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(Research Article)

Establishment of an *in vitro* flow cytometric detection method for lipid-accumulated cells

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Abstract

Eukaryotic cells can store and converse excess lipid to the cytosolic lipid droplets. Adipogenesis of preadipocytes has been often used to study the molecular basis and the effect of obesity drugs on fat cell conversion. Many methods were developed for the detection of the cytosolic lipid droplets as Nile red, BODIPY 493/503 (4, 4-difluoro-1, 3, 5, 7, 8-pentamethyl-4-bora-3a, 4adiaza-s-indacene), BODIPY 665/676, 1,6-diphenylhexatriene (DPH), DAPI, Hoechst, Sudan III, and Oil-red O. The differences in the spectral properties of these lipophilic dyes and their advantages of each are discussed. In this study, an *in vitro* flow cytometric detection method was established for the detection of lipid-accumulated cells. Commonly, the longer the period of adipogenic induction, the greater the quantity of lipid in fat cell can accumulate. Thus, to determine whether increasing the fat stored within a cell would result in the greater granularity. 3T3-L1 cells in culture were hormonally induced for adipogenesis. Then, these cells were dissociated and analyzed in a flow cytometer at 0, 5, and 10 days post-induction. After adipogenic induction, the cells had become increasingly heterogeneous in their cellular granularity. The cells containing greater granular structure were markedly increased, and this increase in granularity positively correlated with the time of the post-adipogenic induction. On the other analysis, the 0 and 10 days post adipogenic induction of 3T3-L1 cells were gated for 4 regions. The R1 region contains cells with a level of granularity similar to that seen in the control cells (non-adipogenic induction), whereas R2 to R4 regions contain cells with increasing granularity. According to all data, we have successfully established an *in vitro* flow cytometric detection method for the detection of lipid-accumulated cells. We wish this method will be applied on the research of obesity drugs and the design of therapeutic strategies for obesity in the future.

Keywords: Establishment; *In vitro*; Flow cytometric detection method; Lipid-accumulated cells

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1 Introduction

According to WHO global estimates, more than 1.9 billion adults aged 18 years and older were overweight in 2016. Of these over 650 million adults were obese. Overall, about 13% of the world's adult population (11% of men and 15% of women) were obese in 2016. The worldwide prevalence of obesity nearly tripled (4% in 1975 to over 18% in 2016) between 1975 and 2016. Over 340 million children and adolescents aged 5-19 years were overweight or obese in 2016. In 2019, an estimated 38.2 million children under the age of 5 years were overweight or obese. Once considered a high-income country problem (obesity), the obesity is now on the rise in low- and middle-income countries, particularly in urban settings. In 2019, almost half of the children were overweight or obese lived in Asia. Obesity is linked to more deaths worldwide than underweight. Globally, there are more people who are obese than underweight. This occurs in every region except parts of sub-Saharan Africa and Asia [1]. Obesity is the excessive accumulation of body fat and it is a consequence of persistent energy intake that exceeds energy expenditure. An increasingly sedentary lifestyle coupled with an energy-rich diet has contributed to a high frequency of obesity and its attendant health problems as type 2 diabetes [1-3].

Obesity is defined as abnormal or excessive fat accumulation that may impair health. The fundamental cause of obesity is an energy imbalance between calories consumed and calories expended. Globally, there has been an increased intake of energy-dense foods that are high in fat and sugars and an increase in physical inactivity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization. Changes in dietary and physical activity patterns are often the result of environmental and societal changes associated with development and lack of supportive policies in sectors such as health, agriculture, transport, urban planning, environment, food processing, distribution, marketing, and education [4-10].

In the recent years, obesity is defined as abnormal or excessive fat accumulation that may impair health. More and more researches on the prevention and treatment of obesity are prevalent. Understanding of the fat cellular molecule mechanisms of obesity was one of the major focuses in obesity field. Evaluation methods for the assessment of fat cell development and maturation of adipogenesis as the cellular lipid droplet formation and cellular lipid staining with Oil Red O by using microscopic examination. On the other hand, body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). Obesity is a BMI greater than or equal to 30. BMI provides the most useful population-level measure of obesity as it is the same for both sexes and for all ages of adults. However, they should be considered a rough guide because it may not correspond to the same degree of fatness in different individuals [11-16]. Thus, an effective method to detect and classify cells with similar fat content will certainly increase analytical precision in monitoring fat cell development and the ability to quantify the effects of therapeutic agents [17-26]. Currently, flow cytometry (FC) is extensively applied in the analysis of many cellular types. In this study, we have explored the feasibility of using FC to assess fat content during fat cell development.

2 Material and methods

2.1 Cellular Culture Condition and Adipogenesis

3T3-L1 cell line (ATCC® CL-173™) was purchased from ATCC (Manassas, VA 20110). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), and adipogenesis reagents were purchased from Sigma-Aldrich. 3T3-L1 cells were cultured in DMEM which supplemented with 10% FBS and 1% penicillin and streptomycin. Adipogenesis was induced by adding 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), and 1 μM dexamethasone. The cells were incubated at 37°C with 5% CO_2. Cells were sub-cultured to replace flesh media per 2-3 days when they became confluent. Cells were continuously maintained in DMEM containing 5 μg/mL of insulin until needed.

2.2 Flow Cytometric Detection

Adipogenically-induced 3T3-L1 cells were analyzed by flow cytometers (FACS Calibur, Becton Dickinson). Cells were briefly rinsed twice with prewarmed 0.25% trypsin-EDTA and then incubated for 5 min at 37°C. Cells were then gently resuspended in PBS, washed twice with PBS, resuspended in cold PBS, and kept on ice prior to flow cytometric analysis. The flow cytometer settings for both side scatters-H (SSC-H) and forward scatters-H (FSC-H) to analyze fat cells were set on the analysis. In general, the voltages and compensation between scatters were set to the degree so that the majority of control cells located below the scale of 200 for the SSC-H and between the scales of 200 and 800 for the FSC-H.

2.3 Statistical Analysis

The data were expressed as mean. WinMDI (version 2.9) software is used for the analysis of flow cytometric data. All graphical representations and statistical calculations were aided by GraphPad Prism (version 8.0).

3 Results

3.1 Increased Granularity and Heterogeneity among 3T3-L1 Cells during Adipogenesis

3T3-L1 cells in culture were induced for adipogenesis. Then, these cells were dissociated and analyzed by using a flow cytometer at 0, 5, and 10 days post- adipogenic induction (Fig. 1A-B). The dot plot of cytometric FSC-H and SSC-H was respectively shown as the X axis and Y axis that respectively reflecting the cell diameter and granular structures within the cell. After adipogenic induction, the cells had become increasingly heterogeneous in their cellular granularity (Fig. 1A-B). The cells containing greater granular structure were markedly increased, and this increase in granularity positively correlated with the time of the post-adipogenic induction (Fig. 1A-B).

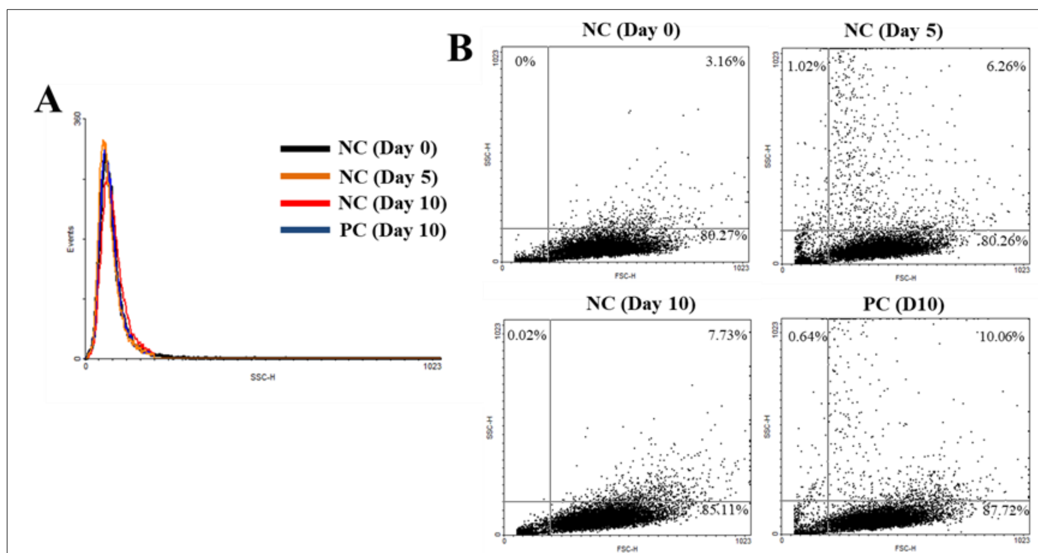

Figure 1 Effects of the granularity and heterogeneity in 3T3-L1 cells during adipogenesis. (A) The overlay of cell distribution (Y axis: Events) versus cellular granular intensity (X axis: side scatter SSC-H). (B) Dot plots of side scatter (Y axis: side scatter SSC-H) versus forward scatter (X axis: FSC-H) of 3T3-L1 cells generated from flow cytometric analysis of levels of granularity at different time points (Day 0, Day 5, and Day 10) of adipogenesis. FSC-H represents the cell size, whereas SSC-H represents cytoplasmic granular intensity. Cells were dissociated and analyzed at 0, 5, and 10 days after adipogenic induction. Abbreviation: NC (normal control); PC (positive control); side scatters-H (SSC-H); forward scatters-H (FSC-H)

3.2 Effects of the Cellular Lipid Content and Granularity of 3T3-L1 Cells after Adipogenic Induction

Commonly, the longer the period of adipogenic induction, the greater the quantity of lipid in fat cells in culture can accumulate. Thus, to determine whether increasing the fat stored within cells would result in the greater granularity seen in SSC-H. Firstly, the 0 and 10 days post adipogenic induction of 3T3-L1 cells were gated and analyzed for four regions as shown in Fig. 2 based on the full scale of SSC-H. The R1 region contains cells with a level of granularity similar to that seen in the control cells (non-adipogenic induction), whereas R2 to R4 regions contain cells with increasing granularity.

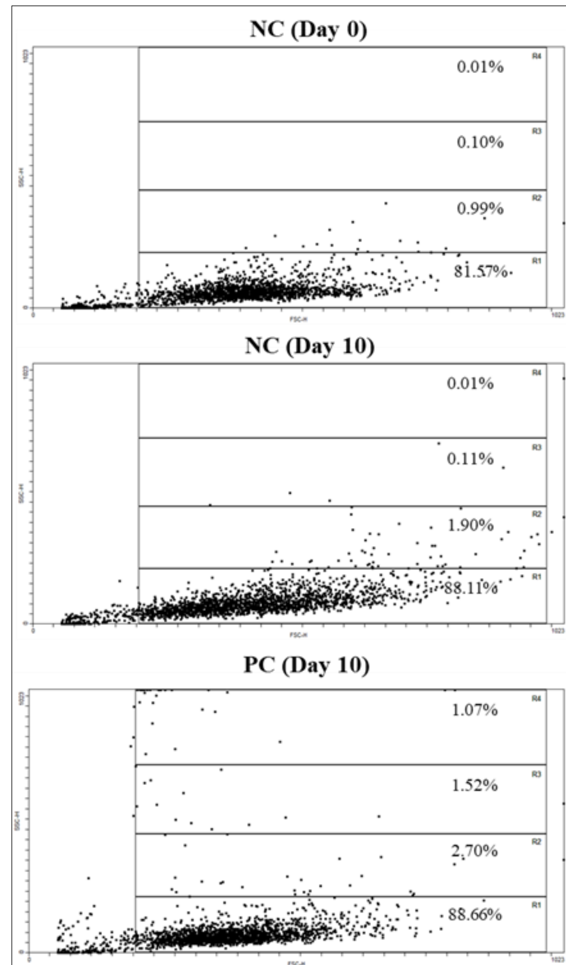

Figure 2 Effects of the cellular lipid content and granularity of 3T3-L1 cells after adipogenic induction. 3T3-L1 cells in the four regions (R1, R2, R3, and R4) were gated and analyzed by using a flow cytometer with WinMDI software (version 2.9). Scatter plot (Y axis: SSC-H) of 3T3-L1 cells with or without adipogenic induction was gated into four regions based on their granularity distribution. The R1 region was gated to include the majority of control cells shown on the SSC-H scale. The R2 to R4 regions were gated to include the remaining range of SSC-H, and each region contained an equal range of SSC-H. The X axis: FSC-H represents the cell size, whereas the Y axis: SSC represents cytoplasmic granular intensity. Abbreviation: NC (normal control); PC (positive control); side scatters-H (SSC-H); forward scatters-H (FSC-H).

4 Discussion

Obesity is an abnormal or excessive fat accumulation that may affect people's health. Recently, many researchers have interest in lipid droplet biology. Lipid droplets link metabolic pathways to affect the basic cell functions and the dysfunction of lipid droplets can be catastrophic for the cell and body. Commonly, BMI (kg/m^2) is a simple index of weight-for-height that is commonly used to classify obesity in adults in life. However, BMI is just a rough guide. Currently, there are many detection methods for the detection of intracellular lipid droplets. These methods include Nile red stain, BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4adiaza-s-indacene) stain, BODIPY 665/676 stain, 1,6-diphenylhexatriene (DPH) stain, DAPI stain, Hoechst stain, Sudan III stain, and Oil-red O stain [20-26; 27-34]. Although the excellent is in detecting the presence of intracellular fat by these methods. However, some methods are ineffective in objectively quantifying the degree of fat accumulation [35-46].

BODIPY 493/503 has been verified a valuable tool for detection of lipid droplets in live and fixed cells. BODIPY 493/503 is very specific for lipid droplets. Other BODIPY analogs as BODIPY 665/676 may also facilitate co-staining with green fluorophores. Despite BODIPY, other lipophilic dye stains were used in the detection of lipid droplet stains. The DPH with DAPI and Hoechst stains to allow simultaneous detection of lipid droplets with green fluorescent protein-tagged proteins. The fluorescence of Nile Red generally appears more intense than BODIPY 493/503. Sudan III and Oil-red O dyes allow detection of lipid droplets by either under conventional light microscopy or fluorescence microscopy. In

addition, another method for visualization of lipid droplets is to supplement with a fluorescent fatty acid analog. Importantly, the specific cellular structures stained with fatty acid analogs may not be the same as those detected with lipophilic dyes and can include endoplasmic reticulum and mitochondria [20-31].

In this study, it was found that the degree of reattachment was negatively correlated with the increased cellular granularity. About 100% of 3T3-L1 cells in the R1 region could reattach, however, about 5% of 3T3-L1 cells from the R4 region could reattach (data not shown). According to the literature [1], cells in the R1 region appeared not to be induced and could proliferate, while cells from other regions continued to accumulate fat as indicated by the growth of their cellular lipid pool. Therefore, our results indicated that FC is a useful tool to detect the granularity of fat cells and also can be easily distinguished via flow cytometric SSC-H. Quantification of distinct cell populations is one of the functions of FC. FC was used to monitor and quantify 3T3-L1 cells based on their degree of fat accumulation at various stages of adipogenesis. Compares the R1 to R4 cell populations of 3T3-L1 cells at various stages of adipogenesis during the 10-day period of adipogenesis.

Taken all data together, the feasibility of directly measuring the fat content of fat cells by using FC was demonstrated in this study. This detection method not only is the system simple, sensitive, and quantitative for its capability of monitoring the degree of intracellular fat accumulation in a precise, fast, and selective manner, but it also offers an advantage over the existing methodology. In this study, an *in vitro* flow cytometric detection method was established for the detection of lipid-accumulated cells. According to all data, we have successfully established an *in vitro* flow cytometric detection method for the detection of lipid-accumulated cells. We hope this method will applied on the research of obesity drugs and the design of therapeutic strategies for obesity in the future.

5 Conclusion

Adipogenesis of preadipocytes has been often used to study the fat cell molecular basis and effect of obesity drugs on fat cell conversion. Many methods were developed for the detection of the cytosolic lipid droplets. In this study, an *in vitro* flow cytometric detection method was established for the detection of lipid-accumulated cells. Commonly, the longer the period of adipogenic induction, the greater the quantity of lipid a fat cell in culture can accumulate. Thus, to determine whether increasing the fat stored within a cell would result in the greater granularity. 3T3-L1 cells in culture were hormonally induced for adipogenesis. According to all data, we have successfully established an *in vitro* flow cytometric detection method for the detection of lipid-accumulated cells. We wish this method will be applied on the research of obesity drugs and the design of therapeutic strategies for obesity in the future.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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