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Background: The present study aimed to investigate the effect of chloroquinone (CQ) on viability, invasive potential, and expression of vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) mRNA in liver carcinoma cell lines to develop an effective treatment strategy. Its effect on the tumor volume in a mouse SNU-182 xenograft model was also investigated.

Material/Methods: Methyl thiazolyl tetrazolium (MTT) assay was used for the determination of cell proliferation. FACSVantage SE flow cytometry system and CellQuest program was used for the analysis of the DNA content of the cells. The total RNA from SNU-182 cells was extracted using TRIzol reagent. The 24-well Transwell cell culture chambers were used for analysis of cell invasion potential.

Results: The results revealed a marked reduction in the viability of SNU-182 and HU-7 carcinoma cells after treatment with CQ. It also caused inhibition of invasive potential and suppression of VEGF and COX-2 mRNA expression. The population of SNU-182 cells in G1 and G2/M phase of cell cycle was significantly increased, with a subsequent reduction in the S phase by CQ treatment. Furthermore, the tumor volume was reduced significantly in the mice tumor xenograft model compared to the untreated group (P<0.01).

Conclusions: CQ treatment inhibits cell viability, reduces the expression of COX-2 and VEGF in liver carcinoma cells, and suppresses tumor volume. CQ can be of therapeutic importance for the treatment of liver cancer.

MeSH Keywords: Cell Cycle • Cell Cycle Checkpoints • Neoplasm Invasiveness

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Source of support: Departmental sources


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Background

Liver cancer is the third leading cause of deaths related to cancer throughout the world and every year more patients with primary liver cancer are detected [1,2]. Patient survival and treatment efficacy are determined by the stage at which the cancer is detected. The average survival or patients with liver cancer remains unaffected by the use of traditional chemical agents [3]. It has been observed that the liver carcinoma incidence rate in China is higher compared to other countries [4–6]. The commonly used treatments for liver carcinoma, including chemotherapy and radiation therapy, have shown very poor results. Liver carcinoma metastasizes to other organs, particularly bones, lungs, lymph nodes, and adrenal glands [7]. Therefore, development of new treatment strategies for liver carcinoma is urgently needed.

Chloroquine (CQ) has shown promising results for the inhibition of autophagy and is administered orally [8]. CQ was developed by Bayer Laboratories and was used as an anti-malarial drug. The U.S. FDA approved CQ for the treatment of malarial patients in clinics in 1947 [9]. Recently, it has been demonstrated that CQ treatment in liver carcinoma cells induces cell cycle arrest in G0/G1, leads to DNA damage, and induces apoptosis [10]. CQ and its derivatives were synthesized and screened for various biological activities. One of the CQ derivatives – hydrated chloroquine – was found to possess anti-inflammatory activity and is used to treat rheumatoid arthritis, lupus erythematosus, and amoebic hepatitis. Currently, studies are being performed to investigate the effect of CQ on inhibition of various types of tumors [9,11]. The present study was performed to investigate the effect of CQ on cell viability, invasive potential, expression of COX-2 and VEGF mRNA, and cell cycle distribution in liver carcinoma cell lines.

Material and Methods

Cell lines and culture

SNU-182 and HU-7, liver carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and antibiotics. The cells were maintained in an incubator with humidified atmosphere of 5% CO₂ at 37°C.

Cell viability assay

SNU-182 and HU-7, liver carcinoma cells were seeded at a density of 4×10⁶ cells per ml into the RPMI-1640 medium in 96-well plates for 48 h. The cells were incubated with 10–50 μM CQ or dimethyl sulfoxide for 48 h. After incubation, 50 μl of methyl thiazolyl tetrazolium (MTT, St. Louis, MO, USA) (5 mg/ml) solution was added to each well and incubation was continued for 4 h more. The supernatant was then decanted and dimethyl sulphoxide (150 μl) was added to each well in order to dissolve the formazan crystals formed. For each of the wells, absorbance was recorded at 490 nm using a microplate reader.

Cell cycle analysis

SNU-182 cells were distributed at a density of 2.5×10⁶ cells into the tissue culture flasks (T-75 flask; Nunc A/S) containing RPMI-1640 medium supplemented with 2% FBS. The cells were incubated for 48 h with different concentrations of CQ or dimethyl sulfoxide. After incubation, the cells were washed with PBS buffer and then subsequently fixed in 70% ethyl alcohol at ~40°C overnight. The contents in each of the flask were centrifuged at 12 000× g for half an hour. Then we added 200 μl PBS supplemented with 1 mM RNase A (San Diego, CA, USA) to each of the flasks, followed by propidium iodide (Sigma-Aldrich) treatment at a concentration of 50 μg/mL for half an hour. We used the FACSVantage SE flow cytometry system and CellQuest program (BD Biosciences) for the analysis of the DNA content of the cells.

Cell invasion assay

SNU-182 cells were seeded at a density of 2×10⁵ cells per mL on to the 24-well Transwell cell culture chambers (Chemicon, Temecula, CA, USA) and treated with CQ or dimethyl sulfoxide. The bottom face of the polycarbonate filters was coated with fibronectin and the top face was coated with Matrigel (Sigma-Aldrich, Carlsbad, CA, USA). The control wells were left without application of any coating. The SNU-182 cells were put into the RPMI-1640 medium supplemented with 0.1% bovine serum albumin. Treatment of the filters with samples for 45 min was followed by addition into the upper chamber. The filters were then incubated for a period of 24 h at 37°C. After incubation, the cells that penetrated into the lower chamber were stained with calcein. The absorbance of the filters was then recorded at 495 and 515 nm.

PCR analysis

The total RNA from the CQ-treated SNU-182 cells was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The 2-μg mRNA samples were reverse transcribed into cDNA according to the primer design principles. Amplification of the first-strand cDNA was performed to obtain 50-μl volumes. Initially, denaturation was performed for 4 min at 95°C. Then amplification of the samples was performed by 8 cycles of denaturation for 50 s at 94°C and annealing was carried out for 50 s at 24°C. The process was extended for 50 s at 72°C. Amplification was again performed for 50 s at 94°C by
30 cycles of denaturation followed by annealing for 1 min at 53°C, extension for 1 min at 72°C, and then extension for 15 min at 72°C. Separation of the PCR products was performed using 1.5% agarose gel following ethidium bromide staining. The quantification of the expression of COX-2 and VEGF mRNA was performed using Cycler software.

**In vivo animal experiment**

Thirty 8-week-old C57BL/6 mice were obtained from the Institute of Laboratory Animals at the Chinese Academy of Medical Sciences (Beijing, China). The animals were housed in air-conditioned rooms with 12 h light/dark cycles and fed standard food and water. The study was approved by the Ethics Committee of the Liaoning Medical University. The animals were subcutaneously injected with 2.5×10⁵ SNU-182 carcinoma cells and were then randomly assigned to 3 groups of 10 each: control, low-dose CQ, and high-dose CQ treatment groups. The animals in the low-dose and high-dose CQ treatment groups were administered 20 and 50 µM doses of CQ, respectively, and those in the control group were given an equal volume of normal saline alternatively for 10 days. To extract the tumors, mice were sacrificed on either day 15 or day 30 after administration of carcinoma cells.

**Statistical analysis**

Student’s t-test and one-way analysis of variance (ANOVA) were used for the statistical analysis of the data. The Newman-Keuls multiple comparison test was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of CQ on SNU-182 and HU-7 liver carcinoma cell lines**

Analysis of the effect of CQ on viability of SNU-182 and HU-7 cells showed a marked reduction in viability in a dose- and time-dependent manner. SNU-182 and HU-7 cells were treated with various concentrations of CQ from 10 to 50 µM for 48 h. The inhibition in viability of both cell lines was significant at all concentrations of CQ, except for 10 µM, compared to untreated cells (Figure 1).

Examination of the invasive potential of SNU-182 cells after treatment with CQ revealed a marked reduction in invasive potential compared to the untreated cells (Figure 2). The
concentration of CQ at which invasive potential of SNU-182 cells was significantly inhibited was 50 µM (Figure 2). The results from RT-PCR analysis revealed a significant reduction in the expression of COX-2 and VEGF mRNA in SNU-182 after treatment with CQ (Figure 3). In the CQ-treated cells the expression of COX-2 and VEGF mRNA was reduced by 5- and 7-fold, respectively, following 48-h treatment compared to the control cells.

**CQ treatment caused cell cycle arrest in G2/M phase in SNU-182 cells**

Treatment of SNU-182 cells with CQ for 48 h induced increase in the proportion of cells in G2/M phase of the cell cycle (Figure 4). However, the proportion of cells in S phase was inhibited.

**Effect of CQ on SNU-182 mice xenograft model**

Treatment of the mice injected with 2×10⁵ mouse SNU-182 liver carcinoma cells with CQ inhibited the tumor size compared to the untreated mice. It was observed that CQ exhibited a dose-dependent inhibitory effect on the tumor volume in the mouse xenograft model. The increase in the dosage of CQ from 10 to 50 µM reduced the tumor volume from 75% to 30% compared to 100% in the control group of mice (Figure 5).

**Discussion**

The present study demonstrates the effect of CQ on cell proliferation, invasive potential, cell cycle distribution, and tumor growth in liver tumor xenograft mouse models. Currently,
studied are being performed to investigate the effect of CQ on inhibition of various types of tumors [9,11]. The 2 most effective compounds for the treatment of myriad non-malarial diseases include chloroquine and hydroxychloroquine. These compounds act as immunomodulatory and immunosuppressive agents and are therefore used for the treatment of rheumatic, lupus erythematosus, and skin diseases, as well as for inhibition of various types of cancer [12]. Results from the present study revealed inhibition of cell proliferation by CQ treatment in both of the tested liver carcinoma cell lines. Arrest of cell cycle in the carcinoma cells inhibits mitosis and prevents cell proliferation. Therefore, cell cycle arrest by use of chemotherapeutic agents is considered a promising strategy for inhibition of cancer growth. In the present study, treatment of liver carcinoma cells with CQ induced cell cycle arrest in G2/M phase of the cell cycle, with subsequent reduction in the S phase.

Malignant tumor cells are characterized by their ability to undergo invasion and metastasis very rapidly. The first step in tumor invasion involves metastasis to lymphatic vessels and nodes, which is responsible for the poor prognosis of patients with malignant tumors [13,14]. However, the mechanism of the carcinoma cell detachment, their invasion into the lymph vessels, and metastasis to lymph nodes are not yet understood. Recent studies have revealed that invasion and growth of cancerous cells can be inhibited by the use of some agents [15,16]. It is reported that some factors, like vascular endothelial growth factor and its receptor, play a vital role in the growth and metastasis of tumors [17]. It is believed that cyclooxygenase (COX)-2 and VEGF interact together in the carcinoma cells

Figure 5. CQ treatment reduced tumor size in the mouse xenograft model. After administration of SNU-182 carcinoma cells, the mice were treated with CQ and then sacrificed to extract the tumor.
and enhance their invasive potential. COX-2 is considered to regulate the expression of VEGF in malignant tumor cells. The present study revealed that treatment of liver carcinoma cells with CQ significantly induced inhibition in the expression of COX-2 and VEGF compared to the untreated cells.

Conclusions

The present study demonstrated that CQ effectively inhibited proliferation and suppressed liver carcinoma cell proliferation. In addition, CQ also inhibited invasive potential of carcinoma cells through reduction in the expression of COX2 and VEGF.

References:

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