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Short-term effects of 7-ketocholesterol on human adipose tissue mesenchymal stem cells *in vitro*

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ABSTRACT

Oxysterols comprise a very heterogeneous group derived from cholesterol through enzymatic and non-enzymatic oxidation. Among them, 7-ketocholesterol (7-KC) is one of the most important. It has potent effects in cell death processes, including cytoxicity and apoptosis induction. Mesenchymal stem cells (MSCs) are multipotent cells characterized by self-renewal and cellular differentiation capabilities. Very little is known about the effects of oxysterols in MSCs. Here, we describe the short-term cytotoxic effect of 7-ketocholesterol on MSCs derived from human adipose tissue. MSCs were isolated from adipose tissue obtained from two young, healthy women. After 24 h incubation with 7-KC, mitochondrial hyperpolarization was observed, followed by a slight increase in the level of apoptosis and changes in actin organization. Finally, the IC_{50} of 7-KC was higher in these cells than has been observed or described in other normal or cancer cell lines.

1. Introduction

Maintenance of the cellular redox balance is crucial for cell survival. Lipids are very sensitive to oxidative modifications [1]. Lipid oxidation is characterized by complex product patterns, including lipid peroxides, aldehydes, and many others. Moreover, molecules with regulatory functions are generated by the oxidation of lipids and fatty acids [2–6]. Oxidized phospholipids are recognized as important mediators of cellular signaling [7]. Oxidative stress, antioxidant efficiency, and lipid oxidizability are known to change in different pathophysiological conditions [8].

Cholesterol is an important structural element of cell membranes and an essential substrate for the biosynthesis of several molecules, such as bile acids and steroid hormones [9]. It is transported in plasma mainly by low-density lipoprotein (LDL). The cholesterol molecule is easily oxidized and may be transformed into numerous oxidation products known as oxysterols. They can be considered a way to route the cholesterol molecule for catabolism.

Oxysterols comprise a highly heterogeneous group derived from cholesterol through enzymatic and non-enzymatic oxidation [10]. Among them, 7-ketocholesterol (7-KC) is one of the most important, and is found in relatively large abundance in oxidized low-density lipoprotein (oxLDL) [11,12].

Oxysterols exhibit several biological activities. They play essential roles in a number of physiological processes, such as cholesterol homeostasis regulation, platelet aggregation, and protein prenylation [13]. They participate in the control of lipid metabolism and regulation of the immune system, and have been associated with several other pathophysiological processes [14].

Oxysterols have potent effects on cell death processes, including apoptosis induction [15,16]; reactive oxygen species (ROS) are reportedly involved in this effect [15]. In fact, oxysterols have been shown to exhibit cytotoxicity in a number of cell lines, including smooth muscle cells, fibroblasts, and vascular endothelial cells [17].

Mesenchymal stem cells (MSCs) are multipotent cells characterized by self-renewal and cellular differentiation abilities [18]. *In vitro*, according to the Society for Cellular Therapy, MSCs are described as cells that are capable of adhering to plastic with fibroblast-like morphology and differentiating into mesenchymal lineages (e.g., chondrogenic, osteogenic, and adipogenic lineages)

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under appropriate culture conditions. They are also characterized by the expression of CD105, CD73, and CD90, as well as by the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II. The property of self-renewal is essential for the expansion of the stem cell pool during fetal development, as well for maintenance of the stem cell pool throughout the organism's lifespan [19].

Several oxysterols exert important effects on cell death, proliferation, and differentiation: 7-ketocholesterol is known to induces monocyte differentiation [11]; and 22(R)-hydroxycholesterol and 25-hydroxycholesterol are potent inhibitors of cell growth in thymocytes, lymphocytes, and keratinocytes, as well as inducers of keratinocyte differentiation [20]. However, very little is known about the effects of oxysterols in MSCs. There are few descriptions, and most are related to their osteogenic effect. In fact, some types of oxysterols can behave as anti-adipogenic signals by diverting pluripotent MSCs away from adipogenic and toward osteogenic differentiation [14].

Here we described the short-term cytotoxic effect of 7-ketocholesterol on MSCs derived from human adipose tissue.

2. Materials and methods

2.1. Human adipose tissue mesenchymal stem cell isolation and characterization

Adipose tissue was obtained from two young, healthy women (20 and 22 year old) that underwent abdominal plastic surgery for esthetic reasons. The Ethical Committee of the Institution approved the protocol for this study, and patients provided written informed consent. With more than 90% of the volume, adipocytes have been described to represent the major cell type in lipoaspirate material [21]. From each patient, 30 ml of fatty material was collected in a sterile flask. The tissue was dissociated with 30 mg of collagenase type IA diluted in 30 ml of Dulbecco's Modified Eagle Medium (DMEM) for 45 min, and then centrifuged to isolate the cells. Medium consisting of 12 ml DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics/antifungal (100 μg/mL streptomycin, 100 UI/mL penicillin, 0.25 μg/mL amphotericin B) was added to the cell pellet. After transference to 75 cm^2 culture flasks, cells were incubated at 37 °C in 5% CO_2 atmosphere [22]. After reaching confluence, cells were detached with a solution containing 0.05% trypsin in 0.02% EDTA and seeded at a density of 5×10^3 cells/cm^3. Cells were used for experiments at the 4th passage.

Total RNA was extracted from undifferentiated adipose tissue mesenchymal stem cells (hAMSCs) using Trizol (Invitrogen, Caltag Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Oct-4 and Nanog gene expression were determined using RT-PCR, as described [23].

Cell surface markers were measured using flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). After trypsinization and washing with PBS, approximately 5×10^5 cells were stained for 15 min in the dark with primary monoclonal antibodies against CD34, CD49d, CD73, and CD90, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrine (PE), all from Invitrogen. The number of acquired events was 10,000 per acquisition using the BD CellQuest Pro software. Finally, hAMSCs were also characterized by their osteogenic and chondrogenic differentiation capability *in vitro*, as described [23].

2.2. Stem cell treatments

The purity of 7-KC (Sigma–Aldrich, St. Louis, MO) was determined to be ~98% by GS/MS. For all experiments, a 7-KC stock solution was prepared at a concentration of 1000 μM in absolute ethanol. The concentrations used in the experiments were in the range of those described to induce cell death on several cell lines [24]. hAMSCs from each donor were plated at a density of 5×10^3 - cells/cm^3 in 96-well Black Flat Bottom Polystyrene Microplates (Corning, MA) and incubated as described above. After 24 h, the medium was replaced with fresh DMEM without FBS for 24 h. Several concentrations of 7-KC (0–100 μM, 200 μl final volume) were added, followed by incubation for another 24 h. At the end of this experimental period, several parameters were determined in each of the two samples, as described hereafter.

2.3. Cell viability assay

Cell viability was determined using MTT (3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) [25]. Briefly, 10 μl MTT reagent (Sigma–Aldrich) were added to each well to a final concentration of 5 mg/ml, incubated for 4 h at 37 °C and centrifuged at 2000 rpm for 10 min. The medium was discarded and 100 μl of dimethyl sulfoxide (Sigma–Aldrich) were added to each well. The experiment was performed using six replicates for each oxysterol concentration and was repeated three times. Cholesterol at the same concentrations was also used as control. The amount of formazan was determined by measuring the absorbance at 570 nm refereed to 630 nm using an Elx800™ Absorbance Microplate Reader (Biotek, Winooski, VT). For IC_{50} calculations, survival data were evaluated by variable slope curve-fitting with GraphPad Prism (GradPad Software, CA).

2.4. Detection of apoptosis

The Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences) was used to determine the percentages of apoptotic cells, as described by the manufacturer. Cells were incubated with 0.5 μl of FITC Annexin V and 0.5 μl PI and incubated for 15 min at room temperature in the dark. The nuclei were counterstained with 0.1 μg/mL Hoechst 33342 (Molecular Probes, NY) for 10 min. The presence of apoptosis was analyzed within 1 h using an ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA). Nine sites per well and three wells per treatment were acquired. Apoptosis (%) was determined by the MetaXpress Cell Health software application.

2.5. Detection of caspase-3/7 activity

Caspase-3/7 activity was measured using the NucView 488 caspase-3 Assay kit for live cells (Biotium, Hayward, CA). The nuclei were counterstained with 0.1 μg/mL Hoechst 33342. Fluorogenic substrates were determined using ImageXpress. Nine sites per well and three wells per treatment were acquired. Caspase-3/7 activity was determined using the cell scoring MetaXpress software.

2.6. Measurement of transmembrane mitochondrial potential

Mitoscreen (BD Biosciences) was used to evaluate the transmembrane mitochondrial potential ($\Delta\psi$). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of $\Delta\psi$. JC-1 can exist in two different states, aggregates (at higher dye concentrations) and monomers (at low concentrations), each with different emission spectra. Uptake of JC-1 monomers into mitochondria is driven by the $\Delta\psi$. The $\Delta\psi$ of normal, healthy mitochondria is polarized, and JC-1 is rapidly taken up by such mitochondria. This uptake increases the concentration of JC-1, leading to the formation of JC-1 aggregates within the mitochondria. JC-1