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Inhibition of autophagy by 3-MA promotes hypoxia-induced apoptosis in human colorectal cancer cells

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Abstract. – OBJECTIVE: Cell autophagy reduces the sensitivity of cancer cells to therapeutic reagents in various types of human cancer. Therefore, the aim of our study was to use human colorectal cancer HCT116 cells to explore whether inhibition of autophagy by 3-Methyladenine (3-MA, an autophagy inhibitor) is able to enhance hypoxia-induced apoptosis *in vitro*.

MATERIALS AND METHODS: HCT116 cells were treated with 3-MA, hypoxia, or 3-MA plus hypoxia, and the autophagy, apoptosis and proliferation of the HCT116 cells were investigated. Western blot analysis was used to detect autophagy specificity protein microtubule-associated protein light chain 3 (LC3) expression. Effects on apoptosis were evaluated by using flow cytometry (JC-1 staining to measure mitochondrial membrane potential) and annexin V-propidium iodide (PI) staining.

RESULTS: The results showed that the treatment of HCT116 cells *in vitro* with hypoxia alone increased autophagy as well as apoptosis, whereas combination treatment with 3-MA and hypoxia markedly inhibited hypoxia-induced autophagy, but increased hypoxia-induced cell apoptosis.

CONCLUSIONS: Autophagy might play a role as a self-defense mechanism in hypoxia-treated colon cancer cells, and its inhibition could be a promising strategy for the adjuvant chemotherapy of colon cancer.

Key Words:
Autophagy, Hypoxia, Apoptosis, Colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death in both men and women in the United States[1]. In China[2] colorectal cancer also is one of the 5 most commonly diagnosed cancers and had a significant upward trend in age-standardized incidence rates from 2000 to 2011. Although surgical reaction currently remains the only curative treatment for CRC, many patients still have to receive adjuvant chemotherapy. The ability of tumor cells to undergo apoptosis is important regarding sensitivity to chemotherapy. Cancer often happens when several various pathways regulating cell differentiation are disturbed. Autophagy is a kind of ubiquitous phenomenon and isolates damaged organelles, allows cell differentiation, increases protein catabolism, and even promotes cell death of cancerous cells[3]. The pathophysiologic roles of autophagy in tumorigenesis may vary among each type of cancer due to the different biological characteristics of cancer cells and their microenvironments. Autophagy is activated in colorectal cancers both *in vitro* and *in vivo,* contributing to the survival of the cancer cells in their microenvironment[4]. The oxygen deficiency, which commonly appears during the process of tumor growth, can increase tumor cell autophagy level and promote the tumor cell survival and tumor growth. In addition, autophagy may also make cancer cells evade apoptosis, continue to grow by removing the damaged organelles, protect cells against damage such as radiotherapy and chemotherapy and so on[5,6]. Furthermore, autophagy and apoptosis can regulate each other. The dual role of autophagy may be associated with the regulation of apoptosis under the hypoxia conditions. Autophagy could block the in-

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duction of apoptosis and attenuate cellular injury. Investigations have found that the moderate autophagy may inhibit the oxidative stress-induced apoptosis[7]. However, in special cases, autophagy or autophagy-relevant proteins may help to induce apoptosis, which could aggravate cellular injury[8]. Thus, the purpose of our study was to investigate the colorectal cancer cell activity via the inhibition of autophagy and induction of hypoxia when compared to individual agents.

Materials and Methods

Cell Culture

The human HCT116 colorectal cancer cell was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 0.03% L-glutamine and incubated in a 5% CO_2 and 95% N2 at 37°C. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Material Co., Ltd. (Hangzhou, Zhejiang, China). Cells in a mid-log phase were used in experiments.

Observation of Cell Morphologic Changes

HCT116 cells were seeded into 96-well flat bottom plates at a density of $2.0x10^5$ cells/well in 2 ml medium. They were divided into four groups: normoxia group, 3-MA (3-Methyladenine, Sigma-Aldrich, St. Louis, MO, USA) group, hypoxia group, and hypoxia+3-MA group. After the cells were incubated for 24 h, cell morphology was observed using a phase-contrast microscope (Olympus, Tokyo, Japan). The photographs were taken at a magnification of x200.

Western Blot Analysis

For extraction of total cell proteins, cells were washed with pre-cooled PBS and subsequently lysed in pre-cooled RIPA lysis buffer [50 mmol Tris-HCl, pH 7.4, 150 mmol NaCl, 1 mmol dithiothreitol (DTT), 0.25% sodium deoxycholate, 0.1% NP-40] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mmol sodium pyrophosphate, 1 mM Na_3VO_4, 1 mmol NaF, 5 mmol EDTA, 5 mmol EGTA, and protease inhibitors cocktail. Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4°C. Protein extraction from four groups cells was performed as previously described. Protein concentration was determined with a Beyotime protein assay kit (Beyotime Biological Technology Research Institute, Shanghai, China). Proteins were resolved on 8.5% polyacrylamide gels and then transferred onto nitrocellulose membranes. For immunoblotting, nitrocellulose membranes were incubated with primary antibody against LC-3 (Sigma-Aldrich, St. Louis, MO, USA) recognizing target proteins overnight at 4°C. The membranes were then incubated with secondary horseradish peroxidase (HRP)-conjugated antibody (1:3000, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature and subsequently analyzed by ECL detection system (Amersham Pharmacia Biotech., Morgan, Canada) and visualized by autoradiography. β-actin (1:5000, Sigma-Aldrich, St. Louis, MO, USA) was used as an internal control for protein loading.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi$) by Flow Cytometry Analysis with JC-1 Staining

Mitochondrial $\Delta\Psi$ was determined using the Beyotime Mitochondrial Membrane Sensor Kit (Beyotime, Shanghai, China). Briefly, the four groups cells were incubated in 24-well plates for 24 h and then treated with 5 μmol/L of JC-1 (Beyotime Biological Technology Research Institute, Shanghai, China) at 37°C for 30 min according to the manufacturer's instructions. Finally, cells were centrifuged and resuspended in 1 mL PBS for flow cytometry analysis.

Detection of Apoptosis by Flow Cytometry Analysis with Annexin V/PI Staining

After the cells were incubated for 24 h, they were washed with PBS, counted and adjusted to 1×10^6 cells/ml. Apoptosis of HCT116 cells was determined by flow cytometry (Epics XL; Beckman Coulter, Brea, CA, USA) using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Beyotime Biological Technology Research Institute, Shanghai, China). Staining was performed in accordance with the method described in the manufacturer's instructions. The rate of apoptosis was analyzed using Multicycle software (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

All data were presented as mean ± SD. Statistical analysis was carried out by ANOVA followed by a Dennett's-test, considering $p < 0.05$ as statistically significant.

Results

Hypoxia and 3-MA Inhibit the Growth of HCT116 Cells

As shown in Figure 1, normoxia culture HCT116 cells appeared as densely packed and disorganized multilayers, after 24 h of incubating in oxygen deficit condition, the cells became scattered and shrunken in morphology. Especially with the addition of 3-MA, which inhibit the autophagy, the cells were decreased apparently compared with normoxia. These photographs demonstrated that hypoxia and 3-MA inhibit the growth of HCT116 cells.

Expression of LC3 In HCT116 Cells in Different Environments

To further investigate the autophagy inducing the effect of hypoxia or 3-MA, we evaluated the expression of the autophagy-related protein LC3 by Western blot. The ratio of LC3-II/LC3-I can reflect the level of autophagy (Figure 2). The ratio of LC3-II/LC3-I was 0.430 ± 1.93% in normal oxygen and reached 0.586 ± 2.5% in hypoxic culture. The ratio was 0.338 ± 0.81% under hypoxia plus 3-MA culture, and it was significantly down-regulated compared to the normoxia group. These data indicate that HCT116 cells autophagy were

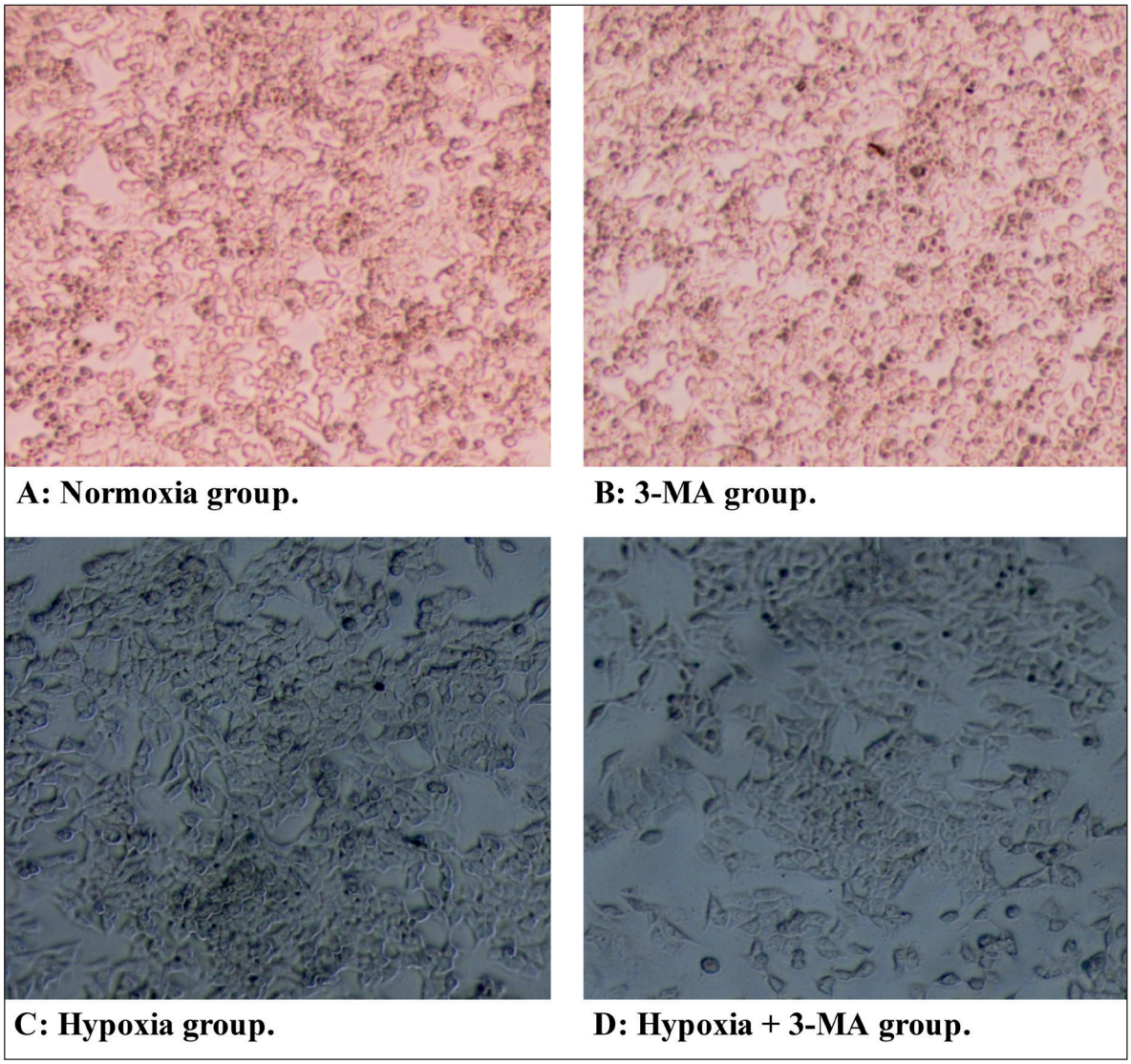

Figure 1. Cellular morphological observation of HCT116 cells: ***(A)*** normoxia group, ***(B)*** 3-MA group, ***(C)*** hypoxia group and ***(D)*** hypoxia+3-MA group by a phase-contrast microscope (Olympus, Tokyo, Japan) (x 200). The cells treated by hypoxia in combination with 3-MA were markedly inhibited.

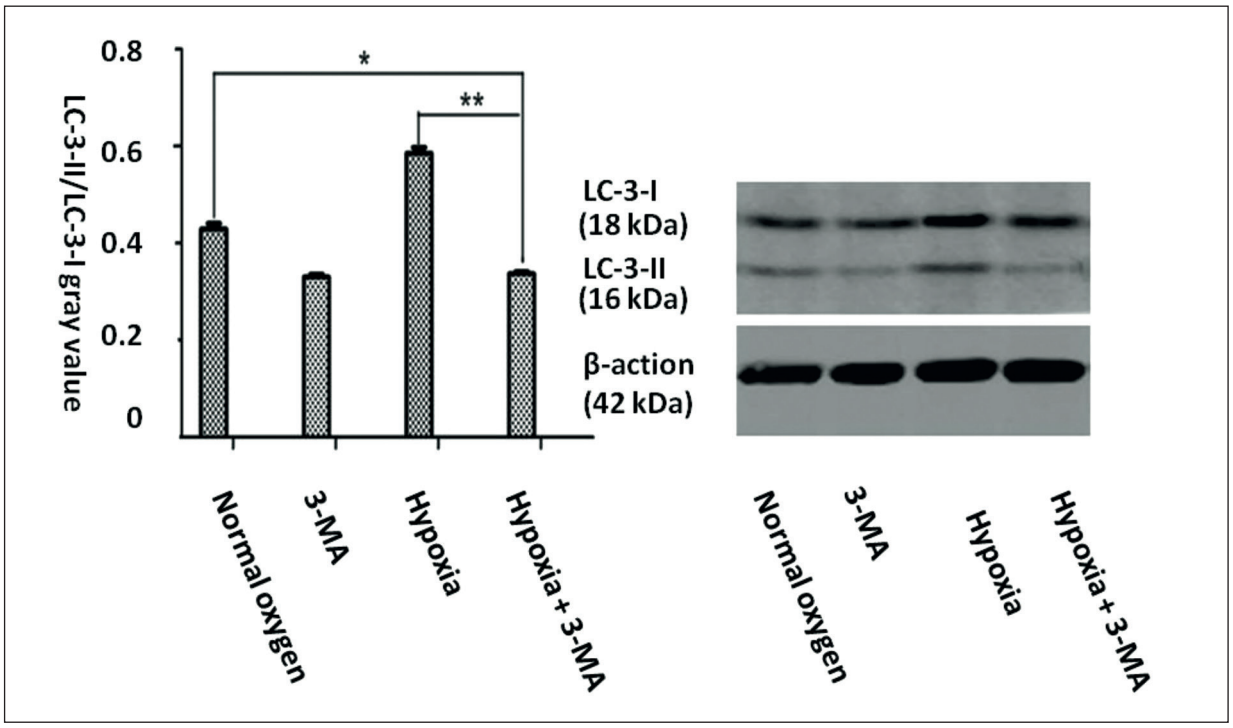

Figure 2. The ratio of LC3-II/LC3-I in Hypoxia+3-MA group was significantly lower than normoxia and hypoxia group, with a statistically significant difference (*$p < 0.05$; **$p < 0.001$), showing that 3-MA inhibited HCT116 cells under hypoxia-induced autophagy.

enhanced by hypoxia alone, but suppressed apparently after hypoxic culture in combination with 3-MA.

Hypoxic Culture with 3-MA-Induced Mitochondrial Dysfunction

In the present study, mitochondrial membrane potential (ΔΨ) was examined using the fluorescent dye JC-1. We detected a collapse in ΔΨ as early as 24 h treatment with different conditions of culture, as indicated by increased emission of green fluorescence. The more of the percentage of grey fluorescence shows the lower mitochondrial membrane potential. This change reached a maximum in the hypoxia+3-MA group (Figure 3). A collapse in ΔΨ is likely to trigger cell apoptosis or necrosis, the results described here showed that inhibition of autophagy under oxygen deficit, significantly reducing mitochondrial membrane potential.

Inhibition of Autophagy By 3-MA Treatment Enhanced Hypoxia-Induced Apoptosis

To evaluate whether the apoptosis of HCT116 cells is associated with the mitochondrial dysfunction, we examined apoptotic activity in HCT116 cells via Annexin V/PI staining. In this assay, Annexin V/PI double-negative stained cells (LL, lower left) indicated live cell population; Annexin V-positive/PI-negative stained cells (LR, lower right) and Annexin V/PI double-positive stained cells (UR, upper right) represented early apoptosis and late apoptosis, respectively. Annexin V-negative and PI-positive stained cells (UL, upper left) showed dead cells. The results revealed that the rate of early apoptosis of HCT116 cells in normal oxygen was 1.90 ± 0.19%. After treatment with hypoxic culture and 3-MA, respectively, increased 4.42 ± 0.20% and 6.57 ± 0.25%, while combination of hypoxia with 3-MA increased the apoptotic ratio to 26.87 ± 1.09%, which was apparently higher than the other groups (Figure 4). These results indicated that 3-MA enhanced hypoxia-induced apoptosis.

Discussion

In this study, through establishing HCT116 colorectal cancer cells model *in vitro*, we observed that both apoptosis and autophagy were

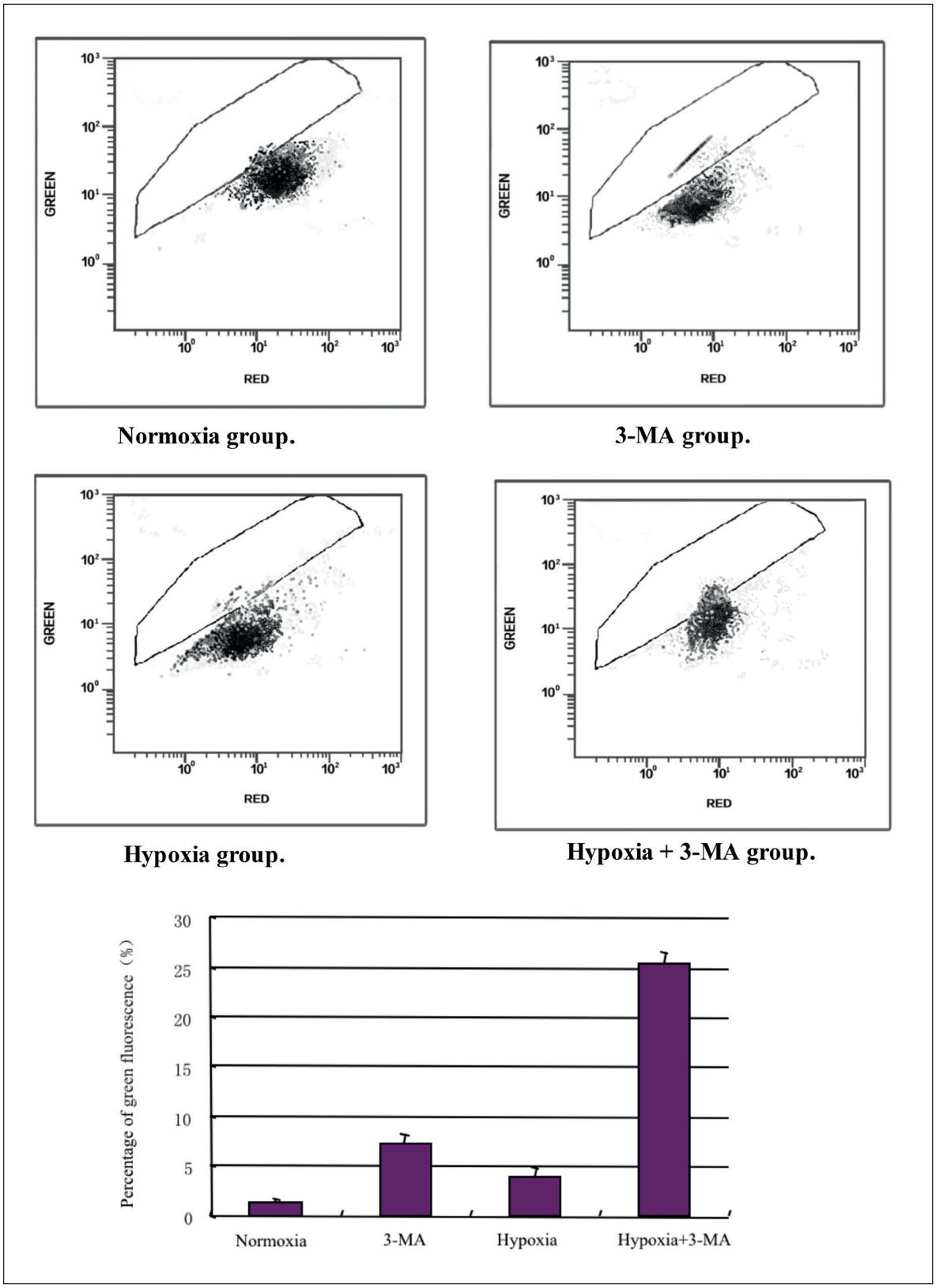

Figure 3. Mitochondrial membrane potential in experimental groups has a different degree of reduction by JC-1 dye using cytometry, and hypoxia +3-MA group of mitochondrial membrane potential is particularly evident difference was statistically significant ($p < 0.05$).

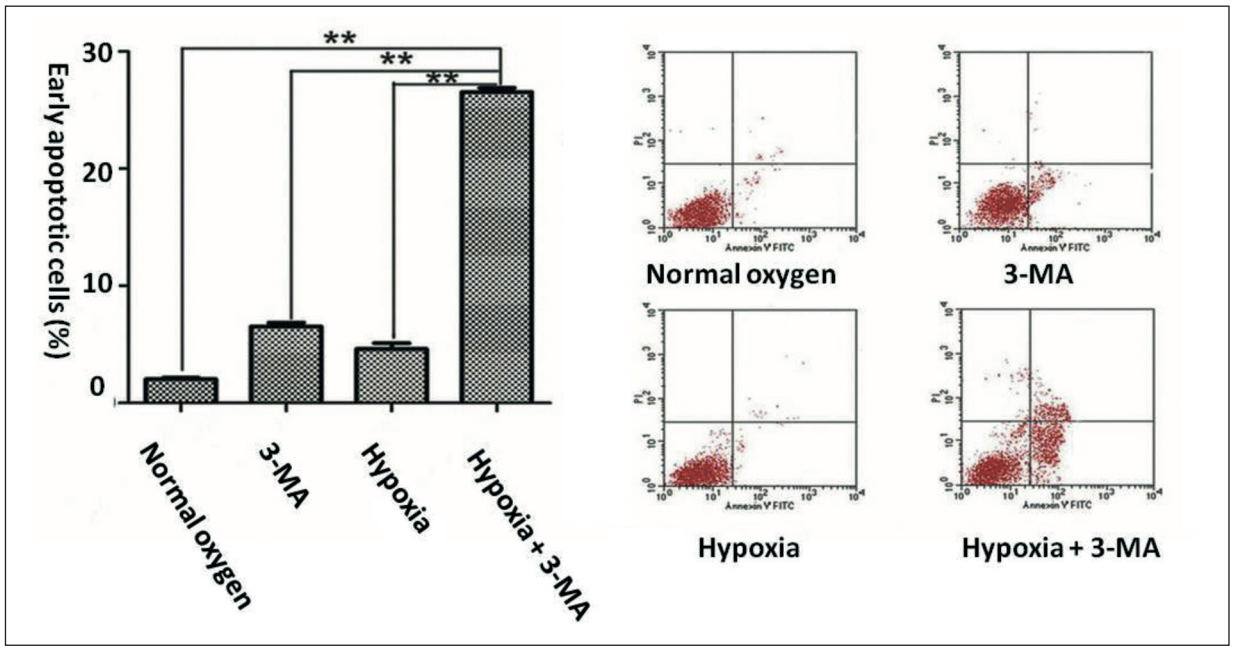

Figure 4. Annexin V/PI staining was performed to examine the apoptosis ratio of different groups. Hypoxia+3-MA group of early apoptosis was significantly higher than the other groups, the difference was statistically significant (*$p < 0.05$, **$p < 0.001$), indicated that inhibition of autophagy under hypoxic conditions significantly promotes apoptosis of HCT116 cells.

increased after treatment of hypoxia. Further experiments showed that combination treatment with 3-MA and hypoxia inhibited autophagy and promoted the occurrence of apoptosis, proving that autophagy may make some resistance effect in antitumor drug, having a certain protective effect[9]. In cell biology, the term autophagy defines the catabolic process regulating the degradation of a cell's own components through the lysosomal machinery[10]. Apoptosis is a process of the programmed cell death in normal physiological conditions and is also an important part of maintaining the stability of the body. As two distinct self-destructive processes, apoptosis ("self-killing") and autophagy ("self-eating") are initiated and regulated respectively by their own molecular mechanisms[11]. On the other hand, a complex relationship exists between autophagy and the apoptotic cell death pathway, where regulators of apoptosis also function as regulators of autophagic activation[12]. In the present study, we have verified the hypoxia-induced apoptosis and autophagy in the human HCT116 colorectal cancer cell. We investigated cell viability of HCT116 cell incubated in hypoxic culture first. The inhibition rate of HCT116 colorectal cancer cells after hypoxic culture was significantly higher than that of normoxia culture (data not showed) by MTT assay. We also found that the cells scattered in morphology and decreased apparently in the hypoxia+3-MA group. Microtubule-associated protein light chain 3 (LC3) is the only credible autophagy marker. LC3 is a cytosolic soluble protein, which is cleaved during autophagic induction and involved in the autophagic vacuole membrane formation. When autophagic process starts, LC3-I (16 kDa) is converted to LC3-II (14 kDa). Through detecting LC3-II, LC3-I level, we obtained LC3-II/LC3-I ratio to reflect the level of autophagy[13]. Our experiment used Western blot to detect LC3 protein expression in HCT116 cells after hypoxia, so as to observe cell autophagy under anoxic condition. Finally, after hypoxia, LC3-II/LC3-I ratio increased apparently, which means hypoxia is significantly associated with the increased expression level of cell autophagy. 3-Methyladenine (3-MA) is a popular inhibitor of the autophagic pathway. It has been reported to inhibit the activity of PI3-Kinase and block the formation of preautophagosome, autophagosome, and autophagic vacuoles[14]. In our experiment, we observed that, in both of colon cancer cells, using 3-MA and hypoxia in combination increased cell death more markedly than using hypoxia alone (Figure 2). Therefore, it appears that 3-MA enhances hypoxia-induced colon cancer death and

that the inhibition of autophagy improves the apoptosis effect in HCT116 colorectal cells. We used inhibitors of autophagy to investigate the relationship between apoptosis and autophagy induced by hypoxia-induced autophagy. Hypoxia-induced autophagy functioned more like a cell survival mechanism, because of the results that the inhibition of autophagy in those hypoxias treated cells induced, more cell apoptosis ratio was detected. The mitochondrion, the energy metabolism center of cell, is the integration of a variety of information transmission and plays an important role in regulating cell death and survival. The drop of mitochondrial membrane potential is considered to be the earliest event in the process of apoptosis; it occurs before the nucleus apoptosis features appear. Once happen the apoptosis is irreversible. In our study, we used 3-MA to inhibit hypoxia-training level of autophagy of colorectal cancer cells, and cells mitochondrial membrane potential decreased significantly. That shows, under the condition of oxygen, that the inhibition of autophagy significantly reduced cell mitochondria membrane potential, leading to apoptosis. Recent reports showed that inhibition of autophagy by the treatment of specific inhibitors for autophagic regulators, 3-MA or suppression of autophagy regulatory pathways, may provoke apoptotic efficiency of chemotherapeutic agents in prostate[15], breast[16], colon[17], lung[18], and HeLa[19] cancer cells. Moreover, there are also other reports indicating that 5-FU and 3-MA combination could also cause a synergistic effect and induce apoptosis dramatically in HT29 colon cancer cells[20]. And 3-MA could attenuate rapamycin-induced death of A549 lung cancer cells with inactivation of Beclin-1 and LC3-II [21]. We examined apoptotic activity in HCT116 cells via Annexin V/PI staining. While the combination of hypoxia with 3-MA increased the apoptotic ratio to 26.87 ± 1.09%, which was apparently higher than other groups (Figure 4). These results implied that the apoptosis induced by hypoxia could be mediated through autophagy in HCT116 colorectal cells, showing that inhibiting to autophagy significantly promotes the occurrence of apoptosis in hypoxia conditions. Thus, this proves that autophagy may play a role in protecting cells in early hypoxia environment. It has been shown that autophagy proteins can play a role in cellular events that occur during apoptosis. On the other hand, inhibition of autophagy leads in most cases to increased susceptibility to apoptotic stimuli, highlighting the pro-survival role of the autophagic process[22,23]. The research results show that the hypoxia can induce HCT116 cells' autophagy, which has a certain protective effect on cancer cells.

Conclusions

We observed that autophagy might play a role as a self-defense mechanism in hypoxia-treated colon cancer cells, and its inhibition could be a promising strategy for the adjuvant chemotherapy of colon cancer. With further research, the mechanism of autophagy and cancer cell self-protection will be further clarified for the development of tumor targeted therapeutic drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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