Autophagy Inhibition by Chloroquine Sensitizes Colorectal Cancer Cells to Concurrent Chemoradiation

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Purpose/Objectives:
Autophagy is an evolutionarily conserved catabolic process in which amino acids and energy produced by this self-digestive process are recycled to support cell survival under stressful conditions (i.e. cancer therapy). Autophagy has been proposed as a mechanism of resistance to radiation therapy (RT) and chemotherapy in multiple cancers. Inhibition of autophagy by chloroquine (CQ) may deprive cancer cells, particularly in hypoxic and radioresistant regions, of this essential survival mechanism and sensitize rectal tumors to RT or concurrent chemoradiation (chemoRT). However, the effects of RT on autophagy and the utility of the addition of CQ to chemotherapy +/- RT is not known in colorectal cancer (CRC) cell lines. We hypothesized that RT would induce autophagy and that the addition of CQ would radiosensitize CRC lines to chemoRT.

Materials/Methods:
In vitro, HCT-116 and HT-29 colorectal cancer (CRC) cell lines were treated as following: (1) PBS; (2) CQ; (3) 5-Fluorouracil (5-FU); (4) RT; (5) CQ and RT; (6) 5-FU and RT; (7) CQ and 5-FU; (8) 5-FU and CQ and RT. Cell viability and proliferative capacity were measured by MTT and clonogenic assays. Western blotting, electron microscopy and fluorescence microscopy assessed autophagy status. Cell death was evaluated by FACS and Annexin V analysis.

Results:
Radiation-Induced Autophagy
Irradiation induced autophagy in both HCT116 and HT-29 cell lines (Figure 1). TEM demonstrated autophagy induction in HT-29 cells 24 hours following irradiation at a dose of 8 Gy (Figure 1A). Compared to non-irradiated controls, RT-treated cells exhibited increased autophagosome formation, as illustrated by increased numbers of double membrane vesicles (Figure 1A, inset).

To further investigate the effects of radiation on autophagic response, HCT-116 and HT-29 cell lines were transiently transfected with GFP-labeled LC3 plasmid and examined for green fluorescent LC3 puncta, representing autophagosomes. Increasing RT doses significantly increased the number of cells with GFP punctate pattern compared to untreated controls for both HCT-116 and HT-29 cell lines (p<0.05) (Figure 1D).

Chemoradiation-Induced Autophagy
Previous studies demonstrated autophagy induction in HCT-116 and HT-29 cells following treatment with 5-FU alone. We now examined the impact of concurrent treatment with 5-FU and RT on autophagy functional status in CRC cells. ChemoRT resulted in increased autophagy induction in HT-29 cells compared to 5-FU alone (Figure 2A and 2B) and may have had a synergistic effect at higher RT doses (8 Gy) (Figure 2B). Autophagy induction in HT-29 cells following chemoradiation was also qualitatively assessed by fluorescence microscopy (Figure 2C). GFP-fluorescent puncta formation confirmed RT-induced autophagy as previously reported. 11,12 demonstrated that chemoradiation was associated with more robust autophagy upregulation.

Radiosensitization of CRC Cell Lines Through Autophagy Inhibition by Chloroquine
To investigate whether autophagy inhibition by CQ increases the radiosensitivity of CRC cell lines, we first used MTT assays (Figure 3A). Cell viability of HCT-116 cells at 72 hours post-RT was significantly decreased upon addition of CQ (10 μM) just prior to irradiation at 4 and 8 Gy (p<0.001). Significant decrease in cell viability in the presence of CQ (10 μM) in HT-29 cells were seen at 8 Gy (p<0.001).

Cancer cell proliferation after treatment was examined by clonogenic survival assays. For HCT-116 cells, clonogenic survival was similar under RT alone or RT and CQ (Figure 3B), whereas HT-29 cells showed decreased survival after combination treatment with RT and CQ (0.5 μM) compared to RT alone at doses of 2 and 8 Gy (p<0.05 and p<0.05, respectively), further supporting the MTT assay results that showed radiosensitization of CRC cell lines by CQ.

Chloroquine inhibits the last phase of autophagy by changing the pH of lysosomes, thus rendering them nonfunctional and unable to process proteins. Effective autophagy inhibition by CQ is manifested as LC3-II accumulation due to failure to re-process LC3-II back into LC3-I. As shown in Fig. 3C, single agent CQ increased LC3-II levels in HT-29 cells compared to vehicle treatment, demonstrating that CQ effectively blocked autophagic flux at the concentration used. Furthermore, HT-29 cells irradiated at 8 Gy after exposure to CQ showed increased LC3-II accumulation compared to cells treated with CQ alone (Figure 3C), indicating that RT induced autophagy.

To investigate the mechanism underlying radiosensitization of HT-29 cells by CQ, cell death by apoptosis and cell cycle progression were assessed. CQ addition to RT (8Gy) increased PARP cleavage, but had little effect on cleaved caspase-3 levels, compared to RT alone (Figure 3C), suggesting that concurrent use of CQ likely increased the RT-induced DNA damage response thensensitizing but alternative cell death pathways, rather than apoptosis were responsible for decreased HT-29 cell survival upon radiosensitization by CQ. Cell cycle analysis demonstrated that CQ did not alter the proportion of cells in any phase of the cell cycle (Figure 3D). Flow cytometry for Annexin V and propidium iodide (PI) at 48 hrs after treatment (Figure 3E) confirmed that CQ did not affect early apoptosis in irradiated HT-29 cells, as the AnnexinV+/PI- cell population remained stable. Of note, addition of CQ to RT significantly increased the number of cells staining positive for both Annexin V and PI, as compared to RT alone (p<0.05). Since Annexin V+/PI+ cells are necrotic or in late apoptosis, the radiosensitization of HT-29 cells by CQ may result from increased necrosis rather than apoptosis or cell cycle arrest.

Conclusions:
RT and 5-FU individually induce autophagy in CRC cell lines and their combination results in synergistic autophagic induction. Autophagy inhibition by CQ increases CRC cell sensitivity to concurrent treatment with 5-FU and RT in vitro, suggesting that addition of CQ to chemoRT improves CRC treatment response.

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