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Lanthanum citrate induces anoikis of Hela cells

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ABSTRACT

Some reports show that lanthanum, a rare earth element, induces apoptosis in certain cancer cells. In the present paper, we report the first observation of anoikis induced by lanthanum citrate (LaCit) in Hela cells at a concentration of 0.001–0.1 mmol/L after 48 h-treatment. Before cell treatment, Hela cells were subjected to anoikis-resistant selection to remove anoikis-sensitive cells and ensure specificity of LaCit-induced anoikis. Anoikis was determined by Annexin/PI, AO/EB staining, cleavage of PARP and soft-agar colony forming assay. Further, findings of decreased mitochondrial membrane potential, the cleavage of caspase-9 and a dose-dependent increase expression of Bax were detected, suggesting that the intrinsic caspase pathway was involved in the anoikis induced by LaCit. In addition, activation of caspase-8 occurred later than that of caspase-9. LaCit also caused reorganization of actin cytoskeleton, and was accompanied by an increase in co-localization of F-actin with mitochondria, implying that both actin cytoskeleton and mitochondria may play important roles in LaCit -induced anoikis.

1. Introduction

Cell–matrix interaction and cell–cell contact play important roles in regulating the proliferation, survival and architecture of mammalian cells including normal cells and non-transformed tumor cells. If either of these two types of associations becomes inadequate or inappropriate, a specific type of apoptosis named anoikis may be triggered. However, transformed cells are often anchorage-independent and lose contact inhibition during cell growth. This acquisition of anoikis resistance is considered to be a crucial step in the tumorigenic transformation of cells [1,2]. Therefore anoikis is a particularly useful tool to study the molecular mechanisms of cancer treatments and provides novel apoptosis-targeted cancer therapeutic approaches.

Rare earth elements (REEs) are widely used in industry and medicine. As an example, radioactive REEs can be used in the diagnosis and treatment of cancer. This therapeutic aspect attracts increasing interest and inspires many researchers to investigate REE effects on tumor development and growth. There is substantial evidence showing that REEs inhibit proliferation and induce apoptosis in certain cancer cell lines [3–6]. Lanthanum is a representative REE, which is of considerable research interest because of strong effect on cell growth and death. However, here has been no published report concerning the effect of rare earth elements on anoikis. In the present study, we report for the first time our investigation on the effect of lanthanum citrate (LaCit), in which lanthanum exists as ionic state (La^{3+}), on inducing anoikis in Hela cells. LaCit was found to induce anoikis at the dose between 0.001 and 0.1 mmol/L. Anoikis appeared to progress by the intrinsic caspase pathway and also involved a reorganization of actin cytoskeleton which occurred after activation of caspase-9 and an increase in co-localization of F-actin with mitochondria.

2. Materials and methods

2.1. Cell culture and reagents

Hela cells and MDCK cells were purchased from Shanghai Cell Bank (Shanghai, China) and maintained in DMEM

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and RPMI-1640, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO_2 incubator. All experiments were performed using cells within 30 passages.

LaCit solution was prepared from lanthanum oxide (purity > 99.9%). The concentration of stock solution was 40 mmol/L. LaCit was diluted to its final concentration directly into the medium before use.

DMEM, RPMI-1640 and FBS were from Hyclone (Beijing, China). The cell Counting Kit-8 (CCK-8) and Mitochondrial membrane potential assay kit With JC-1 were from Beyotime (Shanghai, China). Polyhydroxy-ethylmethacrylate (Poly-HEMA), collagenase, hyaluronidase and trypsin were from Sigma (Guangzhou, China). Antibodies of cleaved caspase-8 and caspase-9 were obtained from ABZOOM (Shanghai, China). Other antibodies were purchased from Santa Cruz Biotechnology (Guangzhou, China). FITC Annexin V – PI kit and AO/EB kit were from Nanjing Keygen Biotech (Nanjing, China). The DAB kit was from Invitrogen (Guangzhou China). Alexa Fluor® 488 phalloidin and MitoTracker Red CMXRos were from Molecular Probes (Shanghai, China). Confocal dish was from Matek (Shenzhen, China).

2.2. Anoikis-sensitivity test

6-Well plates were coated with 10 mg/mL poly-HEMA twice [7]. Briefly, a solution of 10 mg/mL poly-HEMA in 100% ethanol was incubated at 37 °C for 12 h to dissolved particles completely. After 1 mL solution was added into every well and allowed to dry, another 0.5 mL was added, and then plates were left to dry at room temperature. The coated plates were UV sterilized. Before use, the plates were washed with PBS three times. Hela and MDCK cells were seeded at the density of 3.0×10^5 cells/well in coated plates and control plates (the non-coated plates) and were allowed to grow for 24 and 48 h. Hela suspension cells were collected by centrifugation at 1000 rpm for 5 min. The supernatants were discarded and cells were washed gently using D-hanks. Because Hela cells formed compact cell aggregates, a complex of enzymes was added (0.1% trypsin, 0.1% collagenase and 0.1% hyaluronidase) to trypsinize cells. MDCK suspension cells were collected in the same way, but were just gently pipetted without trypsinization to disperse the cells. The dead and live cells were counted by trypan blue staining using hemocytometer.

2.3. Anoikis-resistant selection

In order to remove the proportion of anoikis-sensitive cells, Hela cells were subjected to repeated cycles of selection for survival and growth in suspension culture. The process was similar to the selection method mentioned by Rak [1]. After each 48 h incubation period on poly-HEMA, cells were collected and plated in monolayer culture, and left to recover for 24 h. The surviving cells were expanded and plated in new poly-HEMA coated dishes. Such selection was repeated until the death rate stabilized. After each selection, the dead and live cells were counted as described above. The high anoikis-resistant Hela cells were used for subsequent studies.

2.4. CCK-8 assay

High anoikis-resistant cells were trypsinized and a cell suspension containing 1.0×10^5 cells/mL culture media was prepared. Four concentrations of LaCit: 0.1, 0.01, 0.001 and 0.000 1 mmol/L were used in this study. A volume of 0.5 mL cell suspension containing LaCit was added to each well of a 24-well of poly-HEMA coated plate. Cells were cultured for 48 h. After adding 50 μL of the CCK-8 reagent to each well, the wells were incubated 1 h at 37 °C and 5% CO_2. A volume of 200 μL was transferred to a 96-well plate and the absorbance of each well measured at 450 nm in a microtiter plate reader.

2.5. Annexin V/PI assay

Quantitative apoptotic cell death by LaCit was measured by Annexin V/PI assay. Suspension Cells, after 48 h of treatment in poly-HEMA coated 6-wells, were collected by centrifugation at 2000 rpm for 5 min. Cells were trypsinized and washed twice with PBS (centrifugation at 2000 rpm for 5 min) and then resuspended cells in 500 μL Binding Buffer. After addition of 5 μL Annexin V-FITC and 5 μL of Propidium Iodide (PI) with mixing, the tubes were incubated for 5–15 min at room temperature in the dark. Annexin V-FITC binding was detected by flow cytometry (Ex = 488 nm, Em = 533 nm, FL1 filter for Annexin-V-FLUOS and FL3 filter for PI). The data was analyzed by WinMDI 2.9 software.

2.6. AO/EB staining

Fifty microliter of cell suspension following 12 h of 0.1 mmol/L was stained by 1 μL mixture staining solution of acridine orange (AO) and ethidium bromide (EB) according to manufacturer's instruction. The cells were observed by fluorescence microscopy.

2.7. Soft agar colony forming assay

A total 2000 cells were suspended in 2 mL of DMEM medium containing 0.3% agar and different concentration LaCit. The resulting suspension was added to a 6-well plate covered with a 2 mL layer of solidified 0.6% agar in DMEM medium with 10% fetal bovine serum. Cell colonies were allowed to form for 9 days and counted.

2.8. Mitochondrial membrane potential detection

Cells incubated with 0.1 mmol/L LaCit for 12 h in poly-HEMA coated 6-wells were collected and resuspended in fresh medium. After the addition of 0.5 mL JC-1 working solution, the cells were incubated in a CO_2 incubator for 20 min. The staining solution was removed by centrifugation and cells were washed with JC-1 staining buffer twice. Cells having low membrane potential were quantified by counting 200 cells per microscopic field (at 100×) in ten fields in each sample in triplicate under fluorescent microscope.