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# Retinoic acid induces hypersegmentation and enhances cytotoxicity of neutrophils against cancer cells

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ABSTRACT

Hypersegmentation of nuclei is considered a distinct characteristic of the antitumoral phenotype of neutrophils. Retinoic acid, a metabolite of retinol, reorganizes and induces segmentation of the nucleus during the differentiation of neutrophils. However, the role of retinoic acid in the phenotype polarization of neutrophils has not been fully established. Here, we investigated the effect of retinoic acid on phenotype polarization of neutrophils. Retinoic acid-induced the hypersegmentation of human neutrophils via retinoic acid receptors and mTOR pathways. Retinoic acid-induced hypersegmented neutrophils enhanced neutrophil extracellular traps (NETs) formation in response to phorbol-12-myristate 13-acetate (PMA) and fMLP (N-Formylmethionine-leucyl-phenylalanine) stimulation, and increased cytotoxicity against various tumor cells. Moreover, retinoic acid treatment attenuated tumor growth in a murine model of tumor. Taken together, these results suggests that retinoic acid induces the phenotype polarization of neutrophils to exert antitumor effects.

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## 1. Introduction

Neutrophils are the most abundant circulating leukocytes [1], and play an important role in host defense [2]. Recent studies have highlighted the morphological and functional diversity of the neutrophil subpopulation in the tumor microenvironment [3–6]. Fridlender et al. demonstrated the presence of N1 (antitumoral) and N2 (protumoral) tumor-associated neutrophils (TANs) within the tumor microenvironment [5]. Granot et al. described tumor-entrained neutrophils (TENs), another subpopulation of neutrophils [6]. TENs accumulate in premetastatic lung tissue before the arrival of metastatic cancer cells, and inhibit metastatic seeding through the generation of reactive oxygen species (ROS). These studies clearly indicate the diversity and plasticity of the neutrophil phenotype. The distinct subpopulations of neutrophils in tumors and in circulation have been characterized according to their morphological and functional properties [reviewed in [3]]. Recently, we have found that angiotensin-converting enzyme inhibitors (ACEi) induce the polarization of neutrophils into an antitumoral phenotype by inducing hypersegmentation [4].

Retinoic acid, a metabolite of retinol, is known to play a critical role in neutrophil maturation and differentiation [7,8]. All-*trans*-retinoic acid (ATRA) is currently used as a therapeutic agent in acute promyelocytic leukemia (APL), and induces the differentiation of leukemic cells into neutrophilic cells [8–11]. ATRA also regulates the functional properties of neutrophils such as ROS generation, bactericidal/permeability-increasing protein induction, phagocytosis, and chemotaxis [12–15]. Additionally, retinoic acid is known to neutralize the proinflammatory effects of activated neutrophils, and prevent excessive damage caused by activated neutrophils [16,17]. Moreover, retinoic acid induces hypersegmentation of eosinophil cells and facilitates the sustainment of allergic inflammation [18]. Although these studies clearly suggest a modulatory role of retinoic acid in the differentiation of myeloid cells, the effect of retinoic acid on neutrophil phenotype changes has not been fully studied.

*Abbreviations:* TANs, tumor associated neutrophils; TENs, tumor entrained neutrophils; ROS, reactive oxygen species; NETs, neutrophils extracellular traps; ACEi, angiotensin-converting enzyme inhibitors; ATRA, all-*trans*-retinoic acid; APL, acute promyelocytic leukemia; DCF-DA, 2′7′-dichlorodihydrofluorescein diacetate; RAR, retinoic acid receptor; PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formylmethionine-leucyl-phenylalanine; HDN, high density neutrophils.

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Here, we investigated the effects of retinoic acid on the polarization of neutrophil phenotype. We have hypothesized that retinoic acid polarizes neutrophils into the antitumoral phenotype. To test this hypothesis, we determined the effect of retinoic acid on phenotypic changes of neutrophils and evaluated the antitumoral function of neutrophils.

## 2. Methods

### 2.1. Animals and cells

Animal experiments were approved by the Institutional Animal Care and Use Committee of Hallym University. BALB/c (female, 4 week old) mice were purchased from SAMTAKO (Osan, Republic of Korea). 4T1, COLO-205, Capan-1, and U937 were purchased from the American Type Culture and European Collection of Cell Culture (Manassas, VA). Cells were cultured in DMEM (Gibco, USA, CA) supplemented with 10% FBS (Gibco) and 10 mg/L penicillin/streptomycin (Sigma-Aldrich, USA, St. Louis).

### 2.2. Neutrophil isolation

Human blood experiments were approved by the Institutional Research Board of Hallym University and Kyungpook National University. Neutrophils were isolated from venous blood by double density gradient method using Histopaque 1077 (Sigma-Aldrich) and 5% Dextran (Pharmacosmos, Denmark, Holbeak) [4]. Briefly, heparinized blood was layered over the histopaque solution (1:1) and centrifuged for 30 min at 2500 rpm, RT. The lower layer, rich in RBC and granulocytes, were collected and sedimented using dextran for 45 min at 4 °C. The neutrophil-rich supernatant was collected and remaining RBCs were depleted by hypotonic lysis. The cells obtained were approximately 95% pure (determined by Wright-Giemsa staining) and were finally dissolved in RPMI 1640 (Gibco) supplemented with 5% Fetal Bovine Serum (Gibco).

### 2.3. Wright-Giemsa staining for mean lobe count

Hypersegmentation in neutrophils was determined by Wright-Giemsa staining. Isolated neutrophils ($2 \times 10^6$) were treated with retinoic acid (100 nM, Sigma-Aldrich) for 4 h in the presence or absence of retinoic acid receptor antagonists. The BMS 453 (Santa Cruz, USA, Texas), BMS 493 (Santa Cruz), ER 50891 (Santa Cruz), or MM 11253 (Santa Cruz) retinoic acid receptor antagonists, or the mTOR inhibitor, Rapamycin (Sigma-Aldrich) were used at different concentrations (10 pM–10 μM). Cells were then cytospun and stained with hemacolor stain (Merck Millipore, United States, Massachusetts) and mean lobe count determined.

### 2.4. ROS measurement

Intracellular ROS was determined using a fluorescent probe, 2′7′- dichlorodihydrofluorescein diacetate (DCF-DA, Invitrogen, USA, California) assay. Isolated neutrophils ($2 \times 10^6$ cells/ml) were seeded in 96 well plates in the presence or absence of retinoic acid. Cells were incubated for 4 h and further stimulated with PMA (1 μg/ml, Sigma-Aldrich) or fMLP (1 μM, Sigma-Aldrich) for 1 h at 37 °C. Neutrophils were then treated with DCF-DA (5 μM) for 20 min and washed with PBS, followed by measurement of DCF-DA fluorescence using a fluorescence microplate reader (Spectramax M2/e, Molecular Devices) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

### 2.5. Quantification of NET formation

Extracellular NET formation was measured using Sytox Green (Invitrogen), a cell-impermeable DNA binding dye. Isolated neutrophils ($2 \times 10^6$ cells/ml) were seeded in 96 well plates in the presence or absence of retinoic acid (100 nM, Sigma-Aldrich) for 4 h and further stimulated with PMA (1 μg/ml, Sigma-Aldrich) or fMLP (1 μM, Sigma-Aldrich) for 1 h at 37 °C. Then the cells were treated with SYTOX green (5 μM) for 20 min and washed with PBS, followed by measurement of SYTOX green fluorescence using a fluorescence microplate reader (Spectramax M2/e, Molecular Devices) at an excitation wavelength of 504 nm, and an emission wavelength of 523 nm.

### 2.6. Cytotoxicity measurement

Cell cytotoxicity was determined using Calcein AM (Abcam Inc., UK, Cambridge). Neutrophils were treated with retinoic acid for 4 h. Retinoic acid treated neutrophils were then collected and washed with 1 × PBS. Tumor cells COLO-205, Capan-1, and U937 were pre-stained with 5 μg/ml Calcein-AM for 30 min and exposed to neutrophils at different ratios (Effector: Target ratio 1:1, 5:1, 10:1, 50:1, 100:1) in presence or absence of DNase (10 U/ml). After 18 h, cells were washed with 1 × PBS and remaining fluorescence was measured with a fluorescence microplate reader (Spectramax M2/e, Molecular Devices) at an excitation wavelength of 490 nm, and an emission wavelength of 515 nm. Percentage survival was calculated as "100 × fluorescence of tumors cells/fluorescence of tumors cells without exposure to neutrophils".

### 2.7. Murine tumor model

Tumor inhibition was assessed using a murine tumor model. Five-week old female BALB/c mice were injected with $1 \times 10^5$ 4T1 cells in the right leg and retinoic acid (1.5, 0.15 and 0.015 mg/kg/d) was administered intraperitoneally daily. Tumor growth was measured every three days until day 21.

### 2.8. Statistical analysis

All statistical analyses were performed using two-tailed Student's *t*-test in GraphPad prism 5.0 (Graphpad software).

## 3. Results

### 3.1. Retinoic acid induces hypersegmentation in neutrophils via RAR and mTOR pathways

Neutrophils generally have three to five segmented nuclear lobes, while hypersegmentation denotes neutrophils with more than five distinct lobes. Hypersegmentation of neutrophils has been proposed as a distinct characteristic of antitumoral neutrophils [4,5]. Previously, retinoic acid was reported to induce nuclear segmentation of leukemic cells that results in complete morphological maturation of neutrophils [10,19–27]. Thus, we examined the effect of retinoic acid on the hypersegmentation of neutrophils. Neutrophils were treated with various concentrations of retinoic acid. Then, the percentages of hypersegmentated cells and mean lobe counts were determined. Hypersegmented cells were defined as a cell with mean nuclear lobe counts of five or greater, as previously described [4]. Retinoic acid significantly increased the proportion of hypersegmented neutrophils and mean lobe counts (Fig. 1B). Additionally, retinoic acid-treated neutrophils exhibited reduced cell diameter and area compared to those of control cells (Fig. 1D). We further examined whether retinoic acid induces apoptosis of

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