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Short communication

Influence of membrane cholesterol on monocyte chemotaxis

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ABSTRACT

Cholesterol content influences several important physiological functions due to its effect on membrane receptors. In this work, we tested the hypothesis that cellular cholesterol alters chemotactic response of monocytes to Monocyte Chemoattractant Protein-1 (MCP-1) due to their effect on the receptor, CCR2. We used Methyl-β-cyclodextrin (MβCD) to alter the baseline cholesterol in human monocytic cell line THP-1, and evaluated their chemotactic response to MCP-1. Compared to untreated cells, cholesterol enrichment increased the number of monocytes transmigrated in response to MCP-1 while depletion had opposite effect. Using imaging flow cytometry, we established that these differences were due to alterations in expression levels, but not the surface distribution, of CCR2.

1. Introduction

Chemotaxis is the directed movement of cells in response to chemical stimulus, and plays a vital role in inflammation [1]. Monocyte chemotaxis is of particular importance in homeostatic inflammatory response and also in diseases such as atherosclerosis and cancer [2–4]. Infection or injury trigger the production of pro-inflammatory signals and chemokines, which recruit circulating monocytes from flowing blood to the endothelial surface and transmigration into the extravascular space, respectively [5]. The regulation of these phenomena become particularly crucial in the development and progression of pro-inflammatory diseases such as atherosclerosis.

We have recently shown that cellular cholesterol is an important regulator of monocyte recruitment to atherosclerotic foci [6]. Cholesterol, which is an important risk factor of atherosclerosis alters the distribution of the endothelial adhesion receptor CD44, thus reshaping the dynamics of capture of monocytes from flowing blood [6]. The majority of cellular cholesterol is present on the plasma membrane although it modulates the cell stiffness and hence overall cellular physiology [7]. Despite the striking importance of cholesterol on monocyte function, its role on chemotaxis is not well understood. In this work, we tested the hypothesis that cholesterol content alters the chemotactic behavior of monocytes in response to MCP-1 by altering surface distribution/expression levels of the cognate receptor, CCR2. By depleting or enriching cholesterol levels using MβCD, we examined the response of monocytes to chemotactic stimulus, MCP-1, and changes in the MCP-1 levels and heterogeneity.

2. Materials and methods

2.1. Cell culture and cholesterol treatment

THP-1 (ATCC, Manassas, VA, USA), which is a human monocyte cell line, was cultured in RPMI 1640 (ATCC) supplemented with 10% heat inactivated FBS (Life technologies, Grand Island, NY, USA) and 0.05 mM mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C and 5% CO_2. The cells were allowed to reach a density of 10^6/ml and were then passaged into fresh media. The cell viability was measured by Countess automated cell counter using the trypan blue exclusion assay (Life Technologies).

Cholesterol depletion and enrichment were performed using Methyl-β-cyclodextrin (MβCD; Sigma-Aldrich; stock solution of 200 mM) and MβCD-cholesterol complex (Sigma-Aldrich; stock solution of 25 mM), respectively. Cells were first washed with RPMI and centrifuged for 7 min at 130 × G in room temperature (RT), and finally resuspended in RPMI at a concentration of 1x10^6/ml. Either 10 mM MβCD or 0.5 mM MβCD-cholesterol solution was mixed with the cell suspension, followed by incubation for 30 min. at 37 °C and 5% CO_2. This was followed by estimation of cell viability using trypan blue exclusion assay. The cell suspension was centrifuged at 130 × G for 7 min at RT, and then resuspended at a concentration of 1 × 10^6/ml in RPMI. This was repeated thrice, and cell viability was measured again. After

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the final centrifugation step, the supernatant was discarded and the cells were resuspended as required by the particular assay. At this point, the cell viability was assessed one final time.

2.2. Cholesterol content measurement

Cellular cholesterol content was estimated using Amplex Red Cholesterol Assay Kit (Life Technologies), as per established protocol [6]. Approximately $0.75x10^5$ cells were put in each well of a 96 well plate (Corning Inc., Corning, NY, USA). The Synergy2 plate reader (Biotek, Winooski, VT, USA) was used to measure fluorescence intensities at 540 nm (excitation)/600 nm (emission). The cholesterol contents were determined from a standard curve, using 1–20 µg/ml cholesterol standards.

2.3. Cell chemotaxis

Monocyte chemotaxis was performed using 5 µm transwell inserts (Corning) in 24 well plates (Corning). 30 ng/ml of MCP-1 (R&D Systems, Minneapolis, MN, USA) was used as chemoattractant, and 600 µL of complete media with or without chemoattractant was put in each well. The cells were treated with 1 µM Calcein-AM (Life Technologies), immediately before seeding in the transwell inserts. The transwell inserts were initially primed using complete media without MCP-1 for 30 min at 37 °C. Following the priming, 1,00,000 cells/insert were seeded in a final volume of 100 µL. At least 6 inserts were used per condition. The cells were allowed to pass through the insert pores for 1.5, 3 and 6 h. This was followed by measuring the fluorescence intensity using a plate reader (BioTek). Untreated cells were used as control. Cells from all condition in the absence of MCP-1 was used as negative control. The cell number was estimated from a standard curve for Calcein-AM. After the time points were reached, the inserts were imaged under the microscope at 20X and cell count estimates were obtained to account for the number of cells stuck to the transwell membrane during chemotaxis. At least three inserts were imaged per condition for every experiment.

2.4. Monocyte chemotaxis receptor

Changes in CCR2 distribution and expression due to changes in cholesterol were assessed using multispectral imaging flow cytometry ($Imagestream^X$ Mark II; Amnis corp., Seattle, WA, USA). Fluorescently tagged anti-CCR2 (Miltenyi Biotec, Auburn, CA, USA) was used for visualization and quantification of CCR2. THP-1 cells were initially centrifuged at 130 × G for 7 min at room temperature, and then resuspended in 100 µL PBS (cell concentration of 10^7/ml.) in 1.5 ml. tubes (Eppendorf). 10 µL the antibody reagent was added to the cells and they were incubated for 10 min at 4 °C. The cell suspension was then centrifuged at 130 × G for 7 min at 4 °C. Following this, the cells were fixed using 4% formaldehyde solution in PBS, maintaining a final concentration of 10^7 cells/ml. The intensity and distribution of CCR2 on the plasma membrane were estimated using mean fluorescence intensity and H Variance SD, respectively. A higher mean fluorescence intensity indicates higher expression of CCR2, and vice versa. A lower H variance SD value implies that CCR2 is more uniformly distributed, and vice versa.

2.5. Statistics

All the experiments were performed in triplicates, and each experiment was repeated at least thrice under independent conditions, on different days, unless otherwise mentioned. The results are represented as mean ± SEM from one representative experiment. Statistical differences were evaluated using one-way ANOVA with Tukey's *post hoc* test, and significance was reported at $\alpha = 0.05$.

3. Results and discussion

We have previously established the importance of cellular cholesterol on the tethering and adhesion of monocytes to endothelial-mimetic surfaces, which is the first critical step in its inflammatory response [7]. In this work, we have explored the effects of cellular cholesterol content on monocyte chemotaxis, which remains ill-explored, despite its significant importance in inflammation.

Firstly, we altered the baseline cholesterol levels in human monocytic cell line THP-1 cells and estimated cholesterol levels using cholesterol oxidase based assay (Amplex red cholesterol assay). Depletion resulted in ~ 50% decrease and enrichment caused ~170% increase in cholesterol levels as compared to the untreated cells [6]. We have shown that the MβCD treatment results in comparable changes in cholesterol levels between THP-1 and freshly isolated human peripheral blood monocytes [6,7]. Having successfully altered the cholesterol levels, we investigated the chemotactic response of THP-1 cells to Monocyte Chemoattractant Protein-1 (MCP-1), which is a physiologically relevant chemoattractant for monocytes with established importance in inflammatory response [8,9].

A transwell assay was used to assess the chemotactic response of THP-1 cells to the chemoattractant MCP-1. After 1.5 h, only a few cells passed through the wells towards to the chemoattractant for all the conditions, albeit following a similar trend as longer durations. After 3 h, there was a 40% decrease in the number of cholesterol depleted cells that transmigrated compared to untreated control. In contrast, cholesterol enrichment resulted in a 170% increase in the number of transmigrated cells as compared to untreated control. After 6 h, depletion resulted in 30% fewer cells and enrichment resulted in 90% more cells transmigrated across the membrane in comparison to the untreated control (Fig. 1A). We observed that only a small fraction of the cells were stuck in the transwell membrane during the experiment: 5 ± 1.5% of untreated, 4 ± 0.75% of depleted, and 6 ± 1% of enriched cells, after 3 h of transmigration. This observation indicates that the data obtained is indeed reflective of the true chemotactic behavior of the cells, rather than gravity-driven, non-specific movement.

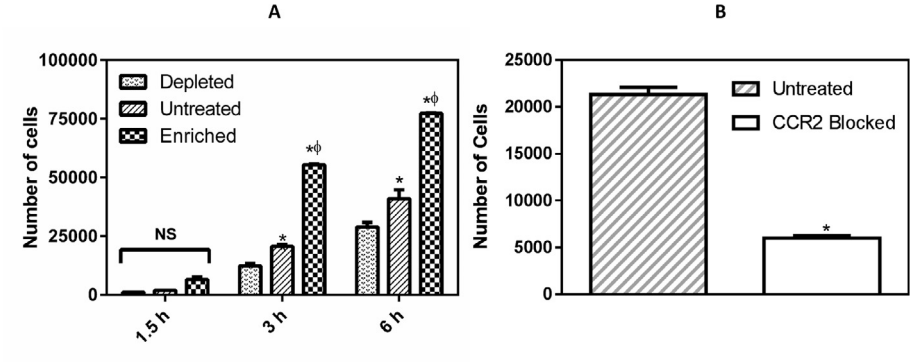

Fig. 1. Monocyte chemotaxis in response to MCP-1 gradient. (**A**) Number of cells transmigrated to the bottom well after 1.5 h, 3 h and 6 h. (**B**) Number of cells transmigrated at 3 h in the presence of anti-CCR2 antibody (25 µg/ml). * and φ represent statistically significant difference ($p < .05$, n = 3) in comparison to depleted and untreated cells, respectively.