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Original Article

Single-Cell Observation of Phagocytosis by Human Blood Dendritic Cells

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SUMMARY: Time-lapse video microscopic observation is useful for analysis of cell biology, especially in rapid response of immune cells. Dendritic cells (DCs) have multiple functions in the immune system, and DCs in peripheral blood play an especially important role at the front line of infection. We have developed a time-lapse video microscopic method for the evaluation of single myeloid DCs (MDC-1) from human peripheral blood. MDC-1 displayed generalized plasma membrane ruffling and phagocytosis of *Pseudomonas aeruginosa*. The morphological changes of MDC-1 increased following stimulation with *P. aeruginosa* but not after stimulation with supernatant from a *P. aeruginosa* culture. The activation of these morphological changes in MDC-1 could be quantitatively analyzed using the time-lapse video microscopy. This novel system may be useful for the evaluation of rapid response with human immune cells against bacterial infection.

INTRODUCTION

Dendritic cells (DCs) play a crucial role in monitoring of the environment, specifically in the recognition of pathogens in non-lymphoid organs and the blood. Immature DCs detect pathogens by taking up soluble pathogenic antigens or by phagocytosing pathogenic macroparticles. This initiates a program of maturation, resulting in migration of the DCs to lymphoid organs and culminating in the enhanced expression of major histocompatibility complex-peptide complexes, costimulatory molecules, and cytokines necessary for T cell activation (1). Because DCs display both major histocompatibility complex class II molecules and costimulatory molecules such as CD80 and CD86, only DCs can induce primary sensitization against specific antigens in naïve T cells (2). Thus, DCs are highly specialized antigen-presenting cells that form a gateway between the innate and adaptive immune systems.

Whole bacteria, yeasts, and microbial products have all been found to induce surface costimulatory molecules and cytokine production by DCs. Immature DCs express surface pattern recognition receptors that bind to microbes and microbial products (3). Much of the response of immature DCs to microbial products is dependent on changes at the transcriptional level, but it is becoming clear that activation of Toll-like receptors can also trigger rapid responses (1). These rapid responses of DCs to invasive microbes are critical for ensuring immunity.

The time-lapse microscopic cultivation system is a useful tool for the single cell level elucidation of rapid response to pathogens in immune cells (4). The observation of single cells over time is important for elucidating how innate immune responses are controlled. Because the rapid response of human DCs to microbes has not been well elucidated, in the current study we used time-lapse video analysis to evaluate how the morphology of human DCs changes upon contact with microbes, and we established a method for quantitatively analyzing these changes in a single-cell-based microcultivation system.

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MATERIALS AND METHODS

Pseudomonas aeruginosa (PAO-1) cells were cultured overnight at 37°C in Brain-Heart infusion (Becton Dickinson Microbiology Systems, Sparks, Md., USA). An aliquot of this culture was transferred to fresh medium and incubated for 3 h at 37°C to obtain bacteria in logarithmic-phase growth. Following the sedimentation of bacteria by centrifugation at 800 × g for 10 min, the supernatant was collected and placed in a separate tube, and the pellet was washed in phosphate-buffered saline (PBS) and quantified by measuring the optical density at 620 nm. Cell pellets containing 1×10^5 colony-forming units of bacteria or supernatant corresponding to culture medium from 1×10^5 colony-forming units of bacteria were then mixed with 500 μl of Macrophage-SFM (Invitrogen, Carlsbad, Calif., USA). Latex beads (Sigma, St. Louis, Mo., USA), 1.1 μm particle size, were used for a control experiment.

We subjected 7 ml of heparinized peripheral blood obtained from healthy volunteers to gradient density separation on Ficoll (GE Healthcare, Piscataway, N.J., USA) to isolate peripheral blood mononuclear cells. Blood DCs were then isolated from the peripheral blood mononuclear cells with Blood Dendritic Cell Isolation Kit II (Milteny Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instructions. The isolation was performed using a two-step immunomagnetic cell sorting procedure. First, B cells and monocytes were magnetically labeled and depleted using a cocktail of CD19 and CD14 MicroBeads. Next, the pre-enriched DCs in the non-magnetic flow-through fraction were magnetically labeled and enriched using a cocktail of anti-

bodies against the DC markers CD304, CD141, and CD1c. The isolated cells were incubated with FITC-labeled monoclonal antibody to Integrin αX (CD11c) (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) for 20 min at 4°C, washed twice in PBS, and then cultured in 1 ml of Macrophage-SFM at a concentration of 1×10^4 per 35-mm glass-bottomed dish (Matsunami Glass, Osaka, Japan).

For microscopic observation, cells were grown at 37°C in a microscope cultivation system (Nikon Instech Co., Ltd., Tokyo, Japan) with a humidified atmosphere containing 5% CO_2 (Fig. 1). Differential interference and fluorescence microscopy were performed using an IX-70 microscope with a ×100 oil-immersion objective lens (numerical aperture = 1.35; Olympus, Tokyo, Japan). Differential interference images were acquired simultaneously using a Retiga2000 charged-coupled device camera and analyzed using QCapture-pro (Nippon Roper, Tokyo, Japan).

Prior to treatment, DCs were incubated for 15 min in the microscope cultivation system. Following treatments, images were collected every 5 s for 3 h. To quantify the duration of phagocytosis, we followed the fate of a single cell cultivated with *P. aeruginosa* for the entire duration of the data collection by analyzing the images one at a time.

Quantitatively assessment of the morphological transformation of DCs was made after handling of each image. The outlines of the DCs were traced with a pen tablet PTZ-630

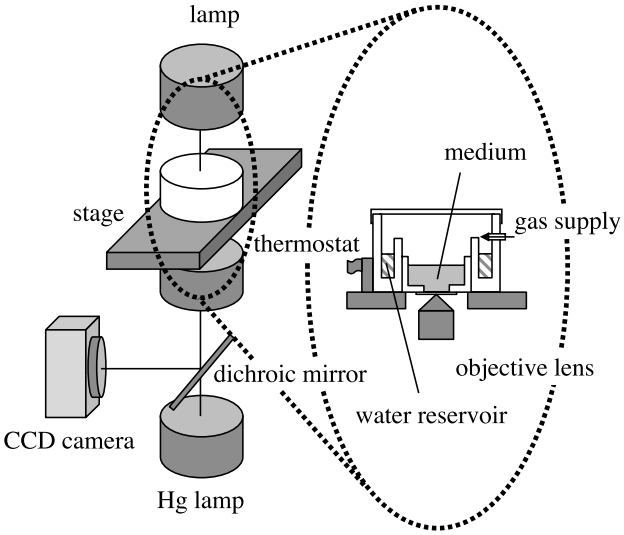

Fig. 1. Schematic drawing of the on-stage microcultivation system. The cells are cultivated at 37°C in a humidified atmosphere containing 5% CO_2 in a glass-bottomed dish on the stage of a differential interference microscope. The images are acquired with a charged-coupled device (CCD) camera.

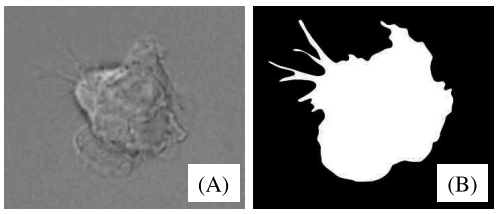

Fig. 2. Single cell images of human blood CD11c^{high} myeloid DC (MDC-1). The differential interference images (A) were acquired by using a charged-coupled device (CCD) camera with an oil-immersion objective lens, and processed images (B) were obtained from original images (A) by outlining with a pen tablet PTZ-630, and silhouetting with Corel® Painter™ Essentials 2.

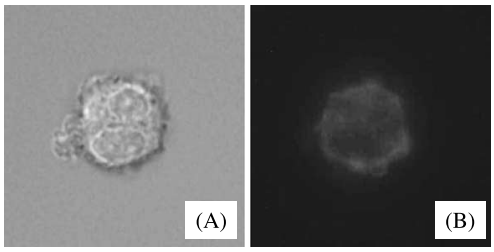

Fig. 3. Single cell images of human blood CD11c^{high} myeloid DC (MDC-1). The differential interference images (A) and fluorescent images with FITC-labeled monoclonal antibody to Integrin αX (CD11c) (B) were acquired by using a charged-coupled device (CCD) camera with an oil-immersion objective lens. Only the experiments with CD11c positive cells were included to this study.

(Wacom Co., Ltd., Saitama, Japan), and the accurate shapes of DCs were silhouetted with Corel® Painter™ Essentials 2 (Corel Japan Ltd., Tokyo, Japan) (Fig. 2). The movement distance of cells, max intercepts of cells, and areas of cells were measured in pixels using LabVIEW software (National Instruments, Austin, Tex., USA). The images were collected every 30 s before and after a 30-min stimulation with *P. aeruginosa* or culture supernatant from *P. aeruginosa*.

At the end of each observation, DCs were observed with fluorescence to determine CD11c positive or negative status (Fig. 3). In this study, the experiments with CD11c^{low} or CD11c^{negative} cells were excluded to analyze the response of CD11c^{high} myeloid DC (MDC-1) to *P. aeruginosa*.

All data were expressed as means ± standard deviation of the mean. Differences between two groups were examined for statistical significance by Student's *t* test. A *P* value of less than 0.05 was considered to indicate a statistically significant difference.

This study was approved by the ethical committee of Nagasaki University Hospital (Nagasaki, Japan). Informed consent was obtained from all volunteers.

RESULTS

DCs obtained form human peripheral blood were observed by differential interference and fluorescence microscopy under conditions of low density so that the cells would not contact each other. Time-lapse video microscopy indicated that the MDC-1 retained a relatively round shape and displayed generalized plasma membrane ruffling (Fig. 4A-C). In culture medium without granulocyte/macrophage colony stimulating factor and other cytokines, MDC-1 could be observed for as long as overnight.

Time-lapse video microscopy also showed phagocytosis by MDC-1 when they were cultivated with *P. aeruginosa*. Specifically, the bacteria were contacted by membrane ruffles, caught within membrane folds, and then internalized by the MDC-1. Occasionally, vacuoles containing bacteria could be observed within the MDC-1 (Fig. 4D-F). Generally, the phagocytosis occurred rapidly: MDC-1 started to transform in 67.8 ± 44.7 s (mean ± S.D., $n = 30$) (Fig. 5B and C) and completed phagocytosis in 137.5 ± 66.7 s ($n = 30$; Fig. 5B-G) after contacting bacteria.

The activation of MDC-1 was evaluated by assessing their quantitative morphological changes. The movement distances of cells were calculated as the change of center coordinates of cells between consecutive images. Although there was some variability in the max intercept (Fig. 6A) and cell area (Fig. 7A) in the absence of stimulation, stimulation with *P.*

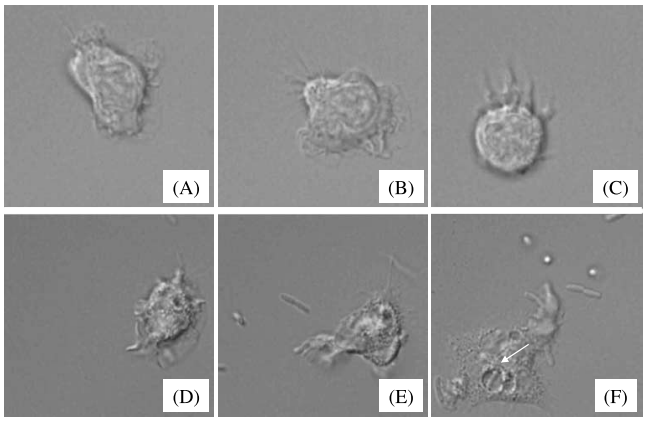

Fig. 4. Single cell images of human blood CD11c^{high} myeloid DC (MDC-1) (A-C), and MDC-1 interacting with *P. aeruginosa* (PAO-1) (D-F). MDC-1 retained a mostly round shape and displayed generalized plasma membrane ruffling (A-D). The morphology of MDC-1 changed notably after contact with *P. aeruginosa* (E). *P. aeruginosa* was caught within membrane folds and ruffles and internalized by MDC-1. Vacuoles containing *P. aeruginosa* could be observed within MDC-1 (F). The arrow indicates a phagocytosed *P. aeruginosa* cell. These images were not consecutive. Their time-lapse images could be seen in the supplement movies.

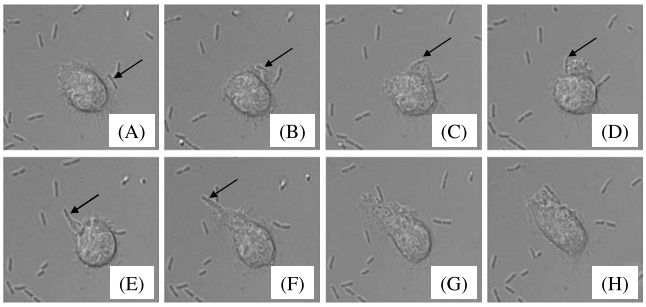

Fig. 5. Consecutive images of phagocytosis by human blood CD11c^{high} myeloid DC (MDC-1). Before an encounter with *P. aeruginosa* cells (A), following an encounter with *P. aeruginosa* cells (B), MDC-1 started to transform (C) and absorbed the bacterial cell by phagocytosis (G). The arrow indicates a phagocytosed *P. aeruginosa* cell. MDC-1 started to transform 67.8 ± 44.7 s after contacting a *P. aeruginosa* cell (B and C), and completed each series of phagocytosis in 137.5 ± 66.7 s (B-G). Images were taken every 20 s.

aeruginosa greatly activated the morphological changes. Stimulation with the supernatant from the *P. aeruginosa* culture did not activate the morphological changes (Fig. 6B and 7B). The average of max intercept (Fig. 6C) and cell area (Fig. 7C) was induced significantly after stimulation with *P. aeruginosa* but not after stimulation with the supernatant from the *P. aeruginosa* culture. To investigate what these results indicated about the pathogen-specific effects on MDC-1 morphology and mobility, we stimulated MDC-1 with latex beads in addition to supernatant of *P. aeruginosa* cultivation. The max intercept and cell area of MDC-1 were slightly increased after stimulation with latex beads in addition to supernatant of bacterial culture, but there was no significant change in the max intercept or cell area between pre and post stimulation (data not shown).

DISCUSSION

Immature DCs have evolved to monitor the environment, detect pathogens, and take up antigens and macroparticles. The capture of antigens or macroparticles triggers the maturation of DCs, which are then targeted to secondary lymphoid organs (4). DCs are highly specialized antigen-presenting cells that form a gateway between the innate and adaptive immune system (3).

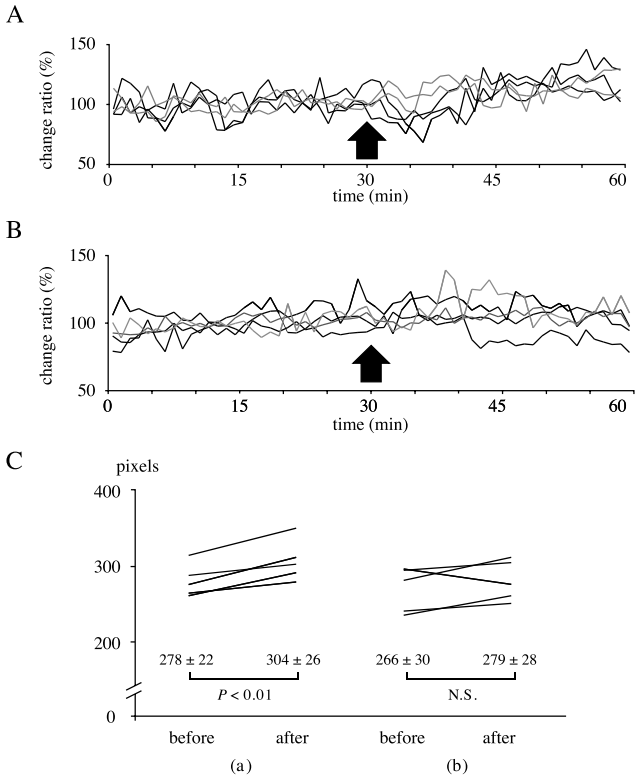

Fig. 6. Quantitative analysis of CD11c^{high} myeloid DC (MDC-1) transformation in max intercept. Images were collected every 30 s before and after stimulation with *P. aeruginosa* (A) or culture supernatant from *P. aeruginosa* (B). Max intercepts were calculated in pixels, and the average of max intercepts before stimulation were defined as 100%. The line with different depth represents individual MDC-1. The arrow indicates the point at which *P. aeruginosa* or culture supernatant from *P. aeruginosa* was added. (C) Each average of max intercept change before and after stimulation with *P. aeruginosa* (a) or culture supernatant from *P. aeruginosa* (b). Data represent the means ± standard deviation of five experiments. *$P < 0.01$.

Time-lapse video microscopy has been used in recent studies of how DCs are activated at the single-cell level. Specifically, the authors of these reports examined the dynamic cell-cell interactions between DCs and T cells (5,6). Single-cell-based microcultivation methods have several advantages: continuous cultivation of single cells under isolated conditions; control of the spatial distribution of cells, interactions between cells, and the number of cells; and continuous observation of single cells under sterile conditions (7). The encounter with a pathogen is known to initiate an immunological response by DCs, but the details of phagocytosis by DCs have not been investigated on a single-cell level. Furthermore, unlike the current study, quantitative analysis of the morphological changes in DCs during phagocytosis was not done in these previous studies.

In this study, we showed time-lapse video analysis of differential interference microscopic images from individual human MDC-1. Using this method, it was possible to evaluate the rapid morphological changes of MDC-1 as well as their phagocytosis of *P. aeruginosa*. The time-lapse images allowed the quantitative analysis of these changes. Our results indicated that activation of MDC-1 by *P. aeruginosa* as a host-pathogen interaction was reflected by an increase in the

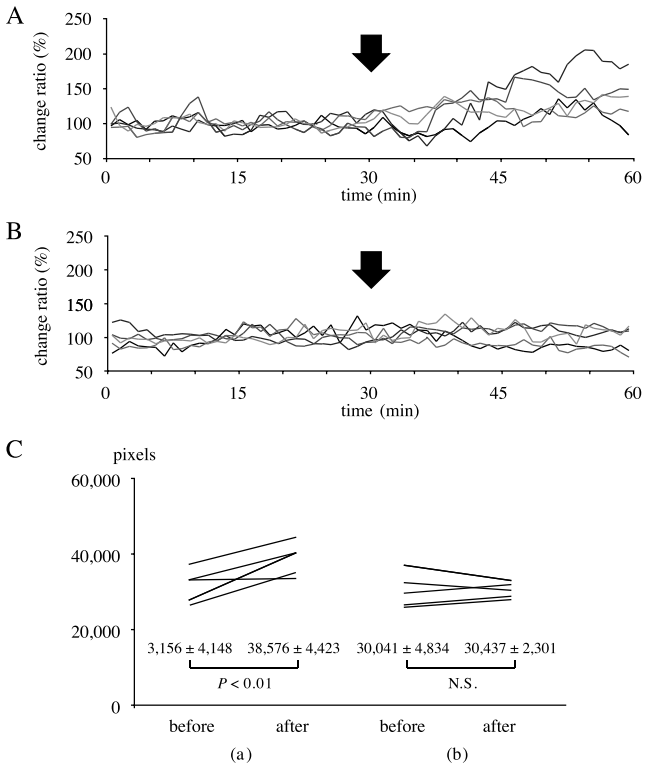

Fig. 7. Quantitative analysis of CD11c^{high} myeloid DC (MDC-1) transformation in cell area. Images were collected every 30 s before and after stimulation with *P. aeruginosa* (A) or culture supernatant from *P. aeruginosa* (B). Cell area was calculated in pixels, and the average of cell area before stimulation were defined as 100%. The line with different depth represents individual MDC-1. The arrow indicates the point at which *P. aeruginosa* or culture supernatant from *P. aeruginosa* was added. (C) Each average of cell area change before and after stimulation with *P. aeruginosa* (a) or culture supernatant from *P. aeruginosa* (b). Data represent the means ± standard deviation of five experiments. *$P < 0.01$.

continuous changes in max intercept of cell and cell area. These morphological changes of MDC-1 are the processes of initializing and phagocytosing the pathogen. Though *P. aeruginosa* increased both parameters significantly, stimulation with supernatant of *P. aeruginosa* cultivation and latex beads in addition to supernatant of bacterial culture did not show a significant increase of max intercept of cell and cell area. These discrepancies might originate from pathogenesis or material property of these stimulations. The meaning of each morphological change and the true mechanism of different reactions of MDC-1 encountering supernatant of bacteria, bacteria, or latex beads was not been resolved in this study. Further investigation is needed.

In conclusion, using a time-lapse video system in conjunction with differential interference microscopy, we were able to precisely evaluate the continuous morphological changes of MDC-1 from a small amount of human peripheral blood. In addition, we were able to quantitatively analyze the morphological changes by MDC-1 following contact with microbes. We are currently using this single-cell-based microcultivation system to evaluate how DCs and other immune cells monitor their environment. This system will allow us to contribute to the establishment of a novel clinical laboratory method of studying the immune system in the future.

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