Targeting autophagy promote the 5-fluorouracil induced apoptosis in human colon cancer cells

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Abstract: Colon cancer is a leading cause of cancer-related death in developed countries. Although 5-fluorouracil (5-FU) has traditionally been studied for its potential to induce apoptosis, its clinical use had been greatly limited as a result of the development of drug resistance in patients. Besides the medicines that could induce apoptosis in cancer cells, several other alternative treatments, which had different mechanisms of action (such as autophagy), had gained more and more attention recently. As for colon cancer, autophagy appeared to have certain protective effects in tumor cells by antagonizing the inhibition caused by chemotherapy and radiotherapy. Therefore, autophagy inhibition seemed to be an effective mean for cancer treatment. In this study, HT29 and SW480 cells were treated with autophagy inhibitors together with/without 5-FU, the proliferation rate, apoptosis and autophagy induction effects were then evaluated. The proliferation rate of cancer cells was analyzed by MTT assay. Apoptosis was quantified by flow-cytometry after the cells were double-stained with Annexin V/PI. Autophagy and apoptosis were both further confirmed by western blot analysis. Finally, to confirm the effects of combinational use of 5-FU with 3-methyladenine (3-MA) and Chloroquine (CQ), the colony formation assay was also performed. Our results demonstrated that 5-FU could induce apoptosis and autophagy in colon cancer cells. Both 3-MA and CQ could enhance the apoptosis induced by 5-FU in colon cancer cells, while CQ had better inhibitory effect against the proliferation of colon cancer cells. Meanwhile, CQ could induce a caspase-dependent apoptosis in colon cancer cells. Cathepsins might function in the synergistic interaction between 5-FU and CQ. Based on the above results, we proposed the combinational use of 5-FU and CQ might be a novel therapeutic method for the treatment of colon cancer.

Keywords: Chloroquine, 5-fluorouracil, colon cancer, autophagy, apoptosis

Introduction

Colon cancer is a leading cause of cancer-related death in developed countries [1]. It was the third most commonly diagnosed cancer diseases in males and second most common in females worldwide in 2012 according to the Global Cancer Statistics [2]. Possibly due to the development of drug resistance during the treatments, five-year survival rate for colon cancer is only 10% in patients with metastases [3]. 5-Fluorouracil (5-FU) is the first choice chemotherapeutic medicine in the treatment of colon cancer. Intracellular metabolites of 5-FU could exert cytotoxic effects through the inhibition of thymidylate synthase, or through its incorporation into RNA and DNA, events that ultimately activated apoptosis [4]. Although 5-FU had traditionally been studied for its potentials to induce apoptosis, its clinical use had been greatly restricted due to the development of drug resistance in colon cancer patients [5]. In order to reduce the toxicity as well as to increase the drug potency, the combinational use of 5-FU with other novel medicines had been tested and reported in several documents [6-8].

Autophagy is a dynamic cellular protective event in response to stressful and abnormal micro environmental conditions [9]. Recently, increasing evidences had suggested the importance of autophagy in human cancer diseases, as it had exhibited a dual function in cancer development. In healthy cells, autophagy acts as a tumor suppressor by removing damaged proteins and inhibiting organelle accumulation to prevent tumorigenesis [10]. However, in cancer cells, autophagy could promote the survival of cancer cells under hypoxic conditions and...
drug treatment, possibly by sustaining the cellular metabolism necessary for survival. Therefore, inhibition of autophagy seemed to be an effective means for colon cancer treatment [11-17]. Autophagy was known to begin with the isolation of double-membrane structures in the cytoplasm. These membrane structures were then elongated and matured, and LC3 (the light chain 3 of microtubule-associated protein) was recruited to the membrane (LC3 aggregation). The elongated double-membranes could form autophagosomes, which could sequester both of the cytoplasmic proteins and the damaged organelles such as mitochondria. After acidification, the matured autophagosomes were fused with lysosomes and became autolysosomes [18]. Eventually, the sequestered contents were degraded by lysosomal hydrolases for recycling. Therefore, the event of autophagy could be divided into three stages; the autophagosome formation stage, the autophagosome and lysosome fusion stage and the autophagy execution stage. 3-methyladenine (3-MA), Chloroquine (CQ), E64d and Pepstatin A [19] were specific inhibitors for certain autophagy stages. Some researchers suggested that the suppression of autophagy by 3-MA [20] and CQ [21, 22] could sensitize 5-FU-mediated cell death in colon cancer, possibly by increasing the apoptosis in the tumor cells. As it was also described in previous text that autophagy might promote the survival of cancer cells under hypoxic conditions and drug treatments, the above inhibitors were expected to be new treatment drugs for colon cancer, especially to be combined with pro-apototic chemotherapy drugs during the treatment.

Before the combinational use of traditional drugs with autophagy inhibitors, it seemed beneficial to confirm whether the synergistic relationship was existed or not between autophagy and apoptosis. Thus the following questions were needed to be answered first. If the autophagy inhibitors were combined used with 5-FU, should the treatment effect be improved or not? Since 3-MA and CQ were autophagy inhibitors on different steps in autophagic process, were their effects equal after combined with 5-FU for the treatment of colon cancer cells? CQ was widely used to treat malaria and rheumatoid arthritis, thus the safety of this molecule had already be confirmed. If the synergistic effect between CQ and 5-FU was demonstrated, it would be a great advantage to combine CQ with 5-FU for the treatment of colon cancer in clinic.

In this study, we aimed to investigate the effect of the combination use of 5-FU with three other kinds of autophagy inhibitors against human colon cancer cells. Our results demonstrated that 5-FU could induce not only the apoptosis but also the autophagy in colon cancer cells. Both 3-MA and CQ could enhance the apoptosis induced by 5-FU in colon cancer cells, while CQ might have better proliferative inhibition effect. Meanwhile, CQ could induce a caspase-dependent apoptosis in colon cancer cells. Cathepsins might function in the synergistic interaction between 5-FU and CQ. Based on these results, we proposed the combination use of 5-FU with CQ might be a novel therapeutic method for colon cancer disease.

Materials and methods

Reagents and antibodies

3-MA, CQ, E64d, pepstatin A, primary antibodies against P62 and McCoy’s 5 A medium were all purchased from Sigma-Aldrich. Z-VAD-FMK was purchased from MBL. RPMI-1640, DMEM medium and fetal bovine serum (FBS) were from Gibco. Primary antibodies against PARP was from Abcam Inc. The GFP-LC3 plasmid was purchased from Miao Ling Inc. Annexin V-FITC/PI Apoptosis Detection Kit was purchased from YEASEN Inc. Primary antibodies against Cathepsin D and B were purchased from Immunoway Inc.

Cell culture and transfection

Human colon cancer cell line HT-29 and SW480 were purchased from the cell bank of Chinese Academy of Sciences. They were maintained in McCoy’s 5A and DMEM medium respectively. The culture medium were supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin and the cells were incubated in a humidified 5% CO₂ incubator at 37°C. The plasmid (GFP-LC3) was transiently transfected into the cells with MegaTran transfection reagent according to the manufacturer’s instructions (OriGene). After 24 hours, the cells were treated with 5-FU and subjected to fluorescent analysis.

Treatment with 5-FU and autophagy inhibitors

Two human colon cancer cells were seeded and cultured until 40-50% confluence were reach-
ed. Then the cells were pre-treated with autophagy inhibitors for 12 hours. After rinsing with PBS, the cells were treated with or without 5-FU for another 48 hours. Based on the IC₅₀ s of 5-FU, CQ and 3-MA against the selected two colon cancer cells, the dosage of CQ was set at 20 µM for SW480 cells, 50 µM for HT-29 cells; the dosage of 3-MA was set at 5 mM both for SW480 and HT29 cells, and for 5-FU, the dosage was 20 µM for SW480 cells and 200 µM for HT-29 cells.

Cytotoxicity assay

The cytotoxicity of chemicals used against colon cancer cells was determined by MTT assay. Generally, cells were seeded into 96-well plates and treated with chemicals at different concentrations. After incubated for 48 hours, 10 µl MTT was added into each well and incubated for 4 hours. The absorbance was then measured using Epoch Microplate Spectrophotometer (Bio-Tek Instruments, Inc.) at 490 nm.

Colony forming assay

Colon cancer cells, which were suspended in fresh culture medium, were plated into 35 mm dishes with 500 cells/dish. The cells were allowed to attach for 24 hours and then treated either with CQ at 20 µM or 3-MA at 5 mM for 12 hours. After that, the cells were rinsed with PBS and treated with/without 5-FU for additional 48 hours. Then the cells were rinsed with PBS, and cultured with fresh culture medium for another 14 days before the colony formation assay was conducted. To visualize the formation of colonies, the culture media was first removed, and the cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Then the cells were rinsed with PBS twice, and were observed under a fluorescence microscope (Olympus. Inc).

Protein isolation and western blots analysis

After treatment with different drugs, cells were rinsed with PBS and then lysed with RIPA buffer with protease inhibitors added. The total protein were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Millipore) and then detected by proper primary and secondary antibodies before visualization with a chemiluminescence kit (Pierce).

Fluorescence microscopy

Cells were transfected with GFP-LC3 plasmids, followed by treatment with 5-FU. Before the detection, the cells were rapidly rinsed with PBS and fixed with paraformaldehyde for 15 minutes at room temperature. Then the cells were rinsed with PBS twice, and were observed under a fluorescence microscope (Olympus. Inc).

Flow cytometry

For flow cytometry analysis, colon cancer cells treated with CQ and 3-MA (with/without 5-FU) were collected, rinsed with PBS and incubated with FITC-labeled annexin V and stained with PI at room temperature for 15 minutes and then analyzed on flow cytometer (Beckman, MoFlo XDP). Annexin V-positive/PI-negative cells were considered to be apoptotic cells at the early stages, annexin V-positive/PI-positive cells were considered to be apoptotic cells at the later stages, whereas PI single positive cells were considered to be necrotic cells.

Statistical analysis

The statistical comparisons were performed by using Student’s T-test. Value of \( P < 0.05 \) was considered statistically significant.

Results

5-FU could induce both autophagy and apoptosis in colon cancer cells

To determine if autophagy and apoptosis could be induced by the challenge of 5-FU in colon cancer cells, changes of autophagy and apoptosis indicators were detected in 5-FU treated cells. SW480 cells were initially transfected with the light chain 3 (LC3) of microtubule-associated protein, which was fused with green fluorescent protein (GFP) and served as a specific marker of autophagosomes. These cells were then treated with 5-FU (200 µM). In contrast to the diffused expression pattern of GFP-LC3 in control cells, after the cells were treated with 5-FU, expression pattern of GFP-LC3 showed punctuated accumulation within the cells (Figure 1A). The number of punctate sites of GFP-LC3 accumulation in each cell was then counted (at least 50 cells were included for each group). Results shown an increased punc-
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The degradation of sequestosome 1 (SQSTM1/p62) was assumed as an accurate indicator for autophagy. Thus, the expression of P62 within 5-FU exposed colon cancer cells was investigated. 5-FU induced P62 degradation was observed to be dose-dependent (Figure 1B), which suggested that 5-FU could induce the autophagy in colon cancer cells.

The poly(ADP-Ribose) polymerase (PARP) was believed to be one of the hallmarks for apoptosis. In this study, the cleavage of PARP within 5-FU exposed colon cancer cells was also investigated. Results shown that the cleavage of PARP could be induced in a dose dependent manner by 5-FU (Figure 1C). Moreover, the number of apoptotic cells after 5-FU was treated was also augmented as 5-FU dosage was increased (Figure 1D). These results further demonstrated the apoptosis could be induced in colon cancer cells by 5-FU.

**3-MA and CQ could enhance 5-FU induced apoptosis in colon cancer cells**

In order to test the possibility of combinational use of autophagy inhibitors with 5-FU, certain autophagy inhibitors that could affect different autophagy stages were selected and combined with 5-FU for the treatment of colon cancer cells. Western blot and flow cytometry were used to evaluate the apoptosis of colon cancer cells. It was observed from the western-blotting results that the cleavage of PARP in colon cancer cells was increased after sequential treatment with 3-MA and 5-FU (Figure 2A). The results from flow cytometry also indicated that...
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Figure 2. 3-MA and CQ could enhance the 5-FU induced apoptosis in colon cancer cells. HT29 and SW480 cells were pre-treated with or without 3-MA for 12 hours followed with or without 5-FU treatment for another 48 hours. Then the expression of PARP was detected by western blot (A). Meanwhile, the cells were rinsed with PBS and incubated with FITC-labeled Annexin V and stained with propidium iodide (PI) at room temperature for 15 minutes. Then the cells were analyzed by flow cytometer (B). HT29 and SW480 cells were pre-treated with or without CQ for 12 hours followed with or without 5-FU treatment for another 48 hours, then the expression of PARP was analyzed by western blot (C) and the percentage of apoptotic cells was analyzed by flow cytometry (D). Results were the representative from 3 independent experiments.
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3-MA pre-treatment could enhance the apoptotic-induction effects of 5-FU in colon cancer cells, as the percentage of apoptotic cells was increased in the 3-MA + 5-FU treatment group (36% in 3-MA + 5-FU treated HT-29 cells compared to 20% in only 5-FU treated HT-29 cells, 36% in 3-MA + 5-FU treated SW480 cells compared to 22% in only 5-FU treated SW480 cells) (Figure 2B). Similar to 3-MA, CQ pre-treatment not only increased the cleavage of PARP in colon cancer cells (Figure 2C), but also enhanced the apoptotic-induction effects of 5-FU. The percentage of apoptotic HT-29 cells was 54% in the CQ and 5-FU combination group compared to 18% in 5-FU group. In SW480 cells, the percentage of apoptotic cells was 58% in CQ and 5-FU combination group and 20% in 5-FU group, respectively (Figure 2D). Furthermore, colon cancer cells combined treated with CQ and 5-FU was prone to the late phase apoptosis (Figure 2D). However, our study also observed that not all the autophagy inhibitors had the synergistic effects with 5-FU, pre-treatment of E64d and pepstain A, inhibitors for the autophagy execution stage, did not enhance the apoptosis induced by 5-FU in colon cancer cells (data not shown).

3-MA and CQ potentiated the growth suppression effects of 5-FU in colon cancer cells

With the dosage of 3-MA, CQ and 5-FU was increased, the growth inhibition was more significant in HT29 and SW480 cells (Figure 3A and 3B). The inhibition effects of 5-FU in HT29 and SW480 cells could be enhanced by pre-
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Figure 4. CQ induced caspase-dependent apoptosis in colon cancer cells. A. HT-29 and SW480 cells exposed to different dosages of CQ were rinsed with PBS and incubated with FITC-labeled Annexin V and stained with propidium iodide (PI) at room temperature for 15 minutes. Then the cells were analyzed by flow cytometry. The Annexin V-positive and PI-negative cells were considered to be apoptotic cells at the early stage. The annexin V-positive and PI positive cells were considered to be apoptotic cells at late stage. B. The expression of PARP was detected by western blot in HT-29 and SW480 cells exposed to different dosages of CQ. C. HT29 and SW480 cells were pre-treated with CQ for 12 hours followed by 5-FU treatment, then a pan-caspase inhibitor, Z-VAD-FMK, was added to the culture medium for another 24 hours. The expression of PARP was detected by western blot afterwards. Results were representatives from 3 independent experiments.

treatment of either 3-MA or CQ for 12 hours prior to 5-FU exposure (Figure 3A and 3B). But the statistically significant effect could only be observed after the cells were sequentially treat-
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Ed with CQ and 5-FU (P<0.05) (Figure 3B). After the combinational treatment of 5-FU either with 3-MA or CQ, the viability of colon cancer cells was further evaluated by colony formation assay. Results had shown that the colony formation of colon cancer cells was significantly reduced in 5-FU and CQ combination group compared to that in 5-FU and 3-MA combination group (Figure 3C).

CQ induced caspase-dependent apoptosis in colon cancer cells

Since our results had shown that the treatment effects of combinational use of 5-FU and CQ was better than that of the combinational use of 5-FU and 3-MA in colon cancer cells, we further detected the mechanism of action of CQ against colon cancer cells. As the dosage of CQ was increased, the apoptosis in HT29 and SW480 cells was exacerbated, which was confirmed by results from flow cytometry (Figure 4A) and western blot (Figure 4B). Moreover, when caspase inhibitor (Z-VAD-FMK) was added, the coordinate effects of CQ and 5-FU could be inhibited accordingly (Figure 4C). These results suggested that a caspase-dependent apoptosis was induced by CQ in colon cancer cells.

Cathepsins might function in the synergistic interaction between 5-FU and CQ

Previous reports had demonstrated that treatment with CQ led to a dramatic increase in cathepsin D expression [23], which could promote apoptosis subsequently through activating caspase pathway and modifying pro-apoptotic molecules such as BAX and BAK [24]. In order to check the role of cathepsin D in colon cancer cells treated with the combination of 5-FU and CQ, cathepsin D expression was detected in HT29 and SW480 cancer cells after CQ and 5-FU were treated. It was observed the expression of cathepsin D could be detected after the cells were treated with 5-FU (Figure 5). However, as different amount of CQ was added, cathepsin D expression was increased in a dose-dependent manner. The expression of cathepsin B was also increased with the increment of CQ dosage (Figure 5). Based on these results, it seemed that cathepsins might function in the synergistic interaction between 5-FU and CQ.

Discussion

Recently, the apoptosis induction through the regulation of autophagy has been proposed as a promising cancer treatment strategy. Various autophagy inhibitors were expected to be used in combination with traditional chemotherapy drugs to enhance their pro-apoptotic effects in the treatment of cancer diseases. It has been reported autophagy inhibition could enhance the anticancer effect of temozolomide for malignant gliomas [25], and potentiated the cytotoxicity effects of lidamycin [26], Gefitinib [27] and cisplatin [28] in the treatment of lung cancer. These results suggested that the cancer cells might be protected by autophagy from therapy-induced apoptosis and concurrently inhibition of autophagy by using autophagy inhibitors could strengthen the efficacy of pro-apoptotic chemotherapeutic drugs. Generally, autophagy could be induced by hypoxia, which was a common phenomenon in solid tumor tissues. As it was described in previous text, the recycled contents in the autophagy process could be used as ultimate nutritional resources for tumor cells to survive from low-nutrient conditions. Thus the suppression of autophagy could potentiate the anti-tumor effects when it was used in combination with other treatment methods [29]. In this study, we had compared the coordination effect between autophagy inhibitors and 5-FU against colon cancer cells. Pepstain A and E64d, the inhibitors in autophagy execution stage, had no coordination effect.
against colon cancer cells when they were used in combination with 5-FU (data not shown) separately. However, inhibitors for autophagosome formation stage and for autophagosomes and lysosome fusion stage had coordinated effects against colon cancer cells after they were combined with 5-FU (Figure 2). As it was shown, pepstatin A was an inhibitor of cathepsins D [30, 31] and E64d was a cell permeable inhibitor of cathepsins B, H and L [32]. It was suggested that the inhibition of cathepsins by E64d could significantly reduce the apoptotic cell number [33]. Cathepsins, particularly cathepsin B, was involved in the molecular switch between autophagy and apoptosis. Our previous results also shown that E64d and pepstatin A could inhibit the autophagy induced by EV71 and decrease the apoptosis in RD-A cells by inhibiting cathepsin D and B [19]. Thus, E64d and pepstatin A could not enhance the pro-apoptotic effect of 5-FU in colon cancer cells, possibly because of its inhibition effect against cathepsins.

Although it had been demonstrated that CQ and 3-MA could potentiate the inhibitory effect of 5-FU against colon cancer cells, the differences between these two autophagy inhibitors had not been clarified. In this study, we compared the coordinated inhibitory effects of 5-FU in combination with CQ and 3-MA separately against the colon cancer cells. Our results demonstrated that the combination of CQ and 5-FU was more potent than 3-MA and 5-FU relate to the proliferation inhibitory effect against colon cancer cells (Figure 3). Li et al [34] had compared the impact of autophagy inhibition at different stages on cytotoxic effect in Glioblastoma cells, they had demonstrated that the inhibition of late process of autophagy (CQ), not the initial step (3-MA), could increase the cytotoxic effect against Glioblastoma cells. In addition, even after six decades of use, CQ is still the drug of choice for the treatment of malaria because of its effectiveness, low toxicity and low price. Its wide therapeutic window makes CQ more usable in the clinic. Our results further demonstrated that CQ was more suitable to be used in combination with 5-FU than 3-MA for the treatment of colon cancer in clinic.

Based on our data, it was shown that the apoptosis induced by CQ in colon cancer cells was dose-dependent (Figure 4A and 4B). A pan-caspase inhibitor, Z-VAD-FMK, could be used to inhibit the pro-apoptotic effects of 5-FU and CQ (Figure 4C), which suggested that the apoptosis in colon cancer cells induced by CQ was caspase-dependent. Thus it was seemed when CQ was combined use with 5-FU, it could inhibit the autophagy induced by 5-FU and moreover, CQ itself could also induce apoptosis in colon cancer cells. It was also shown that the inhibition of autophagy by CQ might have resulted in a bioenergetic shortage, which could trigger apoptosis [35]. In a more subtle fashion, inhibition of autophagosome and lysosome fusion might subvert the cellular capacity to remove damaged organelles or misfolded proteins, which in turn favored the apoptosis. No matter what the mechanisms might be, based on our result, a synergistic effect could be observed when CQ was combined use with 5-FU. Moreover, it could be proposed the resistance of colon cancer to 5-FU might be overcome by the combinational use of CQ during the treatment.

CQ is a weak alkali that can inhibit lysosomal acidification, which prevents the fusion of autophagosomes with lysosomes and subsequently leads to autophagic degradation. It had been shown in our study that the treatment of CQ could resulted a dramatic increase in the expression of cathepsin D protein, which was active at physiological pH and could promote the apoptosis by activating a caspase pathway and modifying pro-apoptotic molecules such as BAX and BAK. Our results suggested cathepsins might participate in the interaction between 5-FU and CQ.

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Disclosure of conflict of interest

None.

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