained with FITC-labeled control Ab or FITC-labeled anti-human activated CD11b Ab for 25 min at 4 °C, flow cytometric analysis was performed with the MACSQuant Analyzer. (A) Figures indicate the process used to determine the number of activated CD11b-positive cells under PMA stimulation. Representative HBSS group images of the flow cytometric analysis used to determine activated CD11b expression in the neutrophil-like cells stimulated with PMA. (B) The dHL-60 or dNB-4 cells were stimulated with 100 ng/ml of PMA in the presence of HRG (1 μM), HSA (1 μM), or HBSS for 1 h at 37 °C in a 5% CO₂ atmosphere. The results are expressed as mean ± SD of 9 experiments. \(*P < 0.05 \text{ vs HBSS, } \star\star\star P < 0.001 \text{ vs HBSS, } \dagger\dagger\dagger P < 0.001 \text{ vs HSA.}

**Figure 5. HRG inhibited extracellular ROS production in neutrophil-like cells**

The dHL-60 or dNB-4 cells were stimulated with 100 μg/ml of PMA in the presence of HRG
(1 μM), HSA (1 μM), or HBSS. Thereafter, extracellular ROS production was determined using isoluminol as a substrate. The intensity of the chemiluminescence in the medium was evaluated using Flexstation 3. HRG significantly decreased ROS production after 20 min of incubation at 37 °C. The results are the mean ± SD of 5 experiments. ***P < 0.001.

Figure 6. HRG inhibited NET formation in neutrophil-like cells

The cells were stimulated with 100 µg/ml of PMA in the presence of HRG (1 μM), HSA (1 μM), or HBSS for 2h (neutrophil) or 4 h (dHL-60 and dNB-4). Thereafter, the cells were stained with SYTOX Orange (nuclei: orange) and anti-cit-histone H3 antibody (NETs: green) for fluorescence detection. (A) White arrowheads indicate NETs. Scale bar, 50 μm. (B, C) NETs were objectively evaluated with the rate of cit-histone H3 positive cell number (%) and cell area (μm²/cell) using the In Cell Analyzer 2000 System. HRG inhibited NET formation. The results are the mean ± SD of 12 experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 7. Neutrophil-like cells can survive longer than purified human neutrophils, and the addition of HRG can extend cell survival rates

The dHL-60 and dNB-4 cells, and purified human neutrophils were pre-stained with Calcein-AM (for Cytosol). Thereafter, the cells were incubated with HBSS or 100 μg/ml of PMA in the presence of HRG (1 μM), HSA (1 μM), or HBSS for 8 h at 37 °C in a 5% CO₂ atmosphere. The live cells (calcein positive sites) in each group were counted with the In Cell Analyzer 2000 System. (A) The dHL-60 and dNB-4 cells survived longer than purified human neutrophils after 8 h of incubation in the unstimulated condition. The results are the mean ± SD of 12 experiments. ***P < 0.001 (B, C, and D) PMA stimulation decreased cell survival rates; however, HRG significantly improved cell viability compared to the other reagents after 6 h of incubation, even with PMA stimulation. The results are the mean ± SD of 12 experiments. *P < 0.05, ***P < 0.001 vs PMA+HBSS, †P < 0.05, ††P < 0.01, †††P < 0.001 vs PMA+HSA.
Figure 1
Figure 2A-D

A

dHL-60  
dNB-4  
neutrophil

HRG concentration (μM)

B

Cell Area Reduction (%)

Log [HRG concentration (μM)]

C

DHL-60  
dNB-4  
neutrophil

HBSS  HRG  HSA  BSA  nMLP

Cell Area (μm²/cell)

HBSS  HRG  HSA  BSA  nMLP

**  ***  ****
Figure 2E-H
Figure 3

A

B

Time [sec]
Figure 4

A

Activated CD11b positive

B

((%)

HBSS  HSA  HRG  ***  HBSS  HSA  HRG  *

dHL-60  dHL-60  dNB-4  dNB-4
Figure 5

The figure shows a bar graph illustrating chemiluminescence [RLU] levels under different conditions. The x-axis represents various treatments: no cell, HBSS, HSA, HRG, HBSS, HBSS, HSA, HRG, zymosan, dHL-60, zymosan, dNB-4. The y-axis indicates the chemiluminescence levels in relative light units (RLU). The data is presented with error bars, and significant differences are indicated by asterisks (***) for each condition.
Figure 6A
Figure 6B-C

B

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C

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Figure 7

A

B

C

D

$\%$

(dHL-60 dNB-4 neutrophil)

$\%$

(dHL-60)

$\%$

(dNB-4)

$\%$

(neutrophil)

(hour)

(hour)

(hour)

- HBSS
- PMA + HBSS
- PMA + HSA
- PMA + HRG