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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.

2.9. Western blotting analysis

Cells were washed with PBS and lysed on ice in RIPA buffer, pH 7.5. The samples were boiled for 5 min, and either used immediately for immunoblotting or stored at −20 °C before use. Total protein (40 μg) were analyzed on polyacrylamide gels and blotted onto PVDF membranes. The blots were blocked overnight at 4 °C in blocking buffer (TBST buffer with 5% BSA) and incubated with anti-human Bax monoclonal antibody (1:900), anti-human cleaved caspase-8 polyclonal antibody (1:500), anti-human cleaved caspase-9 polyclonal antibody (1:500), anti-human cleaved PARP polyclonal antibody (1:500), or anti-human β-actin polyclonal antibody (1:1000) for 2 h at room temperature. The membranes were then washed with TBST twice, each for 10 min and washed with TBS once for 10 min. The membranes were then incubated with peroxidase-conjugated goat anti-rabbit lgG (1:5000) for 2 h at room temperature. Finally, the membranes were washed and stained using DAB kit.

2.10. Double-staining of F-actin and mitochondria

A 200 μL suspension of cells received 0.1 mmol/L LaCit treatment for 3 h and 4 h. Then cells were incubated in CO_2 incubator for 1 h. The MitoTracker medium was carefully aspirated to avoid disturbing the cell pellet. Control cells were gently transferred to the centres of confocal dishes respectively and resuspended in pre-warmed (37 °C) medium containing serum and 300 nm MitoTracker Red CMXRos probe. Pre-warmed (37 °C) PBSA was added and incubated for 10 min to wash away unbound probe. 3.7% paraformaldehyde was pre-warmed (37 °C) and used to fix the cells for 15 min. After fixation, the cells were washed with PBSA three times. Then 0.2% Triton X-100 was added 15 min to permeabilize the cells. After cells were washed with PBSA, blocking buffer (1% BSA, 0.05% Triton X-100) was used to block nonspecific phalloidin binding sites for 1 h. 200 U/mL Alexa Fluor® 488 phalloidin diluted in blocking buffer was added into the cells and maintained for 30 min at room temperature. The cells were then washed with PBSA and left to dry at room temperature protecting from light. Cells were photographed by laser confocal microscopy.

3. Results

3.1. Hela cells were not completely anoikis-resistant

MDCK cells are anoikis-sensitive epithelial cells and served as positive control cells in our anoikis-sensitivity test. A change in cell morphology after anoikis-induction is a known characteristic of MDCK cells [8]. We observed both MDCK and Hela cells formed obvious visible cell aggregates after culturing on poly-HEMA for 4 h, and the size of the aggregates increased with time. The formation of cell aggregates was thought to be a mechanism of self-defense against the anoikis-induced cell death [8]. As shown in Fig. 1A, Hela cells formed larger and more compact cell aggregates than MDCK cells, suggesting stronger cell-cell contact and anoikis-resistance. The result of trypan blue staining agreed with the result with 37% of MDCK cells dead after culturing on poly-HEMA for 24 h. After 48 h, the proportion of dead cells reached 58%. However, the same conditions, caused 6% and 13% decreases in Hela cells respectively (Fig. 1B and C), showing an unacceptably high proportion of anoikis-sensitive cells among the Hela cells population. In addition, we found that when anoikis-induced Hela cells were transferred to regular culture dishes and allowed to reattach for 24 h, the growth rates of the surviving cells were greatly suppressed (data not shown). The presence of anoikis-sensitive cells might cause difficulty in identifying LaCit-induced cell damage and apoptosis. In order to avoid this problem, Hela cells were subjected to anoikis-resistant selection.

3.2. High anoikis-resistant Hela cells are obtained by three cycles of "anoikis-resistant selection"

As shown in Fig. 2A, the proportion of dead cells fell to 5.1% and 2.9% after Hela cells were subjected to 1–4 cycles of anoikis-resistant selection. This finding suggested that Hela cells gradually adapted themselves to the suspension environment and increasingly became anoikis-resistant.

We also used cell reattachment to measure cell survival. After each cycle of selection, an equal number of suspension cells were reattached to normal culture flask for 24 h. Then all dead and/or unattached cells were removed and the surviving cells were detached and counted. The total number of viable cells after the third selection was higher compared to the first cycle (Fig. 2B). In addition, we observed that the non selected cells spread more quickly than the resistant cells (Fig. 2C).

3.3. LaCit inhibits anchorage-independent growth of Hela cells

In the anoikis assay, LaCit treatment at the lowest dose (0.0001 mmol/L) did not produce any effect after 48 h of exposure compared with the control groups, but the higher doses caused strong dose-dependent inhibition of cell anchorage-independent growth resulting in 84.1–66.0% ($P < 0.05 - 0.001$) relative cell survival rate (Fig. 3A). This result was further supported by a soft-agar colony-forming assay by which Hela cells detached from the culture flask were grown on soft agar and colonies enumerated. As shown in Fig. 3B, inhibition of growth was observed with cells exposed to 0.1, 0.01, 0.001 mmol/L LaCit. The lowest dose had no effect on growth in soft agar.

We also examined any possible changes of cell morphology under the light microscope following LaCit treatments. We observed many small and loose cell aggregates with LaCit treatment suggesting that the cells had separated from cell aggregates (Fig. 3C). This effect was strongest at 0.1 mmol/L dose and occurred before exposures of 12 h, suggesting that LaCit might directly prevent cell-cell contact and cell aggregate formation, inhibiting the anchorage-independent growth of Hela cells at the early stage of treatment. In the above experiment, LaCit was added into the media at the beginning of the culture. However, we also added 0.1 mmol/L LaCit after the Hela cells were plated on poly-HEMA for 12 h to further examine this finding. Under the light microscopy, cell aggregates of Hela cells did not remarkably change following 48 h of LaCit treatment (data not shown). This observation showed that LaCit did not inhibit or damage the cell–cell contact of existing cell aggregates.

3.4. LaCit causes anoikis of Hela cells

Using Annexin V/PI staining, we observed that LaCit caused a strong dose-dependent apoptotic death of Hela cells. Treatment with 0.1, 0.01, 0.001 mmol/L LaCit for 48 h resulted in 39.6%, 26.27% and 17.47% ($P < 0.05 - 0.001$) apoptotic cells within there spective populations, accounting for 4.1- to 9.4-fold increase over control, respectively (Fig. 4A).

Cells treated with 0.1 mmol/L LaCit were further examined for AO/EB staining. When examined by fluorescence microscopy after treatment for 12 h, we observed that the nucleoids in control cells were round and stained green,[2] indicating they were normal. Although the nucleoids of LaCit-exposed cells were also green, some had irregular crescent and bursiform shapes, showing that the cells were in early stages of apoptosis (Fig. 4B).

PARP is a marker for apoptosis. It is a classical substrate for caspase-3 and its fragmentation is mediated by caspase-3 activity. LaCit-induced anoikis was detected by PARP analysis. A dose-response cleavage of PARP was observed (Fig. 4C).

Furthermore, whether the induced anoikis was specifically due to LaCit addition or to the change in the pH of culture medium added with LaCit were assessed. The pH of culture medium containing 0.1 mmol/L LaCit was measured. We used sodium citrate to treat cells following in the

[2] For interpretation of color in Figs. 2 and 4–6, the reader is referred to the web version of this article.

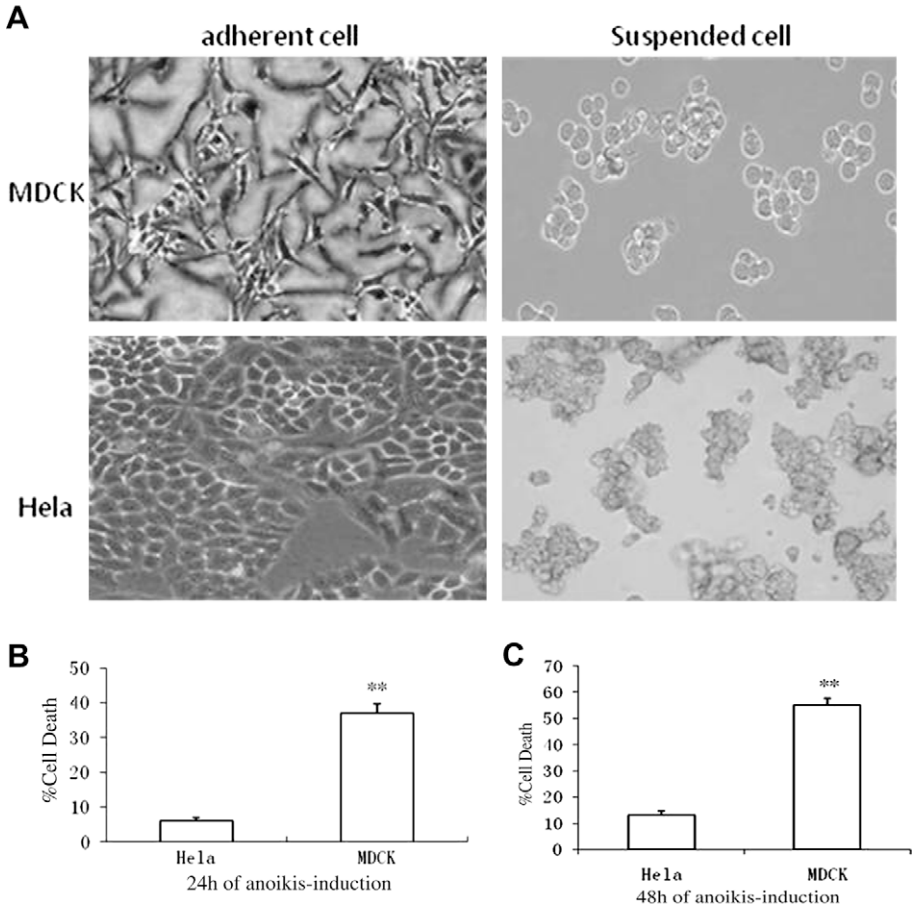

Fig. 1. Hela cells form larger cell aggregates and less anoikis-sensitive than MDCK cells. (A) Hela and MDCK cells were detached for 24 h and cell morphology was examined under a microscopy (magnification, 100×). (B, C) Hela and MDCK cells were collected after 24 h and 48 h of anoikis-induction and cell death rate was counted by trypan blue staining using hemocytometer. Values are presented as mean SE ($P < 0.01$).

same way as LaCit. Sodium citrate showed no effect on anoikis of Hela cells, and medium pH did not change obviously because of LaCit, suggesting that LaCit induced-anoikis is specific and not due to pH change in medium (data not shown).

3.5. Initiation of LaCit-induced anoikis is mediated through caspase-9

As shown in Fig. 5A, number of cells of low $\Delta\Psi m$ increased with time during anoikis induced by LaCit. Caspase-9 is the principal initiator caspase of the intrinsic apoptotic pathway. 48 h of LaCit treatment caused an obvious dose-dependent cleavage of caspase-9 and expression of Bax (Fig. 5B).

LaCit also resulted in a dose-dependent activation and cleavage of caspase-8 at 48 h (Fig. 5B). To further assess which pathway plays a more important role in anoikis, we selected the 0.1 mmol/L group to study the time kinetics of caspase-8 and caspase-9 cleavage. This dose of treatment did not resulted in caspase-8 cleavage until 24 h. However caspase-9 was activated as early as 3 h (Fig. 5C), demonstrating that the initiation of LaCit-induced anoikis is mediated through caspase-9. Caspase-8 might be activated secondarily as apoptosis proceeds or might mediate some of the apoptosis in this system.

3.6. Actin cytoskeleton alterations follow activation of caspase-9 at the early stage in LaCit-induced anoikis

Recent reports surprisingly show that actin dynamics also plays a central role in anoikis [9]. To analyze the dynamics of actin cytoskeleton organization during LaCit-induced anoikis, F-actin was stained with Alexa Fluor® 488 phalloidin and visualized by laser confocal microscopy. In all of the control cells, F-actin was distributed mainly around the cell membrane forming a thick ring staining localized in cytoplasm was diffuse. Treatment cells of 3 h showed no difference from the controls. However at 4 h, thicker and higher levels of F-actin appeared in the cytoplasm of treated cells, forming extensive curved fibers and disordered network (Fig. 6A and B). Surprisingly, we found that F-actin around the cell membrane in 4 h-treatment cells was different compared to that of the controls.

F-actin formed cilium-like structures which radiated from intracellular to extracellular location and densely coated the cell membranes in control cells. These structures also existed in the treated cells at 3 h, however by 4 h, many had disappeared while the remaining ones had became longer (Fig. 6B and C). In addition, those treated cells which had fewer cilium-like structures also had looser cell–cell associations comparing to the controls (Fig. 6C). We even observed the membrane actin cytoskeleton disrupted in some cells which made F-actin spill out (Fig. 6B). In some solid tumor cells such as Hela cells, the cleavage of actin during apoptosis is a downstream event of caspase activation and is activated by caspases. The cleaved actin then leads to the morphologic changes resembling apoptosis [10]. In our study, caspase-9 was activated before alterations of F-actin cytoskeleton in LaCit-induced anoikis cell (Figs. 5D and 6B); however, the relationship between the two is unknown and remains investigation.

3.7. Co-localization of F-actin with mitochondria is accompanied with reorganization of actin cytoskeleton in LaCit-induced anoikis

The increase in mitochondrial association of F-actin is considered to be an early event in apoptosis [11]. Therefore we investigated whether this situation occurred during LaCit-induced anoikis. By double staining,

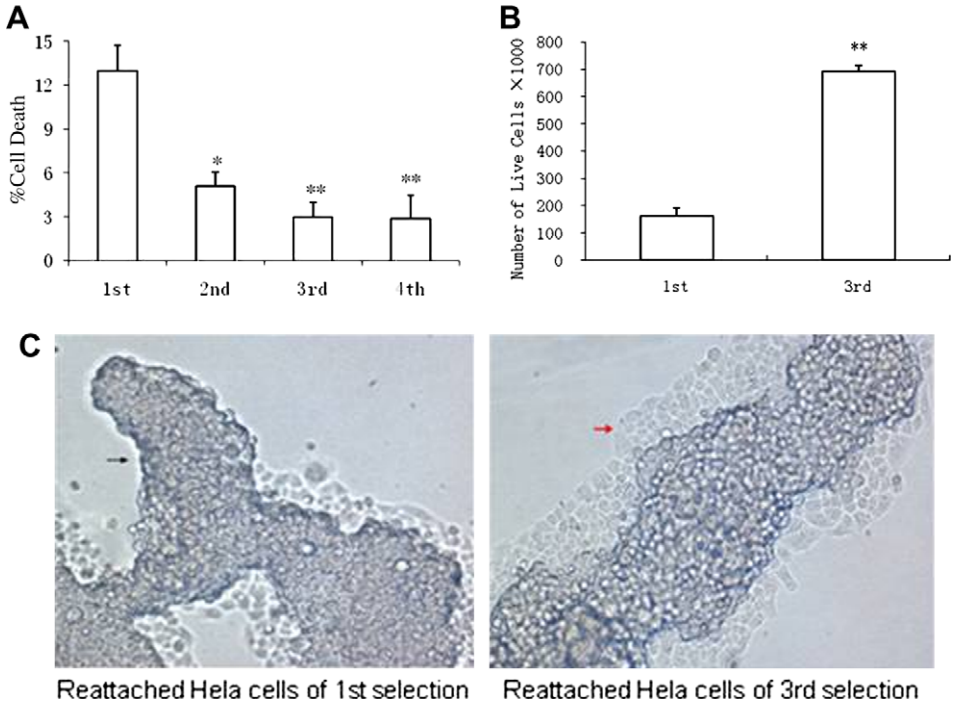

Fig. 2. Hela cells became less anoikis-sensitive gradually after anoikis-resistant selection. (A) Death rate of Hela cells was counted by trypan blue staining after each selection. Values are presented as mean SE ($P < 0.01$). The first selection group was as a control. (B) After reattachment for 24 h, the survival cells of 1st and 3rd selection were detached and counted by trypan blue staining. Values are presented as mean SE ($P < 0.01$). (C) Reattached cells of 1st selection spread less quickly than that of 3rd. The black arrow indicates that reattached cells did not proliferation. The red one shows cells spread (magnification, 100×).

we observed that when F-actin increased in the cytoplasm, mitochondria also distributed increasingly to cytoplasm. Fluorescent signals from F-actin and mitochondria were quantified and the results showed a significant increase in co-localization of F-actin with mitochondria following 0.1 mmol/L LaCit for 4 h compared with that of the control cells, which was accompanied with the reorganization of the actin cytoskeleton described above (Figs. 6A and B and 7). However, this phenomenon did not occur in 3 h treatment cells.

4. Discussion

La^{3+} has been regarded as calcium ion (Ca^{2+}) antagonist because of its similarity in coordination chemistry to Ca^{2+} [12,13]. However, the other biological functions of La^{3+} on cells including apoptosis have been poorly studied. In this study we have demonstrated that a La^{3+} compound, lanthanum citrate (LaCit), is able to induce anoikis in Hela cells. We found that LaCit induced damage and anoikis in Hela cells and much slower growth of the surviving cells was observed when cells were reattached. If those anoikis-sensitive cells were not removed, specific cell death caused by LaCit was difficult to detect. For this reason, we have developed an anoikis-resistant selection method to obtain highly anoikis-resistant cells which can proliferate in anoikis model. Such cells were indeed induced by LaCit to undergo anoikis in our study.

Caspases are cysteine proteases activated in most apoptosis pathways. The intrinsic and extrinsic caspase pathways have been considered to be the two major mechanisms of apoptosis in most cellular systems. Historically, apoptotic responses have been described as "extrinsic" or "intrinsic" based on whether the initiator caspase is caspases-8 or caspase-9. Anoikis is also mediated by the activation of caspases [14]. Which pathway predominates in anoikis seems to be cell-type dependent. Some evidence shows caspase-8 contributes importantly to cell death during anoikis [15], however some reports have disputed this conclusion and suggested caspase-8 activation occurs downstream of cytochrome *c* release in anoikis of human intestinal epithelial cells [16]. We measured mitochondrial membrane potential ($\Delta\Psi m$), cleavage of caspase-9, expression of Bax in order to investigate if LaCit-induced anoikis was involved by intrinsic caspase pathway mediating by mitochondria. Results showed that caspase-8 activation was a downstream event of caspase-9 activation, later by 21 h. Moreover we found that both mitochondrial membrane potential and expression of Bax in treated cells decreased significantly. These results suggest that the intrinsic pathway may be activated directly in this system.

Increasing evidence shows that the actin cytoskeleton performs a crucial role in mediating cell responses to both internal and external signals and apoptosis accompanies the dramatic reorganization of the cytoskeleton [17] and cytoskeletal actin is the substrate of caspase proteases and is cleaved by caspase [18]. We also found reorganization of the cytoskeleton occurred in the early stage of LaCit-induced anoikis and was caused a little later than activation of caspase-9. However we have not further investigated the linkage of the two phenomena in our pres-

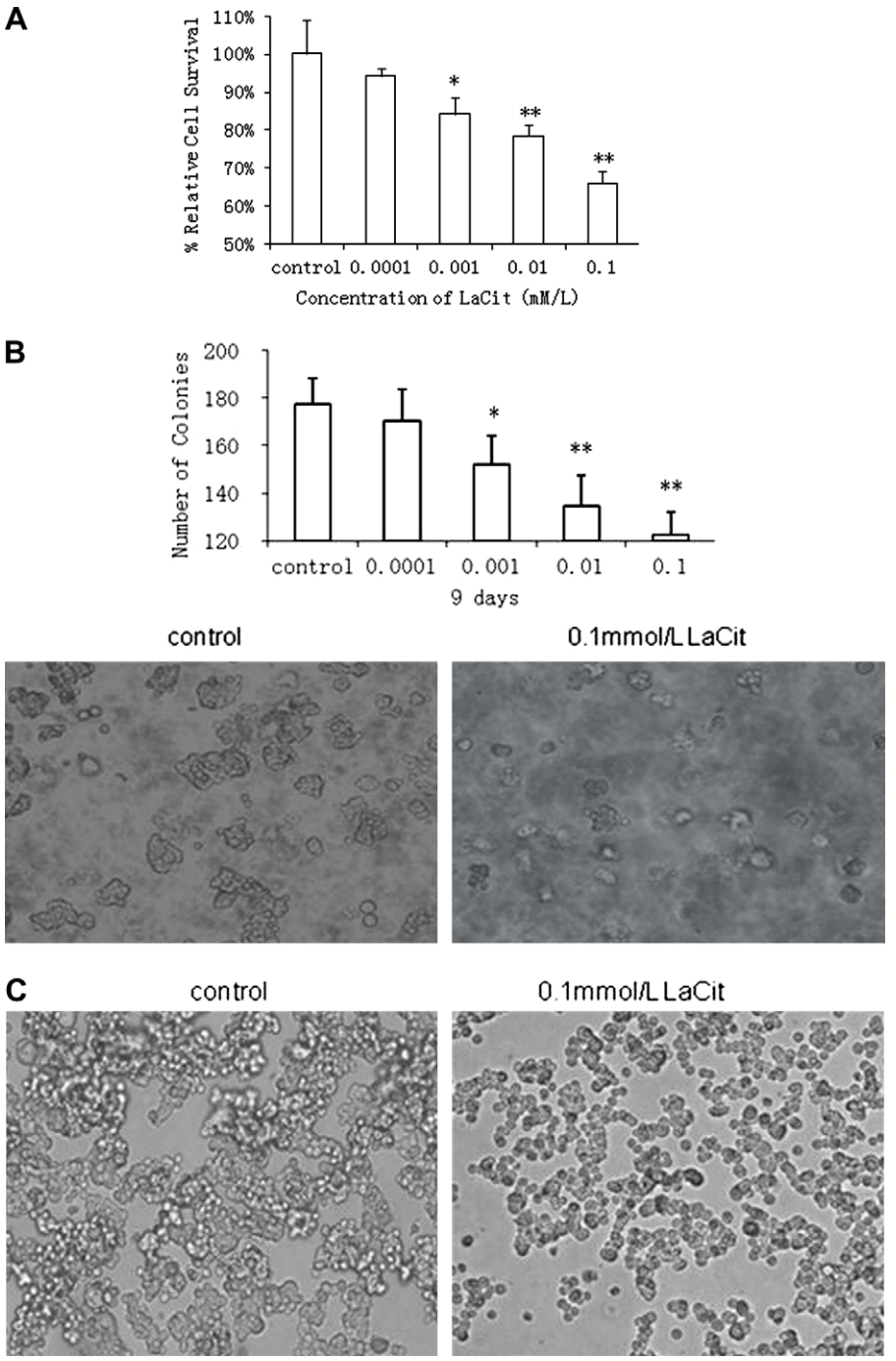

Fig. 3. LaCit inhibits anchorage-independent growth of Hela cells. (A) High anoikis-resistant Hela cells were subjected to LaCit treatment on poly-HEMA plates for 48 h. Cell survival was measured by CCK-8. Values are presented as mean SE ($P < 0.05$). (B) LaCit (0.001–0.1 mmol/L) inhibits Hela cells forming colony on soft agar. Number of colony was counted under microscopy (magnification, 100×) after growing for 9 days. Photograph was taken at 5 days. (C) Small and loose cell aggregates appeared after 12 h of 0.1 mmol/L dose treatment (magnification, 100×).

ent study. The possibility that alterations of actin cytoskeleton are independent of caspase-9 cannot be ruled out.

Rearrangement of the actin cytoskeleton also occurs in anoikis cells [19,20]. As ethanol-induced anoikis progresses, the F-actin cytoskeleton system looses its stress fibers, adopts a more rounded morphology and is reorganized into a peripheral membrane-associated ring in treatment cells [20]. In the present study, F-actin formed similar rings. However such structures existed both in treatment cells and the control cells, suggesting that

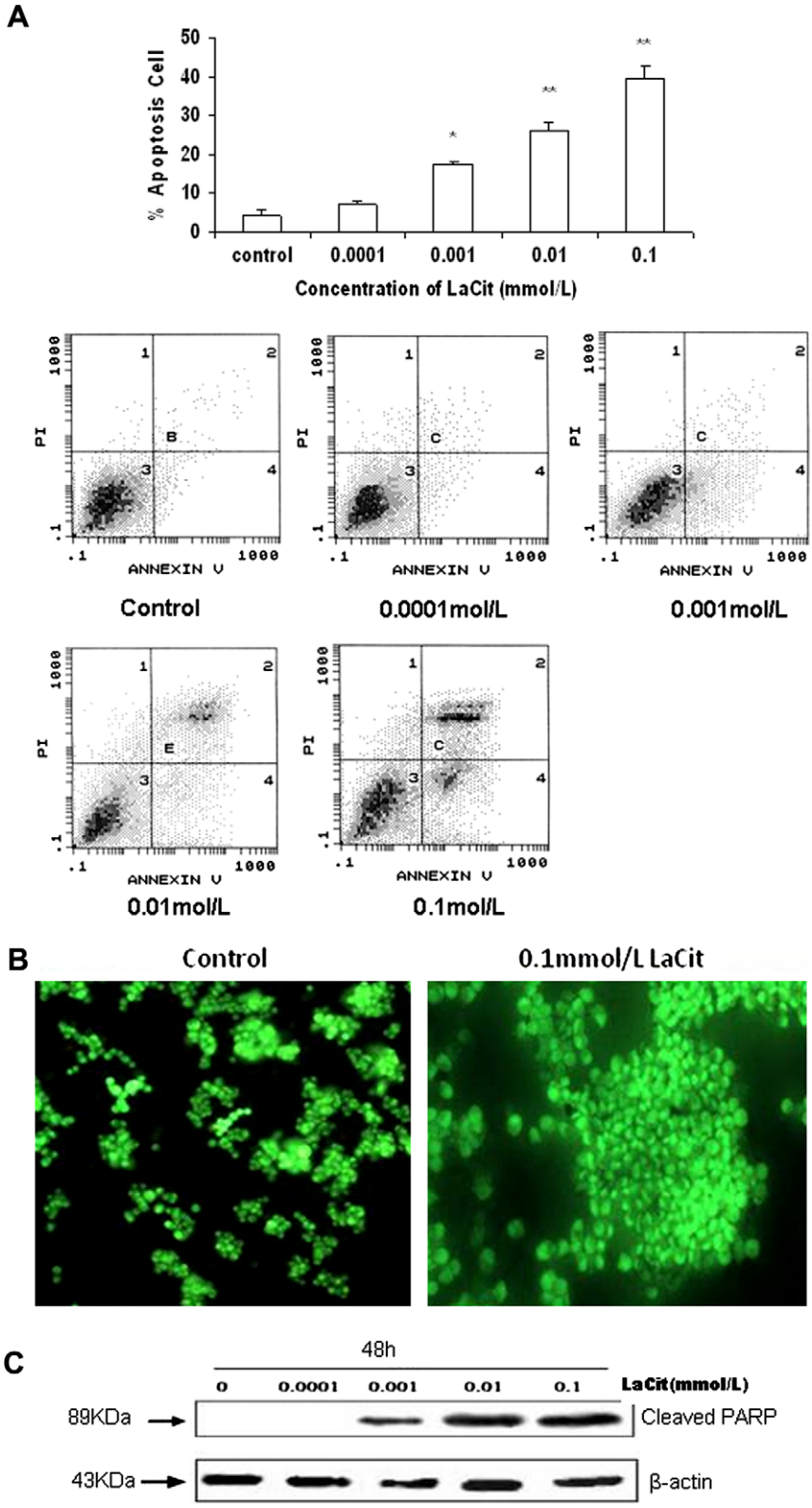

Fig. 4. LaCit induces anoikis of Hela cells. (A) Hela cells were treated with LaCit at different dose (0.0001–0.1 mmol/L) for 48 h and cells stained with Annexin V-PI. The percentage of apoptotic cells was measured by FACScan analysis. (B) Cells subjected to 0.1 mmol/L dose of 12 h treatment were stained by AO/EB. Under fluorescence microscopy, the green and round nucleoids indicate normal cells. The green and irregular shapes nucleoids indicate early apoptosis cells. (C) Hela cells were treated with LaCit (0.0001–0.1 mmol/L) for 48 h and Western blot analysis was done for cleaved PARP. Actin was used as a loading control.

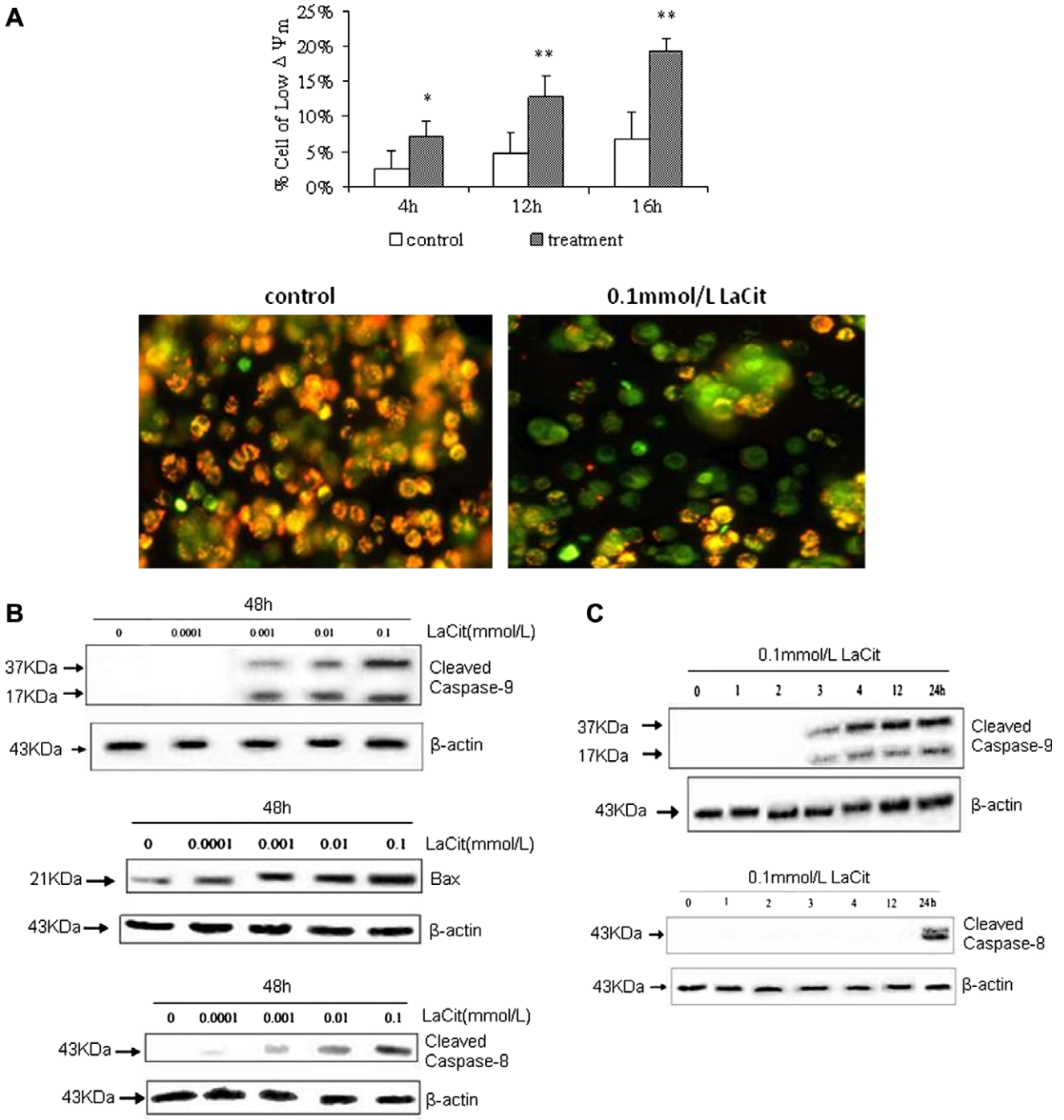

Fig. 5. LaCit induces caspase-dependent pathways in anoikis. (A) Cells subjected to 0.1 mmol/L dose for different time intervals (4, 8 and12 h) were stained by JC-1. Change of ΔΨm was detected by fluorescence microscopy. Normal cells which have high ΔΨm show punctuate yellow fluorescence. Apoptosis cells show diffuse green fluorescence because of decrease in MMP. $^{*}P < 0.001$; statistical significance in LaCit treated groups compared with the controls. (B) Hela cells were treated with LaCit (0.0001–0.1 mmol/L) for 48 h and Western blot analysis was done for cleaved caspase-9, caspase-8 and Bax. (C) Time kinetics of caspase-9 and caspase-8 cleavage following LaCit (0.1 mmol/L) treatment of Hela cells for 0, 3, 4, 12 and 24 h. β-actin was used as a loading control.

formation of actin ring was not specific to anoikis cells. It is worth noting that LaCit caused a special change of F-actin cytoskeleton which has been seldom described. As described above, as F-actin increased in cytoplasm and formed curved fibers and a disordered network, cilium-like structures of the actin ring and the actin connection

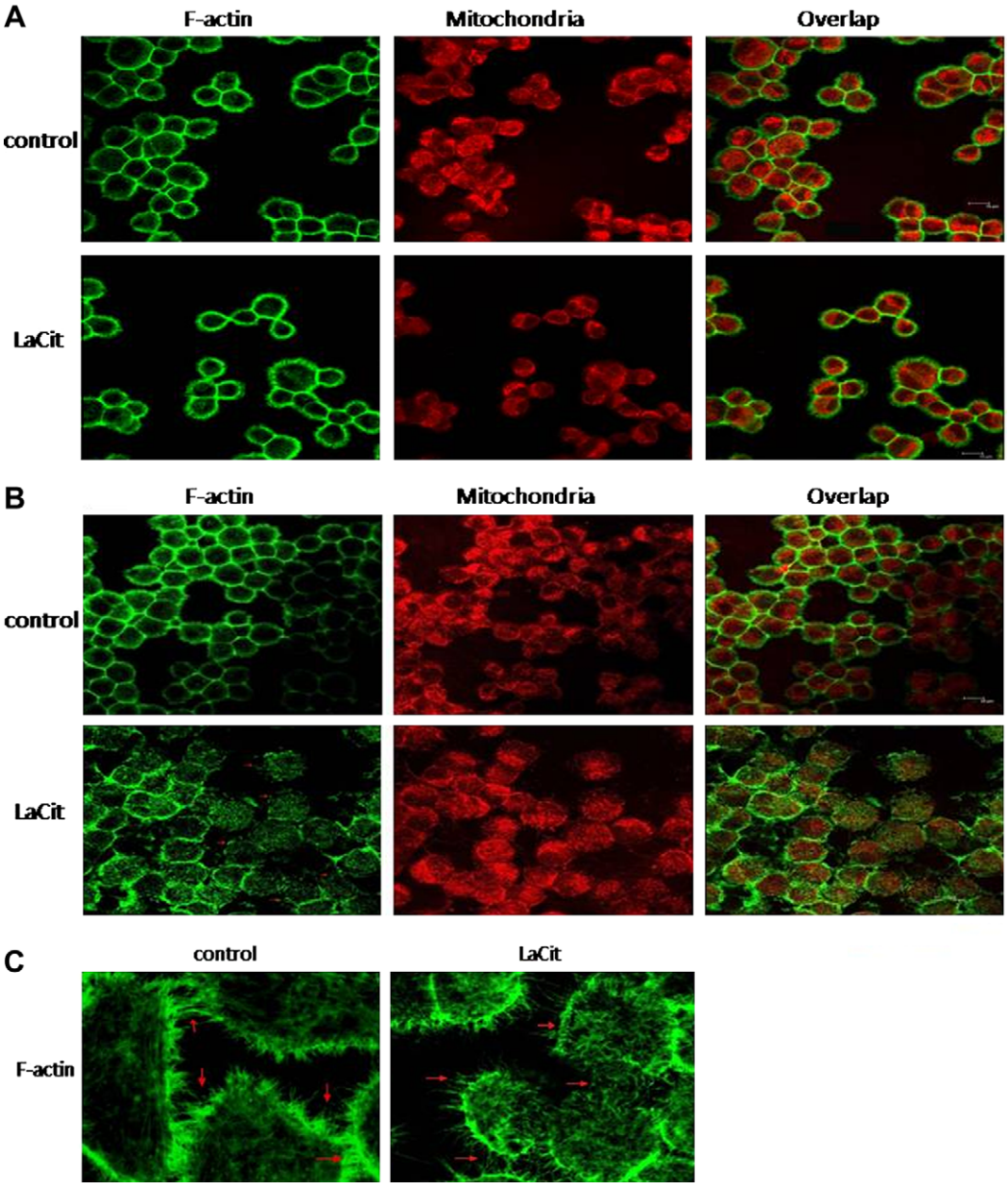

Fig. 6. Changes of F-actin and mitochondria in LaCit-induced anoikis were determined by confocal microscopy. Cells were double-stained by MitoTracker Red CMXRos (red) and Alexa Fluor® 488 phalloidin (green) which respectively label F-actin and mitochondria. (A) Monochromatic and overlaps images of control and 0.1 mmol/L LaCit-treated (3 h) Hela cells. (B) Monochromatic and overlaps images of control and 0.1 mmol/L LaCit-treated (4 h) Hela cells. (C) Cells were single-stained Alexa Fluor® 488 phalloidin. Enlarged fluorescence images of F-actin show the difference between control and 0.1 mmol/L treatment cells at 4 h (magnification, 1000×).

between cells decreased markedly. However whether such change is a consequence or a cause of anoikis is unknown and needs further investigation.

Recent findings indicate that mitochondria–actin interactions contribute to apoptosis [21,22]. Mutations in actin or actin-binding proteins can influence mitochondrial pathways leading to cell death and some cytoskeletal components co-localize with mitochondria or are targeted to the mitochondria during early steps in apoptosis [21]. Ho Lam et al. induced apoptosis of various cells by different stimuli and found that an increase in mitochondrial translocation of actin is a general phenomenon in apoptosis. Perhaps actin could contribute to the initiation of apoptosis by enabling cytosolic pro-apoptotic proteins to be carried to mitochondria by the cytoskeleton-driven trafficking system [11]. The present study indicate that La-

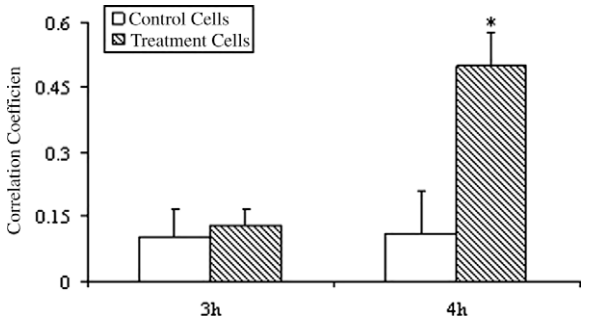

Fig. 7. Increase in co-localization of F-actin and mitochondria at an early stage of LaCit-induced anoikis. Correlation coefficient of the fluorescent signals of F-actin and mitochondria in control and treatment cells at 3 h and 4 h was calculated by Adobe Photoshop. $^{*}P < 0.01$.

Cit resulted in a significant increase in co-localization of F-actin with mitochondria in Hela cells, supporting a link between the actin cytoskeleton, mitochondria and anoikis.

Metastasis is a major factor in the malignancy of cancers, and is often responsible for the failure of cancer treatment. Anoikis has been suggested to act as a physiological barrier to metastasis; resistance to anoikis may allow survival of cancer cells during systemic circulation. Thereby facilitating secondary tumor formation in distant organs [23]. Low concentration LaCit can induce anoikis of Hela cells, which may implied a novel potential clinical application on cancer treatment.

Conflicts of interest

None declared.

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