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Chloroquine inhibits cell growth and induces cell death in A549 lung cancer cells

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Abstract—To investigate the effects of chloroquine diphosphate (CQ) on lung cancer cell growth, we treated A549 cells, a lung cancer cell line, with the drug at various concentrations (0.25–128 μM) for 24–72 h. The results showed that, at lower concentrations (from 0.25 to 32 μM), CQ inhibited the growth of A549 cells and, at the same time, it induced vacuolation with increased volume of acidic compartments (VAC). On the other hand, at higher concentrations (64–128 μM), CQ induced apoptosis at 24 h, while its effect of inducing vacuolation declined. The lactate dehydrogenase (LDH) assay showed that with the treatment of CQ 32–64 μM for 72 h or 128 μM for 48 h, CQ induced necrosis of A549 cells. To understand the possible mechanism by which CQ acts in A549 cells, we further incubated the cells with this drug at the concentrations of 32 or 128 μM in the presence of D609, a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC). The results showed that D609 (50 μM) could inhibit the effects of CQ 32 μM on the viability and VAC, but it could not change the effects of CQ 128 μM on the same. Our data suggested that CQ inhibited A549 lung cancer cell growth at lower concentrations by increasing the volume of lysosomes and that PC-PLC might be involved in this process. The data also indicated that, at higher concentrations, CQ induced apoptosis and necrosis, but at this time its ability to increase the volume of lysosome gradually declined, and PC-PLC might not be implicated in the process.

1. Introduction

Chloroquine is a derivative of quinine and has been widely used as an anti-malarial drug. Recently, several studies have demonstrated that it has extensive biological effects such as mediating radiosensitization in MDA-MB231 cells by increasing necrosis,[1] facilitating premature apoptosis of the infected cells by parasites, and so on.[2] CQ and related anti-malarial drugs appeared to promote apoptosis in T-cells and C6 glioma cells.[3] These results indicated that CQ might cause distinct cell death in different kinds of cells. To address this question, we investigated the effects of CQ on cell growth, apoptosis, and necrosis of A549 lung cancer cells, respectively, and discovered that CQ could inhibit cell growth and induce apoptosis and necrosis in A549 lung cancer cells at different concentrations.

CQ is a well-known lysosomotropic agent. It can increase lysosomal volumes and subsequently enlarge the plasma membrane surface.[4,5] An increase in the total volume of lysosomes is a common event in apoptosis or necrosis.[6] CQ also can concentrate in acidic cytoplasmic vesicles, which increases the cytoplasmic pH in the vesicle. Elevation of the lysosomal pH may influence endocytosis, exocytosis, and phagocytosis.[4,5,7] A recent report revealed that PC-PLC was involved in the death of L929 cells induced by TNF-α.[6] To understand the possible mechanism by which CQ acts in A549 cells, we investigated the role of PC-PLC in CQ inhibiting cell growth and inducing apoptosis and necrosis of A549 lung cancer cells by using D609, a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC).

Keywords: Chloroquine diphosphate; A549 lung cancer cells; Cell death; PC-PLC.
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2. Results

2.1. Effects of CQ on cell morphological changes

When A549 cells were exposed to CQ, from 0.25 to 128 μM, between 2 and 72 h, the cells vacuolated gradually as the concentration increased and the time continued (Fig. 1). As the concentration was higher than 64 μM, besides vacuolation of cells, many cells shrank, plasma membrane blebs occurred, and cell detachment was triggered dramatically.

2.2. Effects of CQ on cell proliferation

We first tested the antiproliferative effect of CQ on A549 cells by the MTT assay which is reliable to detect proliferation of cells.[8] As shown in Figure 2, exposure of cells to CQ 0.25–128 μM for 24, 48, and 72 h resulted in decrease of the cell viability in different degree. We discovered that CQ inhibited the proliferation of A549 cells in a time-dependent manner.

2.3. Drug toxicity of CQ on A549 cells

To confirm the mode of cell death induced by CQ and to examine the drug toxicity, LDH assay was performed. As shown in Figure 3, there was no significant difference ($p > 0.05$, $n = 3$) in LDH release between the cells of control group and cells treated with CQ 16 μM for 72 h, or 16–128 μM for 24 h. But CQ 32 or 64 μM at 72 h, 128 μM at 48 or 72 h promoted LDH release significantly ($p < 0.05$, $n = 3$), which indicated that cell necrosis occurred.

2.4. Nuclear fragmentation assay

When A549 cells were treated with CQ 128 μM for 24 h, the results of acridine orange (AO) staining showed that a part of A549 cells died by apoptosis (Fig. 4). Nuclear condensation and/or fragmentation and apoptotic bodies occurred in the cells. Lysosomal vacuoles appeared. There was no significant nuclear fragmentation in control and CQ 32 μM-treated groups.

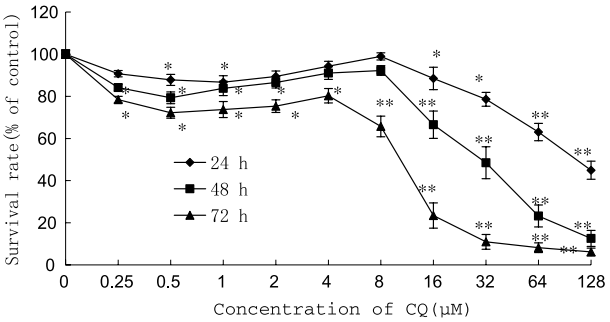

Figure 2. Proliferative inhibition of CQ 0.25–128 μM on A549 cells. The asterisk indicates a significant difference between control group and CQ-treated groups by *t* test ($^{*}p < 0.05$ vs control group, $^{**}p < 0.01$ vs control group, $n = 3$).

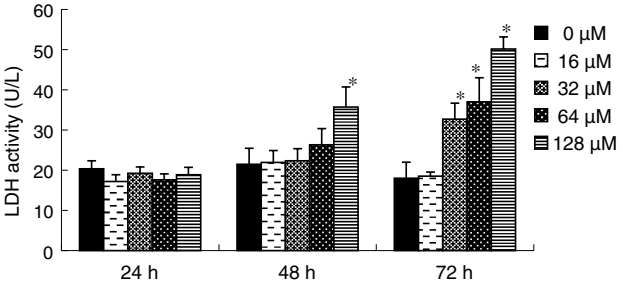

Figure 3. Effect of CQ on the release of LDH from A549 cells. The cell culture medium from the test group and the control groups was collected after 24, 48, and 72 h of treatment with CQ 0, 16, 32, 64, and 128 μM, respectively. Light absorptions were analyzed at 340 nm using a model Cintra 5 UV–visible spectrometer ($^{*}p < 0.05$ vs control group at the same time, $n = 3$).

Nuclear condensation and/or fragmentation and apoptotic bodies could be regarded as a morphological symbol of apoptosis.[9] The results of morphological changes and nuclear fragmentation obtained in our study indicated that CQ 64 and 128 μM initiated the apoptosis of A549 cells.

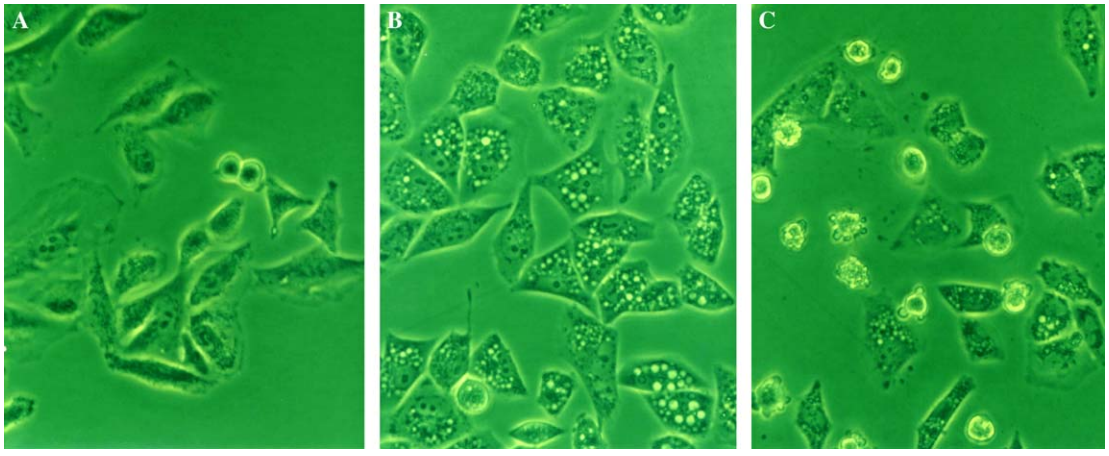

Figure 1. Effect of CQ on A549 cell morphology. Cells were treated with CQ 0, 32 or 128 μM for 24 h, respectively. The figures were obtained by using a phase contrast microscope (×400). (A) Control group. (B) The test group treated with CQ 32 μM. (C) The test group treated with CQ 128 μM.

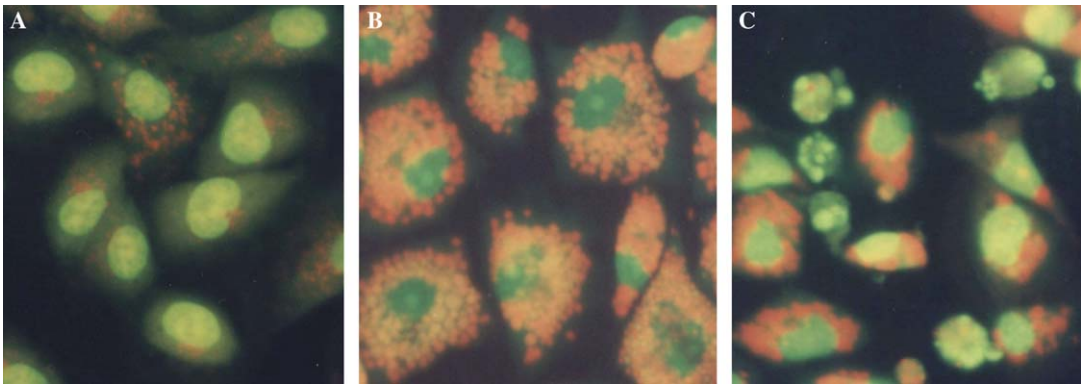

Figure 4. Effect of CQ on nuclear fragmentation of A549 cells. Cells were treated with CQ 0, 32, and 128 μM, respectively, for 24 h, and stained with AO. Orange vacuoles in cells showed that they were acidic. (A) Control group. (B) The test group treated with CQ 32 μM in which the lysosomal vacuolation was obvious. (C) The test group treated with CQ 128 μM in which the nuclear fragmentation was obvious (×600).

2.5. Effects of CQ on VAC of A549 cells

Neutral Red is traditionally used to stain lysosomes and quantify the VAC in cells.[10,11] As shown in Figure 5, we observed that vacuoles in A549 cells were acidic because they were neutral red staining positive.

The VAC of CQ treated A549 cells increased in accordance with the increase of concentration less than 32 μM, but decreased at higher concentrations more than 32 μM, and at this time apoptosis occurred (Fig. 6).

2.6. Effects of D609 on VAC and viability of A549 cells treated with CQ

As shown in Fig. 7, D609 did not influence the viability and VAC of A549 cells in the absence of CQ. D609 suppressed the actions of CQ 32 μM, but had no obvious effects on the VAC and viability of A549 cells treated with CQ 128 μM. The results suggested that PC-PLC facilitated the vacuolation and proliferation inhibition induced by CQ 32 μM in A549 cells.

3. Discussion

In this study, we first elaborately examined the effects of CQ on the growth, apoptosis, and necrosis of A549 lung cancer cells at various concentrations and different treatment time points. We found that, at lower concentrations (from 0.25 to 32 μM), CQ inhibited the growth of A549 cells and, at the same time, it induced vacuolation with increased volume of acidic compartments (VAC). On the other hand, at higher concentrations (64–128 μM), CQ induced apoptosis at 24 h, while its effect of inducing vacuolation declined. Our results also showed that with the treatment of CQ 32–64 μM for 72 h or 128 μM for 48 h, CQ induced necrosis of A549

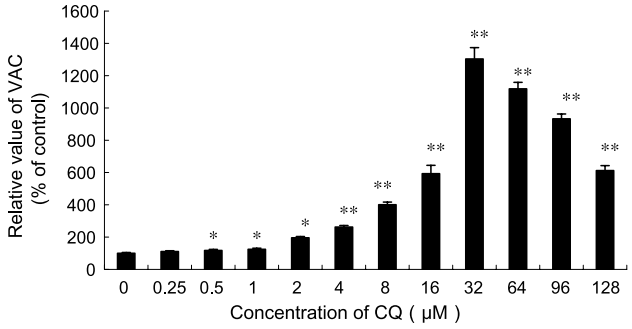

Figure 6. Effect of CQ on VAC of A549 cells. VAC of A549 cells treated with CQ for 24 h increased in accordance with the concentration increasing less than 32 μM, but decreased at concentrations more than 32 μM ($^{*}p < 0.05$ vs control group, $^{**}p < 0.01$ vs control group, $n = 3$).

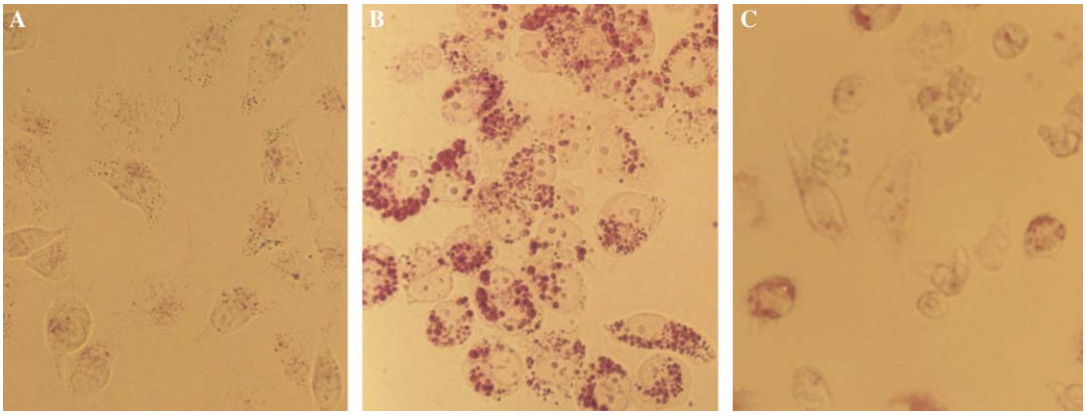

Figure 5. Neutral red staining of CQ treated A549 cells. The images were obtained by using a light microscope with a yellow filter inserted in the light path (×400). (A) Control group. There were no vacuoles in cells. (B) Cells were treated with CQ 32 μM for 24 h. (C) Cells were treated with CQ 128 μM for 24 h. The acidic vacuoles in cells were red.

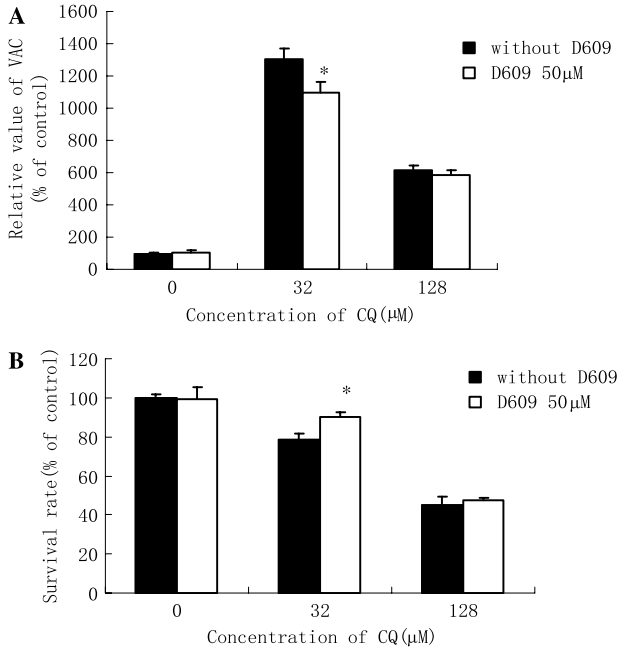

Figure 7. Effects of D609 on survival rate and VAC of A549 cells treated with CQ 0, 32, and 128 μM, respectively. (A) Effect of D609 50 μM on the VAC of A549 cells treated with CQ 0, 32, and 128 μM, respectively. (B) Effect of D609 50 μM on the survival rate of A549 cells treated with CQ 0, 32, and 128 μM, respectively ($^{*}p < 0.05$ vs control group at the same concentration of CQ, $n = 3$).

cells. These data suggested that we might use CQ as a lung cancer therapy drug by carefully preparing its concentrations.

To understand the possible mechanism by which CQ acts in A549 cells, in this study, we further investigated the changes of lysosome volume and the role of PC-PLC in A549 cells treated by CQ. The data suggested that CQ inhibited A549 lung cancer cell growth at lower concentrations by increasing the volume of lysosome and that PC-PLC might be involved in this process. But, at higher concentrations, CQ induced apoptosis and necrosis, at this time its ability to increase the volume of lysosome gradually declined, and PC-PLC might not be implicated in the process. Our data first provided the evidence that PC-PLC might be associated with the action of CQ in A549 lung cancer cells.

It has been reported that the increase of VAC is a common event in many cell death processes including both apoptosis and necrosis.[6] Recently, it was also reported that CQ destabilized the lysosome and plasma membranes, and mediated necrosis of cells treated with irradiation.[1] Our results of VAC assay, LDH assay, and nuclear fragmentation assay showed that vacuolation and apoptosis took place simultaneously, however, necrosis occurred later than vacuolation and apoptosis.

A recent study revealed that the inhibition of PC-PLC by D609 upregulated the viability of L929 cells treated with TNF-α.[6] Accordingly, our data indicated that D609 raised the viability of A549 cells treated with CQ 32 μM which mean PC-PLC promoted the antiproliferative effect. We first discovered that D609 lowered the VAC of A549 cells treated with CQ 32 μM, which suggested that PC-PLC facilitated lysosomal vacuolation.

Recently, it has been reported that D609 is an antioxidant.[12,13] In pancreatic cells, antioxidants could suppress cellular vacuolation.[5] In this study, we also examined the levels of ROS in A549 cells, and the results showed that the ROS levels of A549 cells were too low to measure by the 2′,7′-dichlorofluorescin (DCHF) assay, and that CQ and D609 could not elevate or depress the ROS levels (data not shown). The data indicated that ROS signals might be unimportant elements and D609 did not act as an antioxidant in A549 lung cancer cells.

In summary, the results of this study first showed that CQ was a potent compound that inhibited A549 cell proliferation. The mechanisms of its proliferous inhibition on A549 cells varied at different concentrations. The increase of VAC contributed to the proliferous inhibition at nontoxic concentrations of CQ (below 16 μM). Apoptosis and necrosis occurred when the concentration of CQ was above 32 μM. The data suggested that CQ inhibited A549 lung cancer cell growth at lower concentrations by increasing the volume of lysosome and that PC-PLC might be involved in this process. The data also indicated that, at higher concentrations, CQ induced apoptosis and necrosis, but at this time its ability to increase the volume of lysosome gradually declined, and PC-PLC might not be implicated in the process.

4. Materials and methods

4.1. Reagents, chemicals, and preparation of drugs

RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA) and bovine calf serum was from Beijing DingGuo Biotechnology Co., China. Chloroquine diphosphate was presented by the Epidermic Prevention Bureau of Licheng, Jinan city, China. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (MTT) was purchased from Amresco, lactate dehydrogenase (LDH) assay kit from ZhongSheng Co., Beijing, China, Acridine orange (AO) from Fluka, and tricyclodecan-9-yl-xanthogenate (D609) and 2′,7′-dichlorofluorescin (DCHF) from Sigma Co. (USA).

4.2. Cell cultures

A549 lung cancer cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) bovine calf serum at 37 °C with 5% CO_2, and 95% air. The cells were seeded at the density of 6250/cm^2 into 96-well plates or other appropriate dishes containing the medium.

4.3. MTT assay for cell viability

Cells were seeded in 96-well plates and treated with CQ 0.25–128 μM for 24, 48, and 72 h, respectively. The cell viability was determined by the MTT assay following

the procedure described by Price and McMillan.[8] The light absorptions were measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI Co., USA).

4.4. LDH assay for drug toxicity

The detection was performed as described previously.[9]

4.5. Nuclear fragmentation assay

Nuclear fragmentation was detected with AO staining and observed under a fluorescence microscope. Briefly, the cells were cultured in fresh medium with 0, 32, and 128 μM CQ for 24 h, and stained with 5 μg/ml AO at room temperature. Then the cells were observed and photographed under an Olympus BH-2 fluorescence microscope.

4.6. VAC assay for cell lysosomal vacuolation

VAC assay method was carried out as described previously[10,11] and was appropriately modified. 0.5% neutral red stock solution was prepared in 0.9% saline and filtered. Staining solutions were prepared before each experiment by diluting the stock solution (1:10) in 1640 medium containing 10% fetal bovine serum without $NaHCO_3$. A549 cells had been seeded in 6 cm dishes at the density of 6250/cm^2 24 h before CQ was added. After incubation with CQ for another 24 h, the A549 cells were washed twice with PBS (phosphate-buffered saline) and incubated for 4 min with 4 ml staining solution. The cells were washed twice with PBS, and the neutral red sample was extracted from cells by adding 3 ml acidified alcohol (50% alcohol, 1% acetic acid, and 49% water) per dish. The optical density (OD) at 540 nm of samples was determined by using Cintra 5 UV–vis spectrometer. All assays were performed in triplicate. The OD value of each sample was subtracted from the OD of the dish without cells to yield a net OD. The neutral red uptake readings for each dish would be normalized for total protein.

Then cell monolayers were washed once with 1% PBS and lysed with 100 μl of lysis buffer per dish. The total protein concentration was determined with the Coomassie Plus protein assay reagent by using BSA as a standard. The value of the VAC for each dish was normalized by dividing the neutral red uptake data by the total protein concentration.

4.7. Statistical analyses

Data were expressed as means ± SE, accompanied by the number of experiments performed independently, and analyzed by t test. Differences at $p < 0.05$ were considered statistically significant.

Acknowledgments

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