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Full paper

EGCG synergizes the therapeutic effect of cisplatin and oxaliplatin through autophagic pathway in human colorectal cancer cells

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ABSTRACT

Application of the platinum-based chemotherapy for colorectal cancer is restricted due to its severe cytotoxic effects. In this study we used synergistic strategies by combining (−)-Epigallocatechin gallate (EGCG) with cisplatin or oxaliplatin to minimize the ill effects of platinum-based therapy. MTS assay was used to examine the effect of EGCG, cisplatin and oxaliplatin on the proliferation of human colorectal cancer DLD-1 and HT-29 cells. Autophagic process was evaluated by detection of LC3-II protein, autophagosome formation, and quantification of Acidic Vesicular. Treatment of DLD-1 and HT-29 cells with EGCG plus cisplatin or oxaliplatin showed a synergistic effect on inhibition of cell proliferation and induction of cell death. EGCG enhanced the effect of cisplatin and oxaliplatin-induced autophagy in DLD-1 and HT-29 cells, as characterized by the accumulation of LC3-II protein, the increase of acidic vesicular organelles (AVOs), and the formation of autophagosome. In addition, transfection of DLD-1 and HT-29 cells with siRNA against ATG genes reduced EGCG synergistic effect. Our findings suggest that combining EGCG with cisplatin or oxaliplatin could potentiate the cytotoxicity of cisplatin and oxaliplatin in colorectal cancer cells through autophagy related pathway.

1. Introduction

Colorectal cancer (CRC) is the second most prevalent cancer and the third leading cause of cancer deaths worldwide (1). Globally more than 1 million new cases were reported annually resulting in about 500,000 deaths worldwide per year (2). Although surgical resection currently remains the only curative treatment for CRC, adjuvant chemotherapy after surgical resection was still needed for most patients with metastasis to palliate symptoms and prolong life (3). The platinum-based chemotherapy regimens are adopted widely for treatment of CRC; however, further improvement has been difficult due to their serious adverse cytotoxic events. Oxaliplatin, a third-generation platinum analog, has evolved as one of the most important therapeutic agents in metastatic/recurrent CRC. Although oxaliplatin is generally well tolerated, peripheral sensory neuropathy occurs as an adverse effect of oxaliplatin and is the most common dose-limiting factor for oxaliplatin treatment (4). Despite recent advances in primary prevention, combined chemotherapy with operation, the long-term survival rate of CRC patients has not been substantially improved.

The mode of cytotoxic action of anticancer drugs often involves the induction of several types of programmed cell death (PCD). Apoptosis involves a trial of biochemical events in multicellular organisms, leading to a variety of cellular morphological changes and death (5). Autophagy, another type of PCD, is an evolutionary conserved catabolic process involving the degradation of cytoplasmic constituents by lysosomal activity (6,7). Once initiated, cytoplasmic constituents, including organelles, are first sequestered by a phagophore and subsequently form a double-membraned autophagosome, which subsequently fuse with lysosomes, where the cargo of the autophagosome is degraded by lysosomal enzymes (8). Autophagy is characterized morphologically by the formation of microtubule-associated protein 1 light

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chain 3 (LC3-II)-autophagic vacuoles and the accumulation acidic vesicular organelles in the cytoplasm (9). In addition to the physiological role of autophagy, accumulated evidence has shown that this process is also involved in many pathological conditions, including neurodegeneration, aging, infectious disease, and cancer (10). Although the molecular mechanism underlying autophagy and apoptosis remains to be fully elucidated, autophagic process may serve as an alternative to apoptosis to eliminate transformed cells. Accumulating evidence also indicates that various anticancer agents activate autophagy and autophagic cell death (11). For example, treatment of oxaliplatin and bortezomib induced autophagic cell death of human colorectal carcinoma HCT116 cells (12).

Tea is one of the most widely consumed beverages in the world. Both epidemiological, preclinical and laboratory studies have positively shown an association of tea consumption with lower risk for certain types of cancers (13). Among different types of teas, the non-fermented green tea is of particular interest and has been regarded as the most effective cancer preventive beverage. (−)-Epigallocatechin gallate (EGCG), which is the predominant polyphenolic catechin constituent in green tea, has been recognized as an important chemopreventive agent and as modulators of tumor cell response to chemotherapy to suppress the growth, invasion, metastasis and angiogenesis of various cancer cells by arresting the cell cycle, inducing apoptosis, targeting molecules relating to angiogenesis (VEGF, etc), metastasis (MMP, etc) and many other cellular regulatory pathways (14–16).

Numerous investigations have shown EGCG apoptotic effect on various cancer cell lines, while the study on its autophagic function is poor (17). Our hypothesis was that EGCG, cisplatin and oxaliplatin would act synergistically to induce cell death in colorectal cancer cell lines through interaction between apoptosis and autophagy. Hence, we investigated the synergistic effect of combined chemotherapy by EGCG, cisplatin and oxaliplatin on the proliferation and autophagy of colorectal cancer cells. Our data show that EGCG potently potentiate the cytotoxicity of cisplatin and oxaliplatin in human colorectal cancer DLD-1 and HT-29 cells. To clarify the molecular mechanisms underlying these responses to the combined therapy, the effects of different compounds on autophagy were investigated. Our observations suggest that EGCG enhances the cisplatin and oxaliplatin-induced autophagic death in colorectal cancer cells.

2. Materials and methods

2.1. Cells, chemicals and reagents

The human colorectal adenocarcinoma (DLD-1 and HT-29) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hyclone), penicillin (100 IU/ml), streptomycin (100 μg/ml) at 37 °C in a humid atmosphere with 5% CO_2. EGCG, cisplatin, oxaliplatin, 3-methyladenine (3-MA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies for ATG 5, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), microtubule-associated protein 1 light chain 3 A/B (LC3A/B) were purchased from Cell Signaling Technology. The secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG were purchased from Sigma–Aldrich.

2.2. MTS and synergy analysis

The cytotoxic effect of the compound on cells was determined using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay reagents, according to the manufacturer's instructions (Promega). After compound treatment, cells were incubated with MTS solution for 2 h and submitted for absorbency measurement at 490 nm using an enzyme-linked immunosorbent assay plate reader. All experiments were performed 3 times. The potential synergistic or additive effects of EGCG, cisplatin, and oxaliplatin were evaluated by MTS assay following the protocols as described previously. The combination index (CI) and the degree of interaction was evaluated by computer-based Calcusyn software (version 2.1, Biosoft). According to the CI theorem, CI values of <1, $=1$, and >1 indicate synergism, additive effect, and antagonism, respectively.

2.3. Detection and quantification of acidic vesicular

Autophagy was detected by quantification of acidic vesicular organelles (AVOs), a marker of autophagy, according to published protocol. The non-protonated monomeric form of AO emits green fluorescence and dim red in the cytoplasm and nucleolus, while the acidic compartment (e.g. lysosomes or late endosomes) trapped AO fluoresces bright red or orange. The intensity of the red fluorescence is proportional to the degree of acidity. Briefly, cells were stained with acridine orange (1 μg/ml) at 37 °C for 15 min. After loading, the cells were washed with PBS and immediately analyzed using EPICS flow cytometer.

2.4. Western blot analysis

Western blot analysis was performed as stated previously. The protein concentration was determined by the Bradford assay (Bio-Rad). Equal amounts of the protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred onto nitrocellulose membranes and stained with primary antibodies overnight at 4 °C, followed by the secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). For loading control analysis, blots were stripped and incubated with antibodies to GAPDH. Western results were scanned and quantified using Image J software (http://rsb.info.nih.gov/ij/).

2.5. Knockdown of ATG 5

Autophagy was blocked by knockdown of ATG 5 with ATG 5 siRNA. Cells were seeded into a 12 well plate with 1 ml medium and incubated overnight. Medium without serum, containing 25 nM ATG 5 siRNA, or control siRNA was mixed with 5 μl transfection reagent for 30 min at room temperature and then added to the cells. The final volume was adjusted with medium to 0.5 ml per well and the mixture was incubated for 48 h. Cells were harvested and assays were carried out to evaluate ATG 5 content. Cell viability was assayed by MTS assay after 48 h incubation with EGCG plus different concentration of cisplatin and oxaliplatin.

2.6. Transmission electron microscopy

Electron microscopy was performed as described previously. Briefly, cell samples were washed three times with PBS, trypsinized, and collected by centrifuging at 1000 g for 5 min. The cells pellets were fixed with 2.5% glutaraldehyde and stored at 4 °C overnight. For subsequent processing, the cells were washed in PBS, post-fixed with 1% osmium tetroxide for 1 h at room temperature, dehydrated stepwise with ethanol and embedded in Spurr's plastic resin. Ultrathin sections were cut and then examined under JEM-1230 electron microscope.

3. Statistical analysis

Data were expressed as means ± SD for triplicate experiments. Statistic differences between groups were determined by One-way ANOVA with Bonferroni's multiple comparison-tests. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Co-treatment with EGCG synergistically inhibits the survival of human colorectal cancer cells

To examine the effect of EGCG, cisplatin and oxaliplatin on the proliferation of human colorectal cancer cells, a cell viability assay was performed. DLD-1 and HT-29 cells were exposed to various concentrations of cisplatin, and oxaliplatin alone or in combined with EGCG for 48 h. The 50% inhibitory concentration (IC_{50}) value of EGCG, cisplatin and oxaliplatin were shown in Table 1. Fig. 1 shows a differential response of colorectal cancer DLD-1 and HT-29 cells to different compounds. Cisplatin and oxaliplatin at the concentration of 20 μM had slight inhibitory effect on cell viability. However, a significant, dose-dependent decrease in proliferation was observed in the cells treated with 100 μM EGCG plus 20 μM cisplatin or oxaliplatin (Fig. 1B). A combination of 100 μM EGCG with 20 μM cisplatin caused 66.5% reduction in cell viability than did treatment on cisplatin own. After 48 h co-treatment with 100 μM EGCG, the number of viable cells in the 20 μM oxaliplatin group was 30.0% in DLD-1 cells. Pretreatment with 3-MA, however, blocked the synergistic cytotoxic effect in DLD-1 and HT-29 cells. (Fig. 1B). To further determine whether the inhibitory effects were synergistic, we used the CalcuSyn software to determine the CI values to ascertain synergism (CI < 1), antagonism (CI > 1) or additive effect (CI = 1). The CI values are presented in Table 2. The CI-effect plot showed that CI values for the combination of EGCG with cisplatin and oxaliplatin were all less than 1.0, indicating a synergistic antiproliferative effect. (Table 2).

4.2. Co-treatment with EGCG synergistically induces autophagy in human colorectal cancer cells

Chemotherapy-induced inhibitory effect and cell cycle arrest is usually prerequisite to the demise of cancer cells, which can be mediated by either apoptotic or autophagic pathways. Previously studies indicated that autophagy can both act as an inhibitor or enabler of programmed cell death (11). To investigate whether the synergistic effect of cell death caused by the co-treatment induces autophagic cell death, we examined the conversion of LC3-I to LC3-II, which serves as a marker of the accumulation of autophagic vesicles and autophagic activity. As shown in Fig. 2A, EGCG treatment alone resulted in a dose-dependent increased conversion of the normal LC3-I to the autophagic LC3-II isoform in human colorectal cancer DLD-1 cells. However, inhibition of PI3K (with 3-MA), a well-known inhibitor of autophagic process, impaired EGCG-mediated autophagy induction in DLD-1 cells (Fig. 2B). Following co-treatment with EGCG plus cisplatin and oxaliplatin, the ratio of LC3-II/GAPDH as well as the accumulation of LC3-II was increased (Figs. 2C, D, F). Similar results were obtained by quantification of acidic vesicular organelles (AVOs), a marker of autophagy (Figs. 2E, G). To further confirm that combined-therapy induces autophagy in DLD-1 and HT-29 cells, we used electron microscopy to measure autophagosome formation. Transmission electron microscopy analysis confirmed that the structures of autophagic vacuoles were largely detected in co-treatment group as compared to its monotherapy controls (Fig. 3). These results suggest that the cell death caused by the combined treatment with EGCG and cisplatin or oxaliplatin depends on autophagic cell death at least partially.

Table 1
The inhibitory potentials of EGCG, cisplatin and oxaliplatin on the viability of DLD-1 and HT-29 cells.

Cells	Compound/IC_{50}(μM)		
	EGCG	Cisplatin	Oxaliplatin
DLD-1	196.4	30.5	32.2
HT-29	168.8	32.8	35.6

4.3. Inhibition of autophagy by knockdown of ATG 5 decreased the EGCG-induced synergistic effect

To further investigate the role of autophagy in EGCG-induced synergistic cytotoxicity, ATG5-specific siRNA was transfected into DLD-1 and HT-29 cells. Cells were first incubated with ATG 5 (or control) siRNA for 48 h, EGCG was added and cell viability was assayed after 48 h incubation with 100 μM EGCG plus different concentration of cisplatin and oxaliplatin. Transfection of siRNA against ATG 5 significantly decreased the EGCG-induced synergistic effect with cisplatin from a decline in cell viability of 64.5% in the absence of the ATG 5 siRNA to a decline of 42.3% in the presence of ATG 5 siRNA in DLD-1 cells. The control siRNA had no effect on EGCG toxicity. Both ATG 5 knockdown cell lines showed similar responses to EGCG plus cisplatin and oxaliplatin treatment (Fig. 4). These results further clearly indicate that inhibition of autophagy diminishes DLD-1 and HT-29 cells to EGCG-induced synergistic cytotoxicity.

5. Discussion

The experiments reported in this paper were performed in order to provide a better understanding of the interactive effects of EGCG with cisplatin or oxaliplatin on their therapeutic potential in human colorectal cancer DLD-1 and HT-29 cells.

Historically, consumption of green tea has been associated with health benefits against multiple diseases like cancer. As the predominant polyphenolic catechin in green tea, EGCG has demonstrated remarkable chemopreventive and chemotherapeutic potential against various types of cancers, through interfering and targeting different signaling pathways (18). For example, EGCG could induce apoptosis in pancreatic carcinoma cells, prompting the loss of mitochondrial membrane potential, increasing reactive oxygen species (ROS) formation and cytochrome C release, thereby activating caspase-dependent apoptosis (19). And similar results were observed in human colorectal cancer HT-29 cells through modulating AMP-activated protein kinase (AMPK) pathways (20). The potential role of EGCG in the regulation of autophagy has just recently been investigated. The induction of autophagy is known to maintain cell survival under various cellular stress by degradating injured, or aged proteins and organelles and the subsequent recycling of degraded products in eukaryotic cells. However, persistent activation of autophagy can lead to caspase-independent autophagic cell death, characterized by the excessive depletion of cellular organelles and essential proteins (21). Experiments performed in hepatocellular carcinoma treated with oxaliplatin indicated that autophagy contributes to the tolerance of oxaliplatin via reactive oxygen species (ROS) modulation (22). Activation of autophagy in caco-2 colorectal cancer cells treated with oxaliplatin can also act as cytoprotective response via endoplasmic reticula (ER) stress induced by ROS production (23). However, the role of autophagy in oncogenesis and anticancer therapy is rather contradictory.

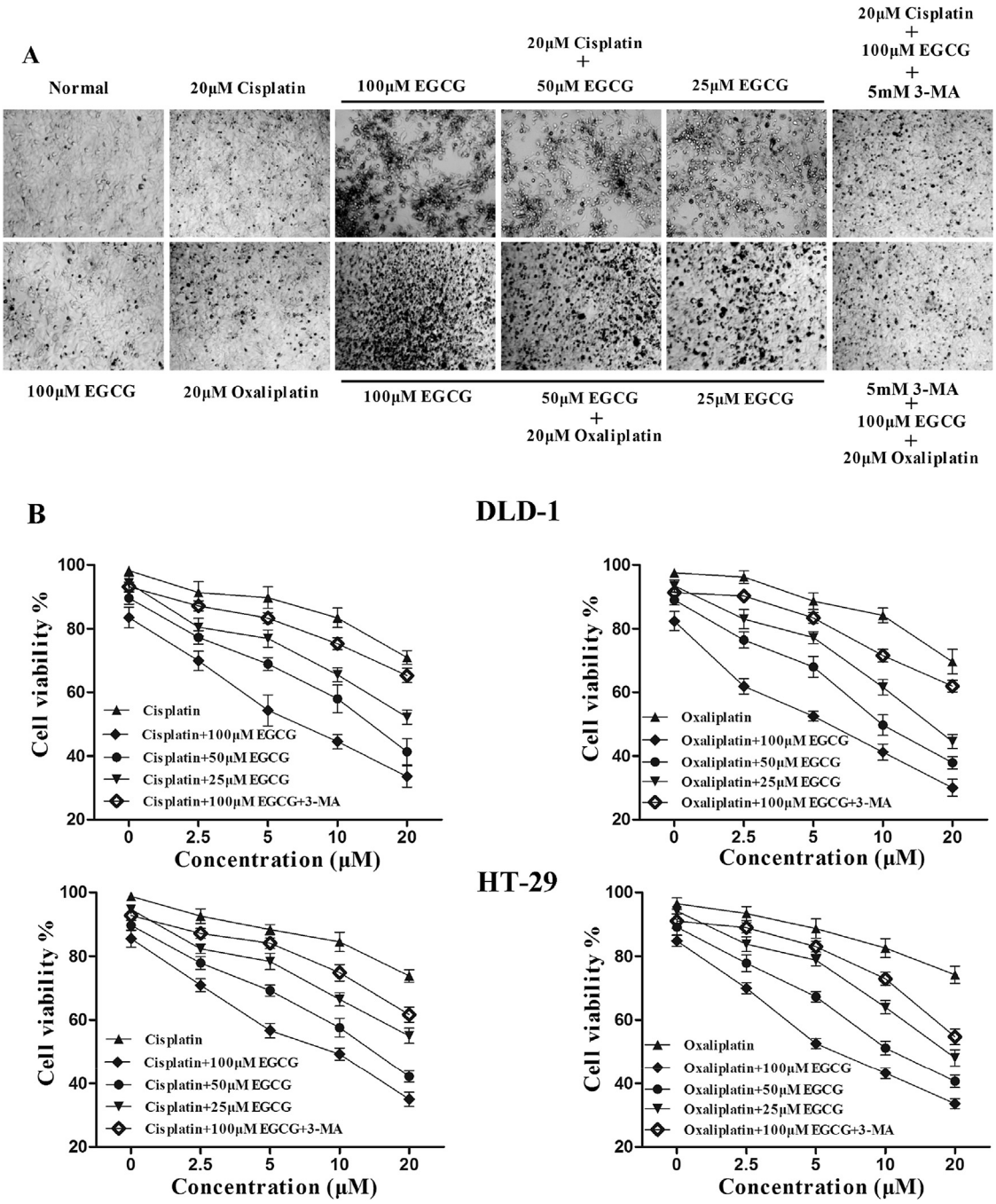

Fig. 1. DLD-1 and HT-29 cells undergo growth inhibition after co-treatment with EGCG plus cisplatin and oxaliplatin. (A) Photomicrographs of DLD-1 cells treated with different compound. DLD-1 cells were treated with cisplatin (20 μM), oxaliplatin (20 μM) and EGCG (100 μM) alone, or both agents with or without 3-MA (5 mM). Photos were taken at 48 h post-treatment (original magnification, × 20). (B) Cell viability was measured by MTS assay after 48 h of different compound treatment in DLD-1 and HT29 cells. Data presented as means ± SD of three independent experiments.

Table 2
The combination index (CI) values of EGCG, cisplatin and oxaliplatin at 50% inhibition in DLD-1 and HT-29 cells.

Cells	Co-treatment	CI values at IC_{50}/EGCG concentration (μM)		
		25	50	100
DLD-1	EGCG + Cisplatin	0.887 ± 0.061	0.825 ± 0.058	0.791 ± 0.047
	EGCG + Oxaliplatin	0.742 ± 0.065	0.714 ± 0.049	0.721 ± 0.063
HT-29	EGCG + Cisplatin	0.931 ± 0.044	0.863 ± 0.049	0.848 ± 0.087
	EGCG + Oxaliplatin	0.811 ± 0.046	0.807 ± 0.053	0.783 ± 0.058

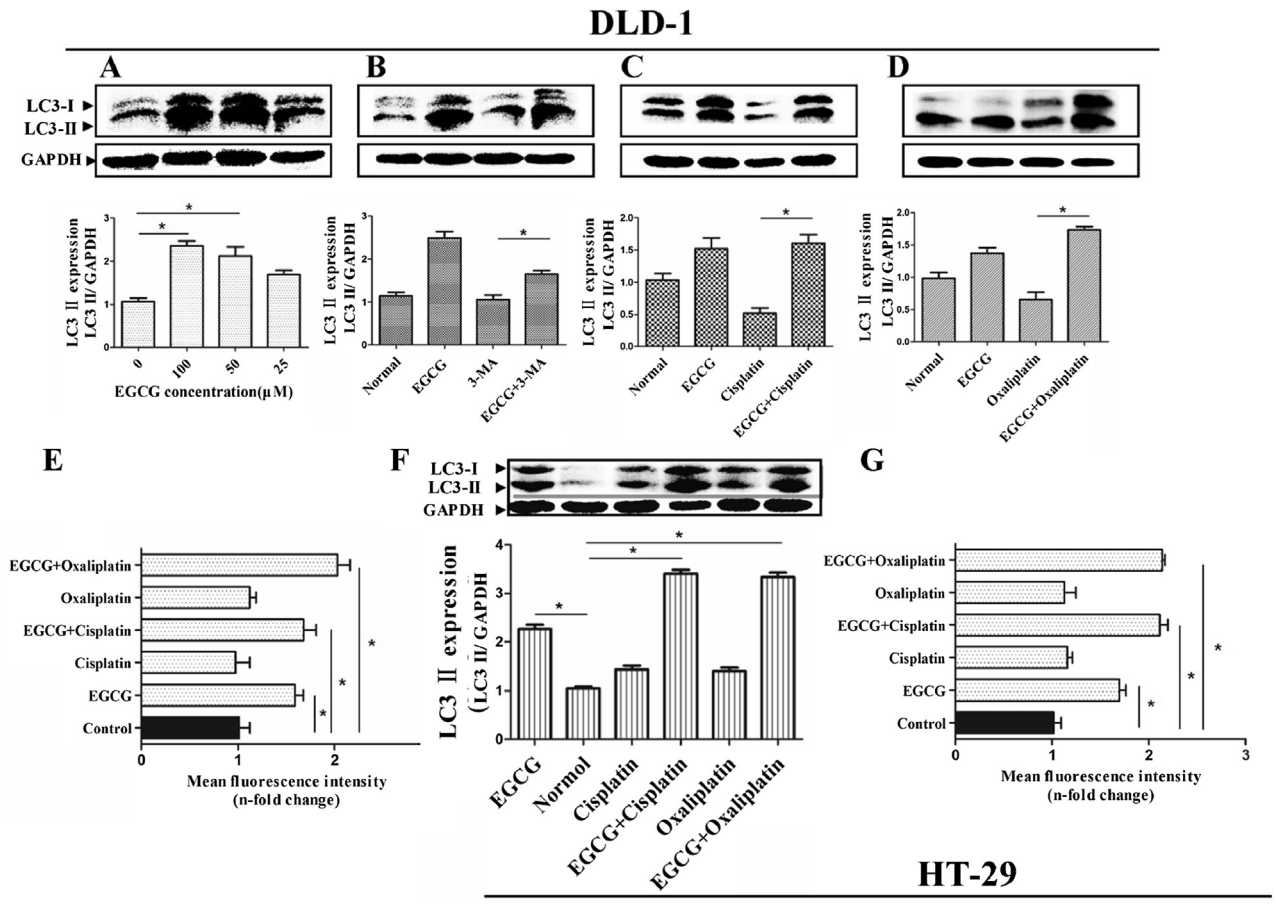

Fig. 2. EGCG enhances the effect of cisplatin and oxaliplatin-induced autophagy. (A–D) DLD-1 cells were treated with EGCG alone (A) pre-treated with 5 mM 3-MA (B) or co-treated with the 20 μM cisplatin (C) or co-treated with 20 μM oxaliplatin (D). After 48 h incubation, total protein extracts were harvested from cells, then was determined by western blotting analysis. The total gray value of each band was determined using Image J. The ratio of LC3-II to GAPDH is presented below. Data shown were the mean ± SD of three independent experiments. (E) Flow cytometry analysis for acridine orange staining in DLD-1 cells treated with different compounds. (F) HT-29 cells were treated with 100 μM EGCG alone or co-treated with 20 μM cisplatin/oxaliplatin. After 48 h incubation, total protein extracts were harvested from cells, then was determined by western blotting analysis. (G) Flow cytometry analysis for acridine orange staining in HT-29 cells treated with different compounds.

The anti-mitogenic and synergistic inhibitory effect of EGCG in our study is associated with the induction of autophagy as evidenced by the up-regulation of LC3-II protein, increased formation of autophagic vacuoles, and accumulation of acidic vesicular organelles, etc. These data are in line with previous studies reporting that EGCG stimulated LC3-II production and autophagosome formation by inducing high mobility group Box 1(HMGB1) aggregation and autophagic degradation in macrophages, thereby alleviating lethal systemic inflammation caused by HMGB1 release (24). Treatment with EGCG could also regulate ectopic lipid accumulation through increased formation of LC3-II and autophagosomes in primary bovine aortic endothelial cells by activation of calmodulin-dependent protein kinase β pathway (25). The mechanism by which EGCG induces or regulates autophagic pathway remains elusive. Previous study indicated that EGCG can form aggregation spontaneously via oxidation reaction which in turn conjugates to proteins either covalently or non-covalently, thus enhancing autophagy-mediated protein degradation (24). Emerging evidence has also suggested a potential link between ROS regulation and autophagic process, because agents capable of inducing (e.g. mitochondrial electron-transport-chain inhibitors) or inhibiting (e.g. N-acetylcysteine) ROS formation affected autophagic process in human embryonic kidney HEK 293 cells and human fibroblast HT 1080 cells (26, 27). ROS are highly reactive oxygen free radicals or non-radical molecules that are generated by multiple mechanisms. Autophagy has been reported regulated by ROS including O_2^- and H_2O_2, and O_2^- is the major form participating in ROS-regulated autophagy (28). Several conventional or natural compounds for cancer treatment have been shown to cause intracellular ROS generation. Sodium selenite, a conventional anti-cancer agent, could lead to the loss of mitochondria membrane permeability and selective autophagic-mediated mitochondria degradation through increasing intracellular ROS formation in malignant glioma cells (29). Platinum-based therapy and EGCG-mediated cell death may also depend on the generation of ROS either directly or indirectly (30). Previous study indicated that autophagy is promoted by AMP activated protein kinase (AMPK), which can be activated directly by EGCG treatment through the generation of reactive oxygen species (ROS) (31). These finding further explain the autophagic induction and synergistic inhibition effect in DLD-1 and HT-29 cells observed in our experiment.

In summary, this study highlights the role of autophagy in EGCG induced synergistic effect with cisplatin and oxaliplatin in DLD-1 and HT-26 cells. Consequently, induction of autophagy by EGCG potentiates the cytotoxicity of cisplatin and oxaliplatin in colorectal cancer cells through autophagy related pathway. As green tea is one of the most widely consumed beverage across the globe, this may offer a new strategy of cancer treatment based on the combination of EGCG and conventional anticancer drugs.

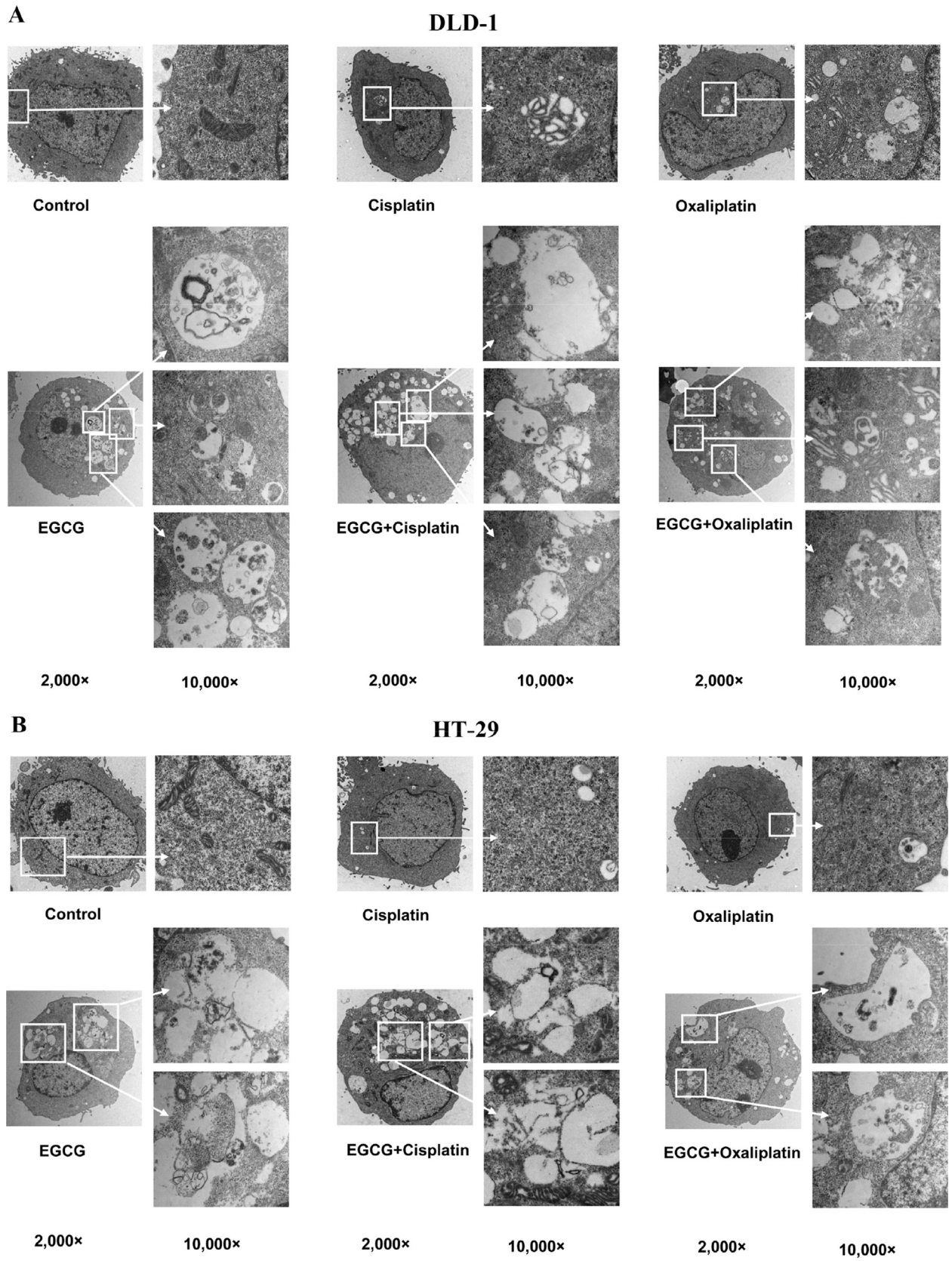

Fig. 3. Electron microscopy analysis of autophagic vacuoles in DLD-1(A) and HT-29 cells (B) treated with different compounds. Representative electronmicroscopic images were shown. The high magnification image showed autophagic vacuolar organelles contained electron dense material and degraded subcellular organelles.

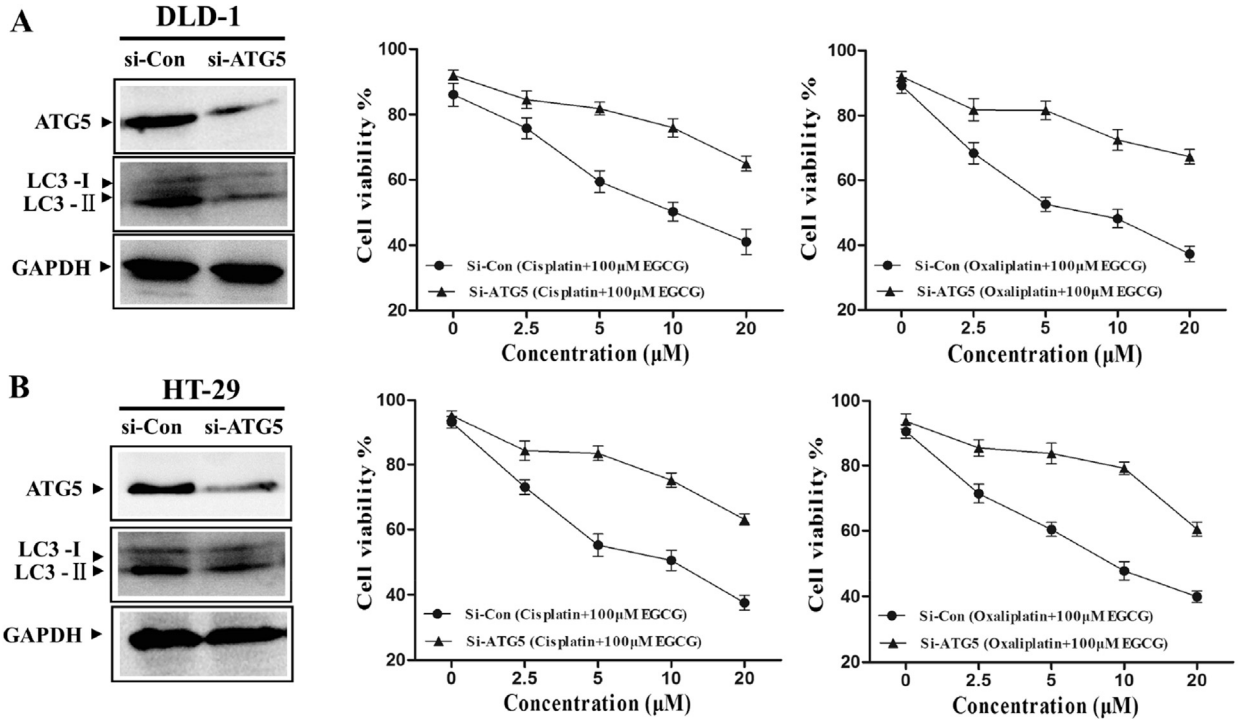

Fig. 4. Effect of ATG5 depletion on EGCG-induced synergistic cytotoxicity. Cells were transfected with control siRNA or siRNA against ATG5 and treated with 100 μM EGCG plus different concentration of cisplatin and oxaliplatin for 48 h, and cell viability was examined. The data are expressed as the mean ± SD of results obtained from three independent experiments. Depletion of ATG 5 reduced EGCG-induced cell death. In transfected cells, LC3 conversion from LC3-I to LC3-II also showed to be lower than that observed in untransfected cells.

Authors' contributions

Hu conceived the idea and performed the experiment. Wei performed the transmission electron microscopy and flow cytometry work. Hu and Wei drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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