Reduced Autophagy in 5-Fluorouracil Resistant Colon Cancer Cells

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Abstract
We investigated the role of autophagy in SNUC5/5-FUR, 5-fluorouracil (5-FU) resistant SNUC5 colon cancer cells. SNUC5/5-FUR cells exhibited low level of autophagy, as determined by light microscopy, confocal microscopy, and flow cytometry following acridine orange staining, and the decreased level of GFP-LC3 puncta. In addition, expression of critical autophagic proteins such as Atg5, Beclin-1 and LC3-II and autophagic flux was diminished in SNUC5/5-FUR cells. Whereas production of reactive oxygen species (ROS) was significantly elevated in SNUC5/5-FUR cells, treatment with the ROS inhibitor N-acetyl cysteine further reduced the level of autophagy. Taken together, these results indicate that decreased autophagy is linked to 5-FU resistance in SNUC5 colon cancer cells.

Key Words: Autophagy, 5-Fluorouracil, SNUC5/5-FUR, Reactive oxygen species, Colon cancer

INTRODUCTION
Over the last three decades, 5-fluorouracil (5-FU)-based chemotherapy has been used as the primary adjuvant treatment for colorectal cancer, and this approach has significantly improved clinical outcome and reduced cancer recurrence (Nordman et al., 2006). To exert its anticancer effect, 5-FU must be converted into its active form, fluorodeoxyuridine monophosphate. The active drug forms a complex with thymidylate synthase, thereby inhibiting its function and impairing DNA synthesis. In its fluorouridine triphosphate form, 5-FU is also incorporated into RNA and interferes with RNA processing (Longley et al., 2003). Moreover, futile cycles of misincorporation excite the nucleotide excision system in cancer cells, causing DNA strand breaks and ultimately leading to apoptosis (Grem, 2000). However, failure of colorectal cancer chemotherapy has recently become more common due to the acquisition of 5-FU resistance by cancer cells. 5-FU resistance can arise due to reduced expression of 5-FU-activating enzymes, including orotate phosphoribosyl-transferase and uridine kinase, or elevated expression of the drug target, thymidylate synthase (Bjønsdorp et al., 2010).

Autophagy, a suite of pathways involved in regulated degradation of cellular components, depends on the hierarchically ordered activity of autophagy-related (Atg) proteins (Kim et al., 2016). The Atg factors are recruited to phagophore assembly sites to form autophagosome, which ultimately fuse with lysosomal compartment (Codogno et al., 2011). Because autophagy is triggered by multiple metabolic stresses and participates in complex signaling networks that govern cell fate, it is now considered to play dual roles in inhibition of carcinogenesis and cancer progression; these roles are mediated by different mechanisms depending on tumor stage (Liu and Ryan, 2012). Accordingly, a growing research effort is devoted to sensitize 5-FU to colorectal cancer by controlling of autophagy. For instance, inhibition of autophagy with 3-methyladenine increases 5-FU-induced apoptosis in colon cancer cells (Li et al., 2009). Another autophagic inhibitor, chloroquine, potentiates the cytotoxic effect of 5-FU in colon cancer cells (Sasaki et al., 2010). Consistent with this, in vivo system, chloroquine resulted in the inhibition of 5-FU-induced autophagy and a significant enhancement in the 5-FU-induced inhibition of tumor growth (Sasaki et al., 2012).

The SNUC5 colon cancer cells, which completely lack nor...
mal p53 function due to missense mutations in p53, have been used for many research purposes, including anticancer drug screening, and studies of multidrug resistance, microsatellite instability, mutations in mismatch repair genes, and the TGF-β signaling pathway (Ku and Park, 2005). Based on the results of a study in an established 5-FU resistant cell line derived from SNUC5, SNUC5/5-FUR, 25-multiplex RT-PCR assay revealed that mRNA expression of four ATP-binding cassette transporter genes is up-regulated, whereas expression of a solute carrier transporter gene is down-regulated in SNUC5/5-FUR (Lee and Choi, 2009). Moreover, production of PGE2 by COX-2 is up-regulated in SNUC5/5-FUR, suggesting that a COX-2 inhibitor might exert an anti-angiogenic effect in colon cancer (Choi et al., 2011). Recently we demonstrated that SNUC5/5-FUR cells were up-regulated the antioxidant system expression such as nuclear factor-erythroid 2-related factor 2 and heme oxygenase-1 via epigenetic modifications of DNA demethylation (Kang et al., 2014).

However, very little research effort has focused on elucidating the role of autophagy in acquisition of 5-FU resistance in colon cancer cells. In this study, we found that the level of autophagy was significantly lower in SNUC5/5-FUR cells than in parent type SNUC5 cells, suggesting a novel approach to overcoming troublesome chemo-resistance in colon cancer.

### MATERIALS AND METHODS

**Reagents**

5-FU, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), N-acetyl cysteine (NAC), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), and primary antibody against actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acridine orange was purchased from Invitrogen (Eugene, OR, USA). Primary antibody against Atg5 was purchased from Abgent (San Diego, CA, USA). Primary antibodies against Beclin-1 and microtubule-associated protein light chain 3 (LC3) were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals and reagents were of analytical grade.

**Cell culture**

The human colon cancer cell line SNUC5 was obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂/95% air. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml), and penicillin (100 units/ml). The SNUC5/5-FUR cell line was obtained from the Research Center for Resistant Cells of Chosun University (Gwangju, Korea) and sub-cultured twice per week in medium containing 140 µM 5-FU for more than 6 months until a stable cell line was established (Jung et al., 2007).

**Cell viability assay**

To evaluate the ability of SNUC5/5-FUR cells to resist 5-FU-induced cytotoxicity, 140 µM 5-FU was added to the medium, and the cells were incubated at 37°C for 48 h. MTT stock solution (50 µl; 2 mg/ml) was added to each well to yield a total reaction volume of 250 µl. After incubation for 4 h, the medium was aspirated. The formazan crystals in each well were dissolved in dimethyl sulfoxide (150 µl), and the absorbance at 540 nm was read on a scanning multi-well spectrophotometer (Carmichael et al., 1987).

**Acridine orange staining**

Acidic intracellular compartments were visualized by acridine orange staining. After seeding, cells were washed with phosphate buffered saline (PBS) and stained with 10 µg/ml acridine orange (Invitrogen, Madison, WI, USA) for 15 min at 37°C. Subsequently, the cells were washed with PBS and viewed under a laser scanning confocal microscope. Microscopic images were collected using the LSM 5 PASCAL software (Carl Zeiss, Jena, Germany). Depending on their acidity, autophagosomes or autolysosomes appeared as orange or red fluorescent cytoplasmic vesicles, whereas nuclei were stained green. Alternatively, acidine orange-stained cells were trypsinized, washed with PBS, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using the CellQuest Pro software (Becton Dickinson).

**GFP-LC3 transfection and detection of GFP-LC3 puncta**

Autophagy was evidenced by the formation of puncta LC3-positive structures, which are essential for the dynamic process of autophagosome formation (Wu et al., 2015). Cells were transfected with green fluorescent protein (GFP)-tagged LC3 plasmid using lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. GFP-LC3 fluorescence was imaged using a confocal microscope equipped with the laser scanning microscope 5 PASCAL program (Carl Zeiss). The number of GFP-LC3 dots was counted within each sample.

**Western blot analysis**

Cell lysates were electrophoresed, and separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibodies, followed by horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (Pierce, Rockford, IL, USA). Protein bands were detected with an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
Fig. 2. SNUC5/5-FUR cells have relatively low autophagy. (A) Cells were imaged by light microscopy after 16 h of incubation (magnification, ×400) and the vacuolated cells/twenty cells were quantified. Arrow indicates vacuolated cell. Cells were then stained with acridine orange. (B) The cells were imaged by fluorescence microscopy and the acridine orange-stained cells/twenty cells were quantified. Arrow indicates acridine orange-stained cell. (C) Also the acridine orange-stained cells were assessed by flow cytometry. FI: Fluorescence intensity. (D) Cells were harvested, and levels of Atg5, Beclin-1, and LC3-I, II were assessed by Western blotting. (E) After 24 h of transfection with GFP-LC3, cells were imaged by fluorescence microscopy, and quantified. (F) Autophagy flux was detected in bafilomycin A1 (1 μM)-treated cells after 24 h. Cells were lysed and the level of LC3 protein was analyzed by Western blotting. *Significantly different from SNUC5 cells (p<0.05).
**RESULTS**

**SNUC5/5-FUR cells are resistant to 5-FU-induced cytotoxicity**

To verify that SNUC5/5-FUR cells were resistant to 5-FU-induced cytotoxicity, we treated both SNUC5 and SNUC5/5-FUR cells with 140 μM 5-FU for 48 h, and then measured cell viability by MTT assay. In SNUC5 cells, 5-FU induced dramatic cytotoxicity, decreasing cell viability to 70%. By contrast, no lethality was observed in SNUC5/5-FUR cells treated with 5-FU (Fig. 1).

**SNUC5/5-FUR cells show relatively reduced autophagy**

Accumulation of vacuolated cells, a marker of autophagy, was diminished in SNUC5/5-FUR cells relative to parent type SNUC5 cells under light microscope (Fig. 2A). The vacuoles were positively stained with the lysosome marker dye, acridine orange. As shown in Fig. 2B, the number of acridine orange-stained vacuolated cells was lower in SNUC5/5-FUR than in SNUC5. In addition, flow cytometry revealed that the fluorescence intensity (FI) of SNUC5/5-FUR cells by acridine orange staining (FI: 120) was significantly lower than that of SNUC5 cells (FI: 189) (Fig. 2C). Autophagy is also characterized by the formation of autophagosome, which is dependent on recruitment of Atg proteins. Expression level of Atg proteins gives main information about the autophagic state of a cell. In SNUC5/5-FUR cells, expression of Atg5, Beclin-1, and LC-II, which are well-characterized hallmark of autophagy, was lower level in SNUC5/5-FUR cells than in SNUC5 cells (Fig. 2D). Transfection of cells with GFP-LC3 plasmid showed that SNUC5/5-FUR cells had lower level of puncta GFP-LC3 localization than in SNUC5 cells (Fig. 2E). It also detected autophagic flux using bafilomycin A1 (BAF), a specific autophagy inhibitor acting as autophagosomal lysosome degradation inhibition, which accumulates the LC3-II. BAF-treated SNUC5/5-FUR cells showed lower level of accumulation of LC3-II than BAF-treated SNUC5 cells (Fig. 2F). Together, this indicated that SNUC5/5-FUR cells exhibited a lower level of autophagy than SNUC5 cells.

**Detection of intracellular ROS**

The level of intracellular reactive oxygen species (ROS) was detected on a FACS Calibur flow cytometer. Twenty-four hours after treatment with 2 mM NAC, cells were loaded with 20 μM DCF-DA for 30 min at 37°C at the indicated times, and the supernatant was removed by suction. Cells were treated with trypsin and washed with PBS, and then the fluorescence of DCF-DA-loaded cells was measured on a flow cytometer using the CellQuest Pro software.

**Statistical analysis**

All measurements were made in triplicate, and all values are expressed as means ± the standard error of the mean. Results were subjected to an analysis of variance (ANOVA) followed by Tukey's post hoc test to analyze differences between conditions. p<0.05 was considered significant.

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**Fig. 3.** Inhibition of ROS attenuates autophagy in SNUC5/5-FUR cells. Cells were treated with 2 mM NAC for 24 h. (A) ROS levels were assessed by flow cytometry after DCF-DA staining. (B) Autophagy was assessed by flow cytometry after acridine orange staining. FI: fluorescence intensity. *Significantly different from SNUC5 cells (p<0.05); **significantly different from SNUC5/5-FUR cells (p<0.05).
these results indicate that SNUC5/5-FUR cells have relatively low level of autophagy, which is related to acquisition of the 5-FU-resistant phenotype.

**ROS regulate autophagy in SNUC5/5-FUR cells**

Based on several lines of evidence from basic research, ROS are considered to be key regulators of autophagy, although their roles are not fully understood (Jisun et al., 2012). ROS level was higher in SNUC5/5-FUR cells (Fi: 157) than in SNUC5 cells (Fi: 52). The higher level of ROS in SNUC5/5-FUR was markedly decreased by treatment with a well-known ROS inhibitor, NAC: upon NAC treatment, ROS level in SNUC5/5-FUR cells was decreased (Fig. 3A). Similarly, after addition of NAC, the level of Fi by acridine orange staining in SNUC5/5-FUR cells was diminished below that in untreated SNUC5/5-FUR cells (Fig. 3B). These results suggest that ROS is related to autophagy in SNUC5/5-FUR cells.

**5-FU reduces autophagy**

Previous study has investigated IC\textsubscript{50} value of 5-FU in SNUC5, a parental cell line of SNUC5/5-FUR, was 9 µM (Kang et al., 2014). So, we examined protein levels of Atg5, Beclin-1, and LC3-I, II in various concentration of 5-FU-treated SNUC5 cells. As shown in Fig. 4, the expression of Atg5, Beclin-1, and LC3-II were decreased in a dose dependent manner. These results showed that 5-FU can affect to reduce autophagy in SNUC5/5-FUR cells.

**DISCUSSION**

Due to the important status of 5-FU as chemotherapy for colorectal cancer, it is essential to overcome 5-FU resistance (Kim et al., 2015). Today, a growing body of evidence links autophagy with chemotherapy resistance, demonstrating that the ability of 5-FU to kill cancer cells is potentiated in a synergistic manner by autophagic inhibitors (Li et al., 2009; Sasaki et al., 2010, 2012). However, most previous studies ignored the role played by autophagy in 5-FU resistance arising in colon cancer cells after long-term 5-FU treatment. To investigate the function of autophagy in this context, we used a 5-FU-resistant SNUC5 colon cancer cell line, SNUC5/5-FUR.

During autophagy, double-membraned vesicles called autophagosome form in the cytoplasm to sequester cytoplasmic materials. These vesicles subsequently fuse with lysosomes to form autolysosome (Yorimitsu and Klionsky, 2005). Acridine orange is a versatile fluorescence dye that stains acidic vacuoles (i.e., lysosome, autophagosome, autolysosome) in living cells (Fan et al., 2006). The present results showed that fewer vacuoles (i.e., autophagosome) were present in SNUC5/5-FUR cells than in SNUC5 cells, indicating a lower level of autophagy in SNUC5/5-FUR cells (Fig. 2).

![Fig. 4. Reduction of autophagy by 5-FU. After 48 h of 5-FU treatment at indicated concentration, the cells were harvested and levels of Atg5, Beclin-1, and LC3-I, II were assessed by Western blotting.](image-url)

The canonical formation of autophagosomes involves four steps, initiation, nucleation, elongation, and recycling, each of which depends on specific proteins (Codogno et al., 2011). Beclin-1, together with its interaction partner Vps34, is critical for the nucleation step in mammalian cells (Glick et al., 2010). Atg5, another component of the core autophagic machinery, forms an ubiquitin-like conjugation system with Atg12 to promote the elongation step during vacuole formation (Gozaucik and Kimchi, 2004). LC3 is conjugated to phosphatidylethanolamine through an enzymatic cascade involving Atg3, Atg7, and the Atg5-Atg12 complex; the resultant lipid-conjugated form (LC3-II) is targeted to the autophagosome membrane (Kabeya et al., 2000). Thus, the level of Atg5, Beclin-1, and LC3-II can be used as specific markers of autophagy. The present results showed that SNUC5/5-FUR cells were observed lower expression level of Atg5, Beclin-1, and LC3-II and lower level of GFP-LC3 puncta than SNUC5 cells. On the basis of these findings, we preliminarily speculate that the 5-FU chemo-resistance of SNUC5/5-FUR cells is related to attenuation of autophagy.

It is widely accepted that ROS engage in a complicated interplay with autophagy and can modulate autophagic function in response to cellular stress (Dewaele et al., 2010). Accumulation of ROS produced by the mitochondria after TNF\textsubscript{α} stimulation of Ewing sarcoma cells, which are defective in NF\textsubscript{κ}B signaling, leads to the sustained activation of JNK1 and induction of autophagy (Djavaheri-Mergny et al., 2006). Moreover, as we reported previously, autophagy can be triggered by generation of ROS induced by compound K, a metabolite of ginsenoside (Kim et al., 2013). In this study, ROS production was significantly higher in SNUC5/5-FUR cells than in wild type SNUC5 cells, however, the autophagic level of SNUC5/5-FUR cells was lower than SNUC5 cells, presenting a controversial results in light of the previous reports between ROS and autophagy. And it has been reported that elimination of damaged mitochondria by autophagy leads to decreased ROS production, thereby limiting tumor-promoting effect of ROS (Morselli et al., 2011). Consequently, autophagy inhibition, following ATG5 or ATG7 deletion, leads to chronic oxidative stress, accumulation of damaged mitochondria, tissue damage and inflammation which all favor tumor initiation (Karantza-Wadsworth et al., 2007; Mathew et al., 2007; Takamura et al., 2011). In addition, the ROS inhibitor NAC significantly diminished the level of acridine orange staining in SNUC5/5-FUR cells. Therefore, we suggest that ROS may not be the key regulator of the reduction in autophagic activity that we propose is responsible for transforming wild type SNUC5 cells into drug resistant SNUC5/5-FUR cells.

In summary, our results demonstrate that a low level of autophagy in SNUC5/5-FUR cells is associated with 5-FU resistance. This finding opens the door to novel approaches for exploring the detailed mechanisms underlying chemo-resistance in cancer therapy. More extensive and comprehensive studies are needed in the future to proceed in order to achieve a better awareness and guidance in this field.
ACKNOWLEDGMENTS

This work was supported by a research grant from the Jeju National University Hospital Research Fund of Jeju National University in 2015.

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https://doi.org/10.4062/biomolther.2016.069