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Palmitic acid, verified by lipid profiling using secondary ion mass spectrometry, demonstrates anti-multiple myeloma activity

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ABSTRACT

Recent studies indicate that lipid metabolic changes affect the survival of multiple myeloma (MM) cells. Time-of-flight secondary ion mass spectrometry (TOF-SIMS), an imaging mass spectrometry technique, is used to visualize the subcellular distribution of biomolecules including lipids. We therefore applied this method to human clinical specimens to analyze the membrane fatty acid composition and determine candidate molecules for MM therapies. We isolated MM cells and normal plasma cells (PCs) from bone marrow aspirates of MM patients and healthy volunteers, respectively, and these separated cells were analyzed by TOF-SIMS. Multiple ions including fatty acids were detected and their ion counts were estimated. In MM cells, the mean intensity of palmitic acid was significantly lower than the mean intensity in PCs. In a cell death assay, palmitic acid reduced U266 cell viability dose-dependently at doses between 50 and 1000 μM. The percentage of apoptotic cells increased from 24 h after palmitic acid administration. In contrast, palmitic acid had no effect on the viability of normal peripheral blood mononuclear cells (PBMCs). The results of this study indicated that palmitic acid is a potential candidate for novel therapeutic agents that specifically attack MM cells.

1. Introduction

Recently, an increasing number of studies have focused on the role of lipid metabolic changes in multiple myeloma (MM) [1–3]. Tirado-Velez *et al.* [1] examined the anti-MM effect of etomoxir, a specific and irreversible inhibitor of carnitine palmitoyltransferase I required for β-oxidation, and orlistat, a specific inhibitor of fatty acid synthase, and showed these two agents reduced MM cell proliferation. Medina *et al.* [2] showed that activators of the PKA/AMPK pathway or inhibitors of acetyl-CoA carboxylase, which catalyzes the first committed step of the fatty acid synthetic pathway, might be useful adjuvants in treating MM. Each of these studies investigated the anti-MM cell proliferative effect *via* the inhibition of *de novo* fatty acid synthesis. They found that lipid metabolic changes affect the survival of MM cells.

Developments in imaging mass spectrometry have facilitated research on the lipid profiles of tumors [4–6]. Recently, we have established sample preparation and analysis techniques for single-cell imaging mass spectrometry that target dispersed cells, particularly hematopoietic cells [7]. These methods use time-of-flight secondary ion mass spectrometry (TOF-SIMS) as a measuring technique. In TOF-SIMS, the sample surface is sputtered with a focused primary ion beam and the ejected secondary ions are analyzed by mass spectrometry [8,9]. TOF-SIMS imaging can be used to analyze the surface of a specimen at a submicrometer resolution, enabling analyses of the subcellular distribution of molecules in individual cells [7,10,11]. TOF-SIMS is thus useful for the analysis of biomolecules such as lipids in biological samples at the single-cell level [12–15]. Therefore, we applied this method to human clinical specimens to analyze the membrane fatty acid composition and determine candidate molecules for MM therapies.

The characteristic fatty acid composition of cancer cell membranes was thought to result from abnormal lipid metabolism, which the survival and proliferation of the cancer cells depend on [5,6,16,17]. The fatty acid composition of MM cells revealed

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by our TOF-SIMS analysis was also considered to reflect an intracellular status suitable for MM cell survival. We thus speculated that disturbing the intracellular status would induce cell death in MM cells. Given that cell death is induced by fatty acid supplementation in some types of cells, mainly non-malignant cells [18–24], we hypothesized that exogenous supplementation of fatty acids, which are less abundant in MM cells, causes cell death. As a cell death assay, we cultured MM cell lines U266 and RPMI-8226 in medium containing fatty acids screened out from the lipid profiling, and evaluated apoptosis by flow cytometry. In this paper, we report 1) the single-cell fatty acid analysis of MM cells by TOF-SIMS, and 2) the anti-MM cell death assay using the fatty acids as candidate anti-tumor molecules.

2. Materials and methods

2.1. Isolation of human clinical samples

Primary patient MM cells were isolated from the bone marrow aspirates of three patients (MM1, MM2, and MM3) using fluorescence-activated cell sorting (FACS), and normal plasma cells (PCs) were isolated from the bone marrow aspirates of two healthy volunteers (PC1 and PC2). After obtaining bone marrow aspirates, red blood cells were lysed using red blood cell lysis buffer (Miltenyi Biotec, Auburn, CA, USA). After incubation, cells were pelleted by centrifugation ($235 \times g$ for 5 min at room temperature), the supernatant was aspirated, and the cells were washed twice in phosphate-buffered saline buffer containing ethylenediaminetetraacetic acid and bovine serum albumin (BSA) (Miltenyi Biotec). The washed cells were incubated with each of the following monoclonal antibodies for 10 min at 4 °C in the dark: anti-CD38 Alexa Fluor 488 (clone HIT2; BioLegend, San Diego, CA, USA), anti-CD138 peridinin chlorophyll A protein (PerCP)-cyanin 5.5 (Cy5.5) (clone DL-101; BioLegend), anti-CD45 allophycocyanin (APC) (clone 5B1; Miltenyi Biotec), anti-CD19 phycoerythrin (PE) (clone HIB19; BioLegend), and anti-CD56 PE-cyanin 7 (Cy7) (clone MEM188; BioLegend). After the incubation, the cells were washed once in phosphate-buffered saline buffer. A BD FACSAria flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to isolate the MM cells and PCs. To separate the MM cells, the cells gated in the $CD38^{high}/CD138^{high}/CD45^{low\sim dim}/CD19^{-}/CD56^{+}$ area were sorted [25–27]. Normal PCs were separated from the healthy volunteers' samples gated in the $CD38^{high}/CD138^{high}/CD45^{+}$ area [25–27]. These isolated cells were stained with the May–Grünwald–Giemsa cytochemical stain (Sigma-Aldrich, St. Louis, MO, USA) and confirmed microscopically. Healthy volunteers' normal peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque density-gradient centrifugation. The Institutional Review Board of the Hamamatsu University School of Medicine approved all the experiments in this study in accordance with the Declaration of Helsinki. Informed consent was obtained in the written form from each patient before starting each experiment.

2.2. Cell lines

The U266 cell line and RPMI-8226 cell line established from human MM cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These cell lines were cultured in RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine (Nissui Pharmaceutical), 100 μg/ml streptomycin (Meiji, Tokyo, Japan), and 200 U/ml penicillin (Meiji). Log-phase MM cell lines were used in subsequent experiments. The HS-5 cell line derived from human bone marrow stromal cells was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS according to the manufacturer's instructions (ATCC). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Reagents

Palmitoleic acid, palmitic acid, linoleic acid, oleic acid, and stearic acid were purchased from Sigma-Aldrich. Bortezomib was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. TOF-SIMS analysis and data processing

The TOF-SIMS analysis and data processing were performed following the methods used in our previous study [7]. TOF-SIMS analyses were performed using a TOF-SIMS instrument, PHI TRIFT V nanoTOF (ULVAC-PHI, Kanagawa, Japan), with a 60 keV Au_3^{++} primary ion beam and a triple focusing time-of-flight (TRIFT) mass analyzer. The beam was focused to a diameter of 500–600 nm with a pulsed current of 0.017 pA in the bunched mode and the ion dose was 2.5×10^{12} ions/cm^2. We analyzed the sample of 50×50 μm, and obtained negative secondary ions in a mass range of *m/z* 0–1850. Mass spectra and ion images were constructed using WinCadenceN software (ULVAC-PHI). For ion counts of individual cells, mass spectra obtained from cells were extracted from the regions of interest (ROIs) along the outline of the cells set according to the total ion counts. From each spectrum, the ion counts of phosphoric acid (*m/z* 79.0), palmitoleic acid (*m/z* 253.2), palmitic acid (*m/z* 255.2), linoleic acid (*m/z* 279.2), oleic acid (*m/z* 281.2), and stearic acid (*m/z* 283.2) were computed. The intensity of each ion was calculated using the area under the curve with a window in the range of *m/z* 0.1 around the peak of each ion. The intensities of fatty acids were normalized to the intensities of phosphoric acid ions, which are fragments of membrane phospholipids. We performed comparisons of mean signal intensities for the five types of fatty acids between MM cells and PCs.

2.5. Cell viability assay

Before the cell viability analysis, cells were re-suspended with fatty acid free-RPMI1640 medium. Fatty acids and bortezomib were dissolved in dimethyl sulfoxide (DMSO) (WAKO, Osaka, Japan) and fatty acid-free BSA (FAF-BSA) (WAKO) in RPMI1640 medium to produce a mixture of 10–1000 μM fatty acid, 0.1% DMSO and 0.5% FAF-BSA medium. The 0.1% DMSO, 0.5% FAF-BSA-RPMI1640 medium was used as a control. Bortezomib was also dissolved in RPMI1640 medium for a final concentration of 0.1% DMSO and 0.5% FAF-BSA. Cells were treated with the following reagents: palmitic acid (0–1000 μM), palmitoleic acid (0–1000 μM), linoleic acid (0–1000 μM), oleic acid (0–1000 μM), stearic acid (0–1000 μM), and bortezomib (0–1000 nM). Cells were plated at a density of 2×10^5 cells/ml in a 48-well plate. The number of viable cells in suspension at 72 h after treatment was determined by the Trypan Blue exclusion test. The concentration of palmitic acid necessary to reduce the cell viability to 50% (IC_{50}) was determined using a probit analysis.

2.6. Co-culture experiments

HS-5 cells (2×10^5 cells) were grown in a 12-well Transwell plate (Corning, Corning, NY, USA) that contained 10% FBS in DMEM medium until development of a confluent monolayer. Cocultures were initiated by seeding U266 cells into the Transwell insert at density of 2×10^5 cells/well. Cells were cultured in 0.1% DMSO, 0.5% FAF-BSA-RPMI1640 medium containing palmitic acid

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