RESEARCH ARTICLE

Aldose Reductase Inhibitor Fidarestat as a Promising Drug Targeting Autophagy in Colorectal Carcinoma: a Pilot Study

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Abstract

Background: Colorectal cancer (CRC) is a leading cause of morbidity and mortality worldwide. Targeting autophagic cell death is emerging as a novel strategy in cancer chemotherapy. Aldose reductase (AR) catalyzes the rate limiting step of the polyol pathway of glucose metabolism; besides reducing glucose to sorbitol, AR reduces lipid peroxidation-derived aldehydes and their glutathione conjugates. A complex interplay between autophagic cell death and/or survival may in turn govern tumor metastasis. This exploratory study aimed to investigate the potential role of AR inhibition using a novel inhibitor Fidarestat in the regulation of autophagy in CRC cells. Materials and Methods: For glucose depletion (GD), HT-29 and SW480 CRC cells were rinsed with glucose-free RPMI-1640, followed by incubation in GD medium +/- Fidarestat (10μM). Proteins were extracted by a RIPA-method followed by Western blotting (35-50 μg of protein; n=3). Results: Autophagic regulatory markers, primarily, microtubule associated protein light chain (LC) 3, autophagy-related gene (ATG) 5, ATG 7 and Beclin-1 were expressed in CRC cells; glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as an internal reference. LC3 II (14 kDa) expression was relatively high compared to LC3A/B I levels in both CRC cell lines, suggesting occurrence of autophagy. Expression of non-autophagic markers, high mobility group box (HMG)-1 and Bcl-2, was comparatively low. Conclusions: GD +/- ARI induced autophagy in HT-29 and SW-480 cells, thereby implicating Fidarestat as a promising therapeutic agent for colorectal cancer; future studies with more potent ARIs are warranted to fully dissect the molecular regulatory networks for autophagy in colorectal carcinoma.

Keywords: Aldose reductase - autophagy - colorectal cancer - drug - fidarestat - therapeutics

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Introduction

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality in the United States; it is the third most common cancer in men and the second in women worldwide (Ferlay et al., 2010). Gastrointestinal (GI) malignancies account for almost one-fourth of cancer-related deaths in the United States and ~30% of all cancer-related deaths worldwide (Desai et al., 2013). Aldose Reductase (AR), initially thought to be involved in secondary diabetic complications, catalyzes the rate-limiting step of the polyol pathway of glucose metabolism (Pandey et al., 2012). Human AR is a monomeric protein of 36 kDa (315 amino acids); it is encoded by AKR1B1 gene mapped at chromosome region 7q35; recent evidence indicates that AR is an excellent reducer of a number of lipid peroxidation-derived aldehydes and their glutathione conjugates, which regulate inflammation and/or subsequent carcinogenesis Aldose Reductase Inhibitor (ARI)-targeted therapies are currently being evaluated in Phase I/II/III studies for diabetes; however, they have limited efficacy and/or unfavorable adverse effects (Pandey et al. 2012). Interestingly, Fidarestat, a novel ARI, appears to be a promising therapeutic target with high specificity and minimal side effects. Autophagy is emerging as an important biological mechanism in targeting human cancers, including colorectal cancer (Pandey and Chandravati, 2012). Autophagy is a process of cytoplasmic and cellular organelle degradation in lysosomes that has been implicated in homeostasis; the metabolic turnover/autophagic flux varies depending on the specific cell and/or tissue type, thereby tilting the cell's fate under stress conditions from survival to death (Chen and Klionsky, 2011). Autophagy ("self-eating") was first described by Christian de Duve in 1963 as a lysosome-mediated degradation process for non-essential or damaged cellular constituents (de Duve, 1963; de Duve, 1966). Microtubule-associated protein light chain 3 (LC3) protein (a mammalian homologue of yeast Atg8) is localized in autophagosomes and autolysosomes after processing; moreover, the amount of LC3-II cleaved product is correlated with the extent of autophagosome

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formation, thereby providing the first molecular marker for detection of autophagic activity (Kabeya et al., 2004; Tanida et al., 2004). Autophagy initiation is centrally regulated by Beclin-1 (mammalian homologue of yeast atg6) (Lee et al., 2012). In this regulatory framework, several mammalian homologues of yeast autophagy-related genes (Atgs) have been identified that participate in the Atg12-binding system and LC3- modifying system during the formation of autophagosome; Atg12 is initially conjugated to Atg5 immediately after its synthesis; this process is regulated primarily by the E1 ligase-like protein Atg7 (Behrends et al., 2010). The Atg12/Atg5 complex thereby leads to the formation of larger protein complexes that are transported onto the membrane, which is necessary for the formation of autophagic vesicles (Kabeya et al., 2004; Tanida et al., 2004). Phosphatidylethanolamine (PE), a lipid molecule considered to anchor Atg8/LC3-II to membranes, is a critical element of LC3-modifying system; eventually, Atg8/LC3-I (mammalian MAP/LC3 proteins) is ligated to PE in a sequential biochemical reaction assisted by Atg7, further transforming into Atg8/LC3-II and ultimately leading to the formation of autophagic vacuoles (Dengjel et al., 2008). A complex interplay between cell death and/or survival including necrosis, apoptosis and autophagy may in turn govern tumor metastasis, and subsequent carcinogenesis.

Inflammation is a known hallmark of cancer; vascular insufficiency in tumors may lead to depletion of glucose and/or oxygen and thus contribute to increased reactive oxygen species production, extracellular acidosis in tumor microenvironment eventually resulting in autophagy, thereby implicating glucose depletion and or deprivation (GD) as a crucial trigger for autophagy (Bensaad et al., 2009; Du et al., 2013; Shoji-Kawata et al., 2013). My study hypothesis was that AR inhibition regulated autophagy in colorectal carcinoma. To investigate how AR inhibition may regulate autophagy in colorectal cancer, I conducted a pilot study examining the effect of ARI Fidarestat in human colon cancer cell lines HT-29 and SW-480 under basal as well as glucose-deprived conditions, determined the expression of regulatory markers in autophagy primarily LC3-I, LC3-II, Beclin-1, Atg5, Atg7 and Bcl-2. The present pilot study provides novel insights into the potential mechanisms underlying colorectal carcinoma using enzyme inhibitors, such as ARI; my findings may be extrapolated and/or replicated in other clinically relevant models of GI/hepatobiliary malignancies, so as to reduce the burden of hepatic diseases in populations worldwide.

Materials and Methods

Reagents and Antibodies: McCoy's 5A growth media, RPMI 1640, PBS, Penicillin/Streptomycin, Trypsin, and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Antibodies against LC3, Beclin-1, Atg5, Atg7 and Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA); antibodies against HMG1and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

ARI Fidarestat was a kind gift from Sanwa Kagaku Kenkyusho Co., Ltd. (Nagoya, Japan). Reagents used in western blot were purchased from Sigma (St. Louis, MO); moreover, all reagents were of analytical grade.

Cell culture: Human colorectal cancer HT29 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and grown to confluence in McCoy's 5A basal media supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37°C under humidified atmosphere containing 5% CO_2.

Human colorectal adenocarcinoma SW480 cells (ATCC) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin solution, 2 mM/L L-glutamine, 10 mM/L HEPES, 1 mM/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate.

For glucose deprivation, HT-29 and SW480 colorectal cancer cells were rinsed with glucose-free RPMI-1640, followed by incubation in GD medium in presence and/or absence of AR inhibitor Fidarestat (10μM). It was strictly ensured that the cells in culture were free from any suspected mycoplasma and/or endotoxin contamination prior to conducting cell-treatments and protein extraction as part of the current exploratory study.

Western blot: To examine the expression of LC3, Beclin-1, Atg5, Atg7, Bcl-2, HMG1 and GAPDH proteins, western blot was performed. Equal amounts of protein from HT-29 and SW-480 cell extracts were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of proteins to nitrocellulose filters and probing with the indicated antibodies. The antigen-antibody complex was detected by enhanced chemiluminescence (Pierce, Piscataway, NJ). All blots were probed with either GAPDH as a loading control, and fold-change was determined using Kodak Image station 2000R (Eastman Kodak Co., Rochester, NY).

Results

Expression of Autophagic markers: GD +/- ARI Fidarestat (10 μM) induced autophagy in HT-29 and SW-480 cells. Expression of autophagic markers LC3, Atg5, Atg7 and Beclin-1 were observed (Figures 1a, 1b and 1c); the relative fold changes in expression levels have been depicted in the figure. Housekeeping protein GAPDH was used as an internal reference. Our data indicates that autophagy flux/metabolic activity in the cell under GD-conditions may be altered, wherein the conversion of the two known isoforms of LC-3A/B, I and II, may be monitored. LC3 II (14 kDa) expression was relatively higher compared to LC3A/B I expression levels in both cell lines, thereby strongly suggesting the initiation and/or occurrence of autophagy under nutrient-starved conditions. Interestingly, LC3 expression (LC3 I as well as II) was relatively stronger in SW-480 cells; this differential expression of the LC3 autophagic marker may be attributed to the different/varying nature of the two CRC cells, viz. SW-480 and HT-29; SW-480 being adenocarcinoma type. However, stimulation of both cell types with the inhibitor Fidarestat (10 μM) did not reveal any marked significant difference in the expression levels

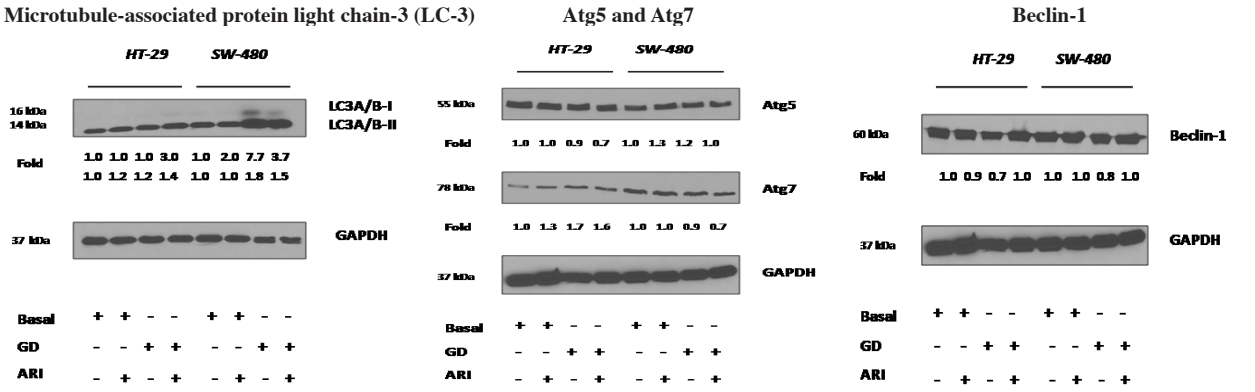

Figure 1. 1A). Autophagic Marker LC-3 Expression in CRC Cells; 1B). Atg5 and Atg7 Expression in CRC Cells; Beclin-1 Expression in CRC Cells

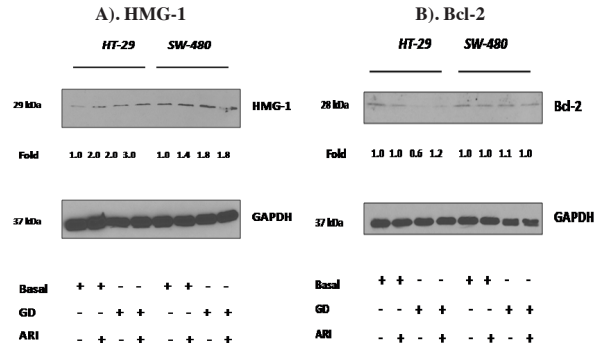

Figure 2. A). Non-autophagic Marker HMG-1 Expression in CRC Cells; B). Non-autophagic Marker Bcl-2 Expression in CRC Cells

of the various autophagic markers, in my experimental *in vitro* system.

Expression of Non-Autophagic markers: To further investigate the occurrence of other form(s) of cell death in my *in vitro* system, I studied the relative expression of necrotic as well as apoptotic marker; it may be noted that autophagy may be inter-related with these conventional cell death pathways, and accordingly the interplay between the components of the 3 cell death pathways, viz., necrosis, apoptosis and autophagy, may in turn govern the fate of the cancerous cell under physiologically stressed conditions, e.g., hypoxia, low nutrient supply, etc., and accordingly the concerned cell type may be predisposed to undergo cell death.

GD +/- inhibitor Fidarestat (10 μM) - treated HT-29 and SW-480 cells revealed the expression of non-autophagic markers high mobility group box 1/HMG1 (necrotic marker) and Bcl-2 (apoptotic marker) (Figure 2a and 2b). However, the expression level(s) of HMG1 and Bcl-2 were not observed to be strong, thereby indicating the relative lower occurrence of non-autophagic forms of cell death in my study. I wish to clearly state that all my experiments were performed in triplicate (n=3), and a representative blot of three independent experiments has been depicted; I used 35-50 μg of protein in my experiments after rigorous initial standardization procedures and used GAPDH to ensure equal loading and thus avoid any experimental error(s) that may possibly lead to misinterpretation of the data while communicating the original findings.

Discussion

The present expression-based brief communication implicates the potential involvement of AR inhibition using Fidarestat in autophagic cell death in colorectal carcinoma. The etiopathogenesis of GI/hepatobiliary malignancies, including colorectal cancer is indeed complex; therefore, a precise molecular dissection of the biochemical pathway(s) regulating carcinogenesis is essential. Autophagy has a pivotal role in defense against microbial infection, neurodegenerative diseases, carcinomas as well as ageing (Srivastava et al., 1995). AR appears to be an important metabolic route for the detoxification of lipid-derived aldehydes (Bhatnagar et al., 1994; Vander Jagt et al., 1995; Srivastava et al., 2000); it is an essential catalyst for the reduction of medium- to long-chain unbranched saturated and unsaturated aldehydes, and their conjugates with glutathione (GSH) (Wang et al., 2011). Inhibiting AR may be efficacious in preclinical models; furthermore, a few ARIs, including Fidarestat, have already undergone clinical trials for diabetic complications. Recent reviews by Pandey et al. (Pandey et al., 2012; Pandey and Chandravati, 2012) provide a more comprehensive understanding of the emerging role(s) of autophagy and ARIs in inflammatory settings, especially human cancers. A novel ARI Fidarestat has been implicated as a pivotal player in inflammatory pathologies (Pandey et al., 2012).

Targeting the complex autophagic machinery with novel agents, such as ARIs, may have long-term clinically relevant therapeutic potential in human diseases, including CRC. Preliminary findings of the present study strongly implicate the potential involvement of ARI Fidarestat in autophagic cell death in colorectal carcinoma. As LC3 protein is an established hallmark of autophagy in diverse cell types (Zhang et al., 2011; Liu et al., 2012), I first investigated whether autophagy was triggered in CRC cell lines HT-29 and SW-480 under normal/basal versus physiologically stressed i.e. nutrient-starved GD conditions by observing the relative expression levels of LC-3. Autophagy indeed occurred in my experimental conditions as demonstrated by the expression of LC3; however, Fidarestat did not alter the relative protein expressions of LC3 in my culture/experimental conditions.

I then explored whether the expression level(s) of other major components of the autophagic machinery could be altered in presence and/or absence of ARI Fidarestat (10 μM); Atg 5, Atg 7 and Beclin1 proteins were expressed under basal as well as GD conditions, highlighting the occurrence of the non-apoptotic mode of cell death i.e. autophagy in CRC cells.

Autophagy is an evolutionarily conserved pathway with diverse roles in carcinogenesis as well as cancer therapy (Fels et al., 2008); it may inhibit the initiation of tumor formation in the vasculature by limiting cytoplasmic damage, genomic instability and inflammation; functional loss of autophagy genes may in turn predispose the cell/biological system towards cancer. Hypoxia and vascular insufficiency in the necrotic core of malignant and/or cancerous cells of the tumor microenvironment contribute to drug resistance and subsequent cancer progression (Kreuzaler et al., 2012). As there may be a possibility of cross-talk between autophagy and other inter-related pathways such as apoptosis and necrosis in regulating colorectal cancer initiation, subsequent metastasis and cancer progression, I further decided to investigate the expression of non-autophagic markers, primarily Bcl-2 and HMG1, that are known markers for studying apoptosis and necrosis under altered physiological mileu, respectively; the relative expression levels of these apoptotic and necrotic mediators/markers was lower than the expression of autophagic markers in presence and/or absence of Fidarestat under basal and GD conditions.

These preliminary findings strongly suggested the role of GD-triggered autophagy in my study. Moreover, cell death-related research in the past decade has substantially enhanced my current understanding of non-apoptotic programmed cell death events, primarily lysosomal-mediated cell death, necroptosis and autophagy (Amravadi, 2009; Tengku Din et al., 2014) and the complex cross-talk among diverse components of each of these cell death pathways further regulates subsequent cancer progression under stressed/physiologically altered conditions.

The emerging role of autophagy has been well demonstrated in colorectal carcinoma in recent times (Yang et al., 2011; Shi et al., 2012; Han et al., 2014). Distinct patterns of expression of autophagy related proteins, viz. Atg 5, Beclin 1 and LC3 have been related to prognosis and tumorigenesis in colonic mucosa (Cho et al., 2012; Groulx et al., 2012). Thus, identification of novel compounds and/or inhibitors, appears to be a logical idea for dissecting the molecular complexities associated with colorectal carcinoma. Suppression of cell proliferation by induction of apoptosis and autophagy in human colorectal cancer cells has been demonstrated by a novel compound C, AMPK inhibitor, and proteasome inhibitor (Wu et al., 2012; Yang et al., 2012). Cetuximab efficacy in CRC has been predicted by autophagy related proteins LC3 and Beclin1 (Guo et al., 2011). Autophagy is an important contributor to cell death when combined with targeted therapy. Moreover, nutrient deprivation is one of the essential triggers of autophagic cell death in malignant/cancerous cells in the colonic epithelia (Sato et al., 2007; Koukourakis et al., 2010). However, it should be noted that the specificity and binding affinity of a particular inhibitor, including ARI Fidarestat, may vary in different stress/altered metabolic conditions in different cell types, including HT-29 and SW-480, and accordingly the metabolic flux in the tumor microenvironment may be altered, thereby tilting the cell's fate from survival to death.

To conclude, it may be suggested that inhibition of AR may be a clinically relevant therapeutic strategy for regulating inflammation, and novel AR inhibitors manipulating autophagy-mediated signaling may prove efficacious in treating patients with inflammatory diseases, including colorectal cancer. Therefore, identifying novel anti-cancer agents that specifically activate or inhibit autophagy by targeting regulatory molecules of the complex autophagic pathway may further enhance our understanding of the complex etiology of colorectal carcinogenesis and other GI/hepatobiliary ailments in disease-susceptible populations worldwide. My exploratory study findings in ARI-mediated autophagy may be replicated in other hepatobiliary malignancies, including hepatocellular carcinoma.

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