Down-regulation of Mitochondrial F1F0-ATP Synthase in Human Colon Cancer Cells with Induced 5-Fluorouracil Resistance

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Abstract

5-Fluorouracil (5-FU) is widely used for treatment of advanced colorectal cancer. However, it is common for such patients to develop resistance to 5-FU, and this drug resistance becomes a critical problem for chemotherapy. The mechanisms underlying this resistance are largely unknown. To screen for proteins possibly responsible for 5-FU resistance, cells resistant to 5-FU were derived from human colon cancer cell lines and two-dimensional gel electrophoresis–based comparative proteomics was done. Two-dimensional gel electrophoresis data showed there was lower expression of the α subunit of mitochondrial F1F0-ATP synthase (ATP synthase) in 5-FU–resistant cells compared with parent cells. Western blotting showed that expression of other ATP synthase complex subunits was also lower in 5-FU–resistant cell lines and that these resistant cells also showed decreased ATP synthase activity and reduced intracellular ATP content. The ATP synthase inhibitor, oligomycin A, strongly antagonized 5-FU–induced suppression of cell proliferation. When 5-FU sensitivity was compared with ATP synthase activity in six different human colon cancer cell lines, a positive correlation has been found. Furthermore, suppressed ATP synthase α-subunit expression by siRNA transfection increased cell viability in human colon cancer cells and indicates a link between mitochondrial F1F0-ATP synthase (ATP synthase) and thymidylate synthesis de novo (11). Understanding 5-FU resistance mechanisms at a molecular level seems essential to design strategies to overcome this resistance.

Introduction

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic treatments for advanced colorectal cancer patients and is still considered a mainstay of therapy (1). However, 5-FU produces major responses in only about 10% of such patients (1). Many attempts to enhance its therapeutic effectiveness (2, 3) have resulted in only minor improvements in patient survival (4, 5). A common critical issue for patients responding to 5-FU is the development of resistance to the drug, which has become a major obstacle in chemotherapy (6, 7).

Numerous efforts have been made to identify mechanisms underlying 5-FU resistance (8–10). Some mechanisms seem to involve thymidylate synthase, which is crucial in de novo synthesis of thymidylate (11). Understanding 5-FU resistance mechanisms at a molecular level seems essential to design strategies to overcome this resistance.

To identify proteins involved in 5-FU resistance, we did comparative proteomics using two-dimensional gel electrophoresis to screen proteins from human colon cancer cell lines with induced 5-FU resistance. In the present study, we found that mitochondrial F1F0-ATP synthase (ATP synthase) was down-regulated in 5-FU–resistant human colon cancer cells. ATP synthase is a complex of 16 different subunits, with α3β3γδε and comprising the F1 component, and α, b, c, d, e, f, g, A6L, oligomycin sensitivity-conferring protein (OSCP), and coupling factor 6 comprising the F0 and stator (12, 13). Also associated in the complex under some conditions is an intrinsic inhibitor protein F1I (14, 15).

Recently, down-regulation of ATP synthase β-subunit expression has been reported in liver, kidney, colon, squamous oesophageal and lung carcinomas, as well as in breast and gastric adenocarcinomas (16–18). These findings provided compelling evidence that bioenergetic dysfunction of mitochondria is a hallmark of these types of cancers, which is consistent with the Warburg hypothesis (19). In the present study, we show that altered mitochondrial bioenergetic features are also a characteristic of 5-FU–resistant human colon cancer cells and indicate a link between mitochondrial ATP synthase and 5-FU resistance.

Materials and Methods

Human colon cancer cell lines and establishment of 5-fluorouracil–resistant cell lines. Human colon cancer cell lines, SNU-81, SNU-769A, SNU-769B, SNU-1033, SNU-C4, and SNU-C5 (20, 21), were obtained from the Korean Cell Line Bank (Seoul, Korea). Three individual cell lines resistant to the anticancer agent 5-FU (Choongwae Pharma Corporation, Gyeonggi, Korea) were derived from SNU-C4, SNU-769A, and SNU-769B cells as previously described (22).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A colorimetric assay using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was used to assess suppression of cell proliferation by 5-FU (Dong-A Pharmaceutical Co. Ltd., Seoul, Korea) or ATP synthase inhibitors, oligomycin A and aurovertin B (Sigma, St. Louis, MO). Single-cell suspensions were prepared and cell density was measured. MTT assays were done as previously described (22). Briefly, an equal number of cells was added into each well in 0.18-mL culture medium, to which 0.02 mL of 10× normal concentration of drug or PBS (for untreated 100% survival control) was added. After 4 days of culture, 0.1 mg of MTT was added to each well and incubated at 37°C for a further 4 hours. Plates were centrifuged at 450 × g for 5 minutes at room temperature and the medium removed. DMSO (0.15 mL) was added to each well to solubilize the crystals and the plates were immediately read at 540 nm using a scanning multwell spectrometer (Bio-Tek Instruments, Winooski, VT).
Inc., Burlington, VT). All experiments were done thrice and the mean and SD of the IC_{50} (µg/mL) values were calculated.

Two-dimensional gel electrophoresis–based comparative proteomics. Two-dimensional gel electrophoresis analysis was done as previously described (22). Briefly, 0.15-mg protein sample was applied to 13-cm immobilized (pH 3-10) nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). Proteins were focused at 8,000 V within 3 hours. The second-dimension separation used 12% polyacrylamide gels (chemicals from Serva, Heidelberg, Germany and Bio-Rad, Hercules, CA). Two-dimensional gel electrophoresis gels were stained with Colloidal Coomassie Blue (Invitrogen, Carlsbad, CA) for 24 hours and then destained with deionized water.

Melanie 4 software (Swiss Institute of Bioinformatics, Geneva, Switzerland) was used for image analysis of two-dimensional gel electrophoresis gels. Proteins showing abnormal expression were subjected to matrix-associated laser desorption ionization-mass spectroscopy (MALDI-MS) analysis for identification.

MALDI-MS analysis of two-dimensional gel electrophoresis protein spots was done as previously described (22). Briefly, pieces of gel containing protein spots of interest were excised, destained with 50% acetonitrile in 0.1 mol/L ammonium bicarbonate, and dried in a Speedvac evaporator. The dried gel pieces were reswollen with 3 µL of 3 mmol/L Tris-HCl (pH 8.8) containing 50 ng trypsin (Promega, Madison, WI) and, after 15 minutes, 3 µL of water were added. One microliter was applied to the dried matrix spot. The matrix consisted of 15% mg nitrocellulose (Bio-Rad) and 20% mg α-cyano-4-hydroxycinnamic acid (Sigma) dissolved in 1-mL acetonitrile/isopropanol (1:1, v/v). Matrix solution, 0.5 µL, was applied to the sample, and samples were then analyzed using a QSTAR XL Hybrid liquid chromatograph/ternary mass spectrometry system (Applied Biosystems, Foster City, CA) with a 20-kV accelerating voltage. Peptide masses were matched with theoretical peptide masses of all proteins from all species in the SWISS-PROT database.

Mitochondria subcellular fractionation and measurement of mitochondrial inner membrane potential. For isolation of an enriched, functional mitochondrial fraction from cells, a “Mitochondria isolation kit” (Sigma) was used as recommended by the manufacturer. The fluorescent carbocyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carboxyanine iodide) was used to measure the mitochondrial inner membrane potential as it is actively taken up by respiring mitochondria.

Enzyme activity assays. Mitochondrial fractions were used for determination of mitochondrial enzyme activity (i.e., ATP synthase and serine hydroxyethyltransferase activity). ATP synthase activity, measured in the direction of ATP hydrolysis (ATPase activity), was assayed using the continuous spectrophotometric assay of Rosing et al. (23), except that 2 mmol/L Tris (pH 8.0), 3.0 mmol/L DTT, 0.25 mmol/L MgCl_{2}, 2 mmol/L ATP, 2 mmol/L EGTA, 1 mmol/L KCN with 0.1 mmol/L NADH, 5 units/mL pyruvate kinase, and 5 units/mL lactate dehydrogenase (Sigma). The total volume in the cuvette was 1 mL. The blank cuvette contained water. The linear reaction was followed for 2 minutes at 340 nm at 25°C. One unit of activity was defined as that required for oxidation of 1 µmol NADH min^{-1} mg^{-1} at 25°C, pH 7.4, in the conditions specified above.

Serine hydroxyethyltransferase was assayed according to a modified procedure described by Geller and Kotb (24). A complete assay reaction mixture contained 50 µmol/L Tris (pH 8.0), 3.0 µmol/L DTT, 0.25 mmol/L pyridoxal phosphate, 2.5 mmol/L EDTA, 2.0 mmol/L tetrahydrofolic acid (Sigma), 0.4 mmol/L L-serine containing 1-[^3]H-serine (Amersham Biosciences), and mitochondrial fraction (enzyme source). Weighed amounts of tetrahydrofolic acid were added to the assay mixture immediately before starting the incubation with L-serine/[^3]H-serine (Amersham Biosciences), and mitochondrial fraction (enzyme source). Weighed amounts of tetrahydrofolic acid were added to the assay mixture immediately before starting the incubation with L-serine/[^3]H-serine. The reaction was carried out at 37°C and stopped by freezing a 25-µL aliquot of the assay mixture onto a 2.3 cm in diameter disc of DE-81 (Whatman) filter paper. The filter was washed five times with 15 mL distilled water and transferred to a scintillation vial containing 10 mL scintillation cocktail for liquid scintillation counting. One unit of activity was defined as 1 pmol of hydroxyethyl group of serine transferred to tetrahydrofolic acid per-minute per-milligram at 37°C, pH 8.0, in the conditions specified above.

Glyceralddehyde-3-phosphate dehydrogenase (GAPDH) activity was determined as previously described (25). Briefly, cell protein extract was added to a reaction mixture containing 10 mmol/L sodium pyrophosphate (pH 8.5), 20 mmol/L sodium phosphate, 0.25 mmol/L NAD, and 3 µmol/L DTT. The reaction was initiated by addition of glyceralddehyde-3-phosphate (substrate) and was allowed to proceed for 5 minutes. Activity of GAPDH was determined by formation of NADH as measured by a gain in absorbance at 340 nm.

Measurement of intracellular ATP. Intracellular ATP content was measured using an ATP Bioluminescence Assay Kit HS II (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer.

Western blot analysis. Western blot analysis was done as previously described (22). Briefly, 4,000 × g supernatants of cell homogenates containing equivalent amounts of protein were subjected to SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), which were blocked by incubating overnight at 4°C in 1% Tween 20-TBS buffer containing 1.5% nonfat dry milk (Bio-Rad, Richmond, CA) and 1 mmol/L MgCl_{2}. Membranes were incubated for 2 hours at room temperature with primary antibody against either ATP synthase α subunit (BD Biosciences, San Jose, CA), β subunit, d subunit, OSCP subunit, IF1 (Molecular Probes, Eugene, OR), Hsp60, poly(ADP-ribose) polymerase (Abcam, Cambridgeshire, United Kingdom), or actin (Sigma). Membranes were washed 3 × 15 minutes with blocking solution and incubated with diluted horseradish peroxidase–conjugated secondary antibody (SouthernBiotech, Birmingham, AL) for 1 hour at room temperature. Membranes were then washed again (3 × 15 minutes) with blocking solution and incubated with WEST-ZOL (plus) chemiluminescence reagent (INBRON Biotechnology, Seoul, Korea) for 1 minute and exposed to film (Kodak Blue XB-L, Rochester, NY).

Fluorescence-activated cell sorting assay. Cells were stained with propidium iodide (Sigma) and analyzed using a FACScan machine (BD Biosciences). Fluorescence-activated cell sorting (FACS) analysis was done as previously described (26). The percentage of propidium iodide–positive cells was quantified.

siRNA synthesis and transfection. The following target sequences were used to generate siRNAs (Qiagen, Chatsworth, CA): 5′-TAAATAATTATACGTTAA-3′ (nucleotides 607-625 of Genbank accession no. NM_006356) for ATP synthase d subunit and 5′-ATTTCTCGAATGGTCACTG-3′ for nonsilencing control. The synthetic siRNA duplexes were transfected to SNU-81 cells via electroporation. Briefly, subconfluent monolayers of SNU-81 cells were detached from the culture dishes by trypsin treatment, washed with PBS, and adjusted to a concentration of 10 × 10^6 cells/mL of OPTI-MEM I culture medium (Invitrogen). Then, 0.3 mL of the cell suspension (3 × 10^6 cells) was mixed with siRNA (final concentration, 5 µmol/L) by gentle pipetting, transferred to a 0.4-cm-wide electroporation cuvette (Bio-Rad), and subjected to an electric pulse at 0.9 mF and 170 V using a Gene Pulser (Bio-Rad).

Statistical analyses. Between-group differences were calculated using the nonparametric Mann-Whitney U test and within-group correlations were calculated using the Spearman rank coefficient. Significance was set at the P < 0.05. Antagonism was assessed by the method of Chou and Talalay (27) using a nonfixed ratio experimental design and the CalcuSyn software (Biosoft, Ferguson, MO). A combination index value >1.0 indicates antagonism.

Results.

Down-regulation of ATP synthase in human colon cancer cell lines with induced 5-fluorouracil resistance. 5-FU–resistant cell lines were established from the human colon cancer cell lines SNU-C4, SNU-769A, and SNU-769B. The 5-FU IC_{50} values for both resistant and parent cell lines are shown in Table 1.

Whole cell protein extracts from SNU-C4 and its 5-FU–resistant derivative line, SNU-C4R, were analyzed using two-dimensional gel electrophoresis (Fig. 1A). A protein spot with a molecular size of 70 kDa and pI 10.0 was observed to be expressed at lower levels in SNU-C4R cells compared with the parent cell line (Fig. 1A). The spot was excised from the two-dimensional gel.
Down-regulation of ATP synthase α subunit in SNU-C4R cells was confirmed by Western blot analysis (Fig. 2A). Protein levels of other subunits comprising the ATP synthase complex were also determined. Like the α subunit, the d subunit, OSCP, and the endogenous inhibitor protein of ATP synthase, IF1, were at lower levels in SNU-C4R cells compared with parent cells. Expression of the β subunit and the mitochondrial marker protein Hsp60 was similar in both lines (Fig. 2A).

Consistent with down-regulation of ATP synthase subunits, both ATP synthase activity, normalized to mitochondrial serine hydroxymethyltransferase, and intracellular ATP content were significantly decreased in SNU-C4R cells compared with parent cells (both \( P = 0.0286 \); Fig. 2B and C). In contrast, neither mitochondrial membrane potential nor GAPDH activity was altered (Fig. 2B, right, and D).

Using Western blotting, we investigated expression of ATP synthase subunits in other human colon cancer cell lines with induced 5-FU resistance. We found that expression of α, β, d, and OSCP subunits was lower in both SNU-769AR and SNU-769BR cells compared with their respective parent cells (Fig. 3). IF1 and Hsp60 expression was the same in both resistant and parent cells (Fig. 3).

5-Fluorouracil suppression of cell proliferation is overcome by ATP synthase inhibitors. We investigated whether cell proliferation was affected by the ATP synthase inhibitors oligomycin A and aurovertin B. Cells were incubated with an ATP synthase

| Table 1. 5-FU IC\(_{50}\) values for human colon cancer cell lines and their derivative 5-FU–resistant cell lines |
|-----------------------------|----------------|-----------------|
| **Cell lines**               | IC\(_{50}\) (µg/mL)* | Relative resistance† |
| SNU-C4                      | 0.93 ± 0.06      | 43.18           |
| SNU-C4R (5-FU resistant)    | 40.16 ± 29.93    | 25.30           |
| SNU-769A                    | 0.42 ± 0.08      |                 |
| SNU-769AR (5-FU resistant)  | 10.63 ± 1.80     | 14.10           |
| SNU-769B                    | 0.05 ± 0.03      |                 |
| SNU-769BR (5-FU resistant)  | 0.71 ± 0.37      |                 |

*IC\(_{50}\) is defined as the drug concentration that produced a 50% reduction in 540-nm absorbance compared with untreated controls in MTT assays.
†Fold changes in 5-FU IC\(_{50}\) in resistant cells compared with parent cells.
‡Higher resistance has been induced from 5-FU–resistant SNU-769A cells used in our previous study (45).

**Figure 1.** Expression of the mitochondrial ATP synthase α subunit is lower in human colon cancer cells with induced 5-FU resistance. A, two-dimensional gel electrophoresis gels of protein extracts from parental SNU-C4 cells and their 5-FU–resistant derivative cells, SNU-C4R. Typical electrophoretic patterns are shown. Enlarged partial gel images and three-dimensional protein spot images highlight a 70-kDa protein with p\( \_I \) 10.0. B, MALDI-MS analysis. The protein was identified as the α subunit of mitochondrial F\(_1\)F\(_0\)-ATP synthase (ATP synthase). Theoretical and approximate observed Mr, and pl values, as well as the number of matching and total peptides, are given.
inhibitor for 4 days, after which cell proliferation was determined by MTT assay. We found that both ATP synthase inhibitors dose-dependently inhibited proliferation in both SNU-C4 and SNU-C4R cells, with dose-response being similar for both resistant and parent cells (Fig. 4A). Aurovertin B at 0.1 μmol/L failed to suppress proliferation of either SNU-C4 and SNU-C4R cells (Fig. 4A).

The 5-FU concentrations suppressing cell proliferation by 50% and 80% were determined and defined as IC50 and IC80, respectively. SNU-C4 and SNU-C4R cells were incubated at the IC50 or IC80 for 5-FU in the presence of either oligomycin A or aurovertin B in the range 0.1 to 1 μmol/L, and proliferation was monitored (Fig. 4B). In all cases, the combination index values between 5-FU and ATP synthase inhibitor were greater than 1 (data not shown). These results show strong antagonism between ATP synthase inhibitor and 5-FU on suppression of cell proliferation. In the presence of 5-FU IC80, both SNU-C4 and SNU-C4R cells treated with either 0.1 or 1.0 μmol/L oligomycin A showed significantly increased proliferation compared with cells treated with 5-FU alone (Fig. 4B, bottom left; ***p = 0.0003 and **p = 0.00222, respectively, for SNU-C4 cells, both ***p = 0.0002 for SNU-C4R cells). A similar effect was also observed when 5-FU-resistant cells were incubated with 0.1 μmol/L aurovertin B at 5-FU IC50 (Fig. 4B, top right; *p = 0.0028). In parallel with the above proliferation experiments, apoptosis was examined in SNU-C4 parent cells incubated with 0.1 μmol/L oligomycin and 5-FU IC80. FACS analysis of propidium iodide–stained nuclei to assess the sub-G1 fraction and Western blot analysis for poly(ADP-ribose) glycohydrolase (GAPDH) activity in SNU-C4 and SNU-C4R cells. Columns, mean of three independent experiments; bars, SD.

Figure 2. Mitochondrial ATP synthase is dysregulated in SNU-C4R cells. A, dysregulated expression of ATP synthase complex protein subunits. Levels of mitochondrial ATP synthase subunits were determined in SNU-C4 cells and their derivative 5-FU-resistant SNU-C4R cells using Western blot analysis. Protein levels were normalized using the mitochondrial marker, Hsp60. B, decreased ATP synthase activity in SNU-C4R cells. SNU-C4 and SNU-C4R cells were assayed for ATP synthase activity, mitochondrial serine hydroxymethyltransferase (SHMT) activity, and mitochondria membrane potential. C, intracellular ATP content. D, GAPDH activity in SNU-C4 and SNU-C4R cells. Columns, mean of three independent experiments; bars, SD.

Figure 3. Expression of mitochondrial ATP synthase complex protein subunits is decreased in human colon cancer cell lines with induced 5-FU resistance. Western blot analysis was done to investigate expression of mitochondrial ATP synthase complex subunits in SNU-769AR and SNU-769BR 5-FU–resistant cells.
polymerase cleavage showed that oligomycin had no antagonistic effect on 5-FU–induced apoptosis (Fig. 4C).

**Positive correlation between ATP synthase expression and 5-fluorouracil sensitivity in human colorectal cancer cell lines.** Western blot analysis was done to determine levels of each ATP synthase subunit in six different human colon cancer cell lines (Fig. 5A). We found that in each line, levels of all subunits except IF1 positively correlated with each other; the expression of IF1 did not correlate with any other subunit (Fig. 5F).

We compared 5-FU sensitivity and ATP synthase complex expression and activity in six different human colon cancer cell lines. We found that 5-FU sensitivity positively correlated with ATP synthase subunit expression and with ATP synthase activity (both \( P = 0.0028 \); Fig. 5C, bottom, and D). Although expression of the ATP synthase subunit also seemed to show a positive correlation with 5-FU sensitivity, this correlation did not quite reach the level set for statistical significance (\( P = 0.0583 \); Fig. 5C, top). Neither mitochondria membrane potential nor intracellular ATP content correlated with ATP synthase activity (Fig. 5F).

**Effect of suppressed ATP synthase subunit expression on 5-fluorouracil sensitivity.** To verify a causative role of ATP synthase for 5-FU resistance, SNU-81 cells were transfected with siRNA specific to ATP synthase d subunit and cell viability was determined in the presence of 5-FU. The expression of ATP synthase d subunit was efficiently suppressed at 24 hours after transfection with ATP synthase d-subunit siRNA, and this suppression was continued up to 72 hours regardless of the presence of 5-FU (100 \( \mu \)g/mL; Fig. 6A). However, the expression of mitochondrial marker Hsp60 was not affected by transfection of siRNA (Fig. 6A). When cell viability was determined at 72 hours after transfection (48 hours after 5-FU treatment), cells transfected with ATP synthase d-subunit siRNA showed higher viability in various concentrations of 5-FU (0.1, 1.0, 10 or 100 \( \mu \)g/mL) compared with cells transfected with either nonsilencing control siRNA or PBS buffer (Fig. 6B).

**Discussion**

Mitochondria play a pivotal role in providing energy for cells and in execution of apoptosis (28–30). However, the role of mitochondria in cancer is not clear. In 1930, Warburg (19) proposed the hypothesis that cancer cells may have impaired mitochondrial function and that this alteration would result in an elevated rate of glycolysis. Glycolytic characteristics of cancer cells have been studied at both biochemical and molecular levels (31, 32) but impaired mitochondrial function is not established in cancer biology (33). Recently, Warburg’s hypothesis was supported by studies from Cuezva and coworkers (16–18) which provided two alternative pathways by which cancer cells down-regulate mitochondrial activity (16). In liver cancer there seems to be a general reduction in mitochondrial components, consistent with repressed mitochondrial proliferation (34). In contrast, in kidney, colon, squamous oesophageal and lung carcinomas, as well as in lung, breast and gastric adenocarcinomas, expression of the \( \beta \) subunit of ATP synthase was lower, consistent with selective repression of the expression of components involved in mitochondrial bioenergetic function (16–18).

Because of oxidative phosphorylation coupled with mitochondrial respiration, the down-regulation of ATP synthase may inhibit the electrons flux to the respiratory chain. This limited electron flux would increase the level of superoxide radical, which causes DNA damage and mitochondrial mediated cell death. Cuezva et al. (16), therefore, showed that cells with down-regulation of ATP synthase would be prone to establishing a transformed phenotype. However, the role of down-regulated ATP synthase for carcinogenesis still remains to be clarified.

Cuezva et al. (16) suggested that a defect in mitochondrial ATP synthase is not only a bioenergetic feature of many cancer types but may also be a link to chemotherapy and radiotherapy resistance because the overall oxidative phosphorylation capability of the cell is diminished and, thus, the apoptotic potential of the cancer cell is hampered (35, 36). Interestingly, we found that dysregulated expression of ATP synthase complex protein subunits resulted in decreased ATP synthase activity in 5-FU–resistant human colon cancer cells (Figs. 1 and 2). Furthermore, the sensitivity of human colorectal cancer cell lines to 5-FU correlated with ATP synthase activity (Fig. 5). However, neither GAPDH activity, which represents cellular glycolytic potential (16), nor membrane potential, which is crucial for ATP synthesis by ATP synthase (37), was altered in 5-FU–resistant cells compared with parent cells (Fig. 2B and D). These findings show there is a link between 5-FU chemoresistance and ATP synthase down-regulation.

Because ATP synthase activity and protein expression were both down-regulated in human colon cancer cell lines with induced 5-FU resistance, we investigated whether expression of an endogenous natural ATP synthase inhibitor, IF1, was altered in 5-FU–resistant cells. Although IF1 is considered part of the in vivo control mechanism proposed to regulate the ATP synthase complex (38), we found expression of IF1 was not altered in 5-FU–resistant human colon cancer cells (Figs. 2A and 3). Furthermore, expression of IF1 did not correlate with either 5-FU sensitivity or ATP synthase activity in human colorectal cancer cells (data not shown). These results suggest IF1 does not play critical roles in either regulation of ATP synthase activity or induced 5-FU resistance in human colon cancer cell lines.

We examined whether 5-FU resistance was affected by addition of exogenous ATP synthase inhibitors. Oligomycin A is an inhibitor of the F0 part of ATP synthase (39) whereas aurovertin B inhibits the ATP synthase F1 catalytic sector (40). Interestingly, the combination index values between 5-FU and ATP synthase inhibitors were greater than 1 (data not shown), demonstrating strong antagonism between the two compounds on suppression of cell proliferation. In the presence of 5-FU IC50, cells treated with oligomycin A recovered cell proliferation compared with cells treated with 5-FU IC80 alone (Fig. 4B, bottom left). Similar effects were observed when cells were incubated with aurovertin B and 5-FU IC50 (Fig. 4B, top right). These findings further support a link between ATP synthase activity and 5-FU resistance in human colon cancer cells.

Experiments examining the effect of 5-FU and ATP synthase inhibitors on apoptosis were run simultaneously with those examining proliferation (Fig. 4C). These apoptosis studies showed that at an oligomycin concentration which significantly decreased intracellular ATP content, apoptotic death induced by 5-FU was not antagonized in spite of suppression of 5-FU–induced growth inhibition. Reduction of ATP can also block apoptosis and/or switch apoptotic death mechanisms to necrosis, and this effect was assumed to underlie the antiapoptotic action of oligomycin during apoptosis induced by such anticancer drugs as etoposide and dexamethasone (41, 42). Oligomycin is reported to trigger several antiapoptotic events, including reduction of ATP, blocking...
of Bax dimerization (39, 41, 42), and inhibition of cytochrome c release (43, 44). Interestingly, Shchepina et al. (39) showed that oligomycin also has an antiapoptotic function not involving inhibition of ATP synthesis. Whereas the precise antiapoptotic mechanisms invoked by oligomycin remain undetermined, the present data indicate that increased resistance to 5-FU (i.e., increased cell proliferation) was not due to antiapoptotic effects of oligomycin.

Although our data showed the link between ATP synthase and 5-FU resistance in human colorectal cancer cells, the causative role of ATP synthase for 5-FU resistance had to be clarified. In this sense, SNU-81 cells, which are sensitive to 5-FU and have high level of ATP synthase d-subunit expression, were used for siRNA approaches. Transfection with ATP synthase d-subunit siRNA efficiently suppressed its expression (Fig. 6A) and allowed SNU-81 cells a higher viability in the presence of 5-FU (Fig. 6B).

Figure 4. Mitochondrial ATP synthase inhibitors antagonize 5-FU-induced suppression of cell proliferation. A, cell proliferation is suppressed by the ATP synthase inhibitors, oligomycin A, and aurovertin B. After 4 days of incubation with an ATP synthase inhibitor, cell proliferation was determined by MTT assay. B, antagonism between ATP synthase inhibitors and 5-FU on suppression of cell proliferation. 5-FU concentrations suppressing cell proliferation by 50% and 80% were defined as IC_{50} and IC_{80}, respectively. After 4 days of incubation with 5-FU with or without an ATP synthase inhibitor, cell proliferation was determined by MTT assay. In all cases, the combination index values between 5-FU and ATP synthase inhibitors were greater than 1 (data not shown). *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with proliferation suppressed by 5-FU alone. C, effect of oligomycin A on intracellular ATP content and apoptosis induced by 5-FU. Cells were incubated with 0.1 μmol/L oligomycin in the presence or absence of 5-FU IC_{80}. ***, P < 0.001 when compared with cell proliferation suppressed by 5-FU alone. Columns, mean of three independent experiments; bars, SD.
Figure 5. Mitochondrial ATP synthase activity is positively correlated with 5-FU sensitivity in six human colon cancer cell lines. A, expression of ATP synthase complex protein subunits in human colon cancer cell lines. Western blot analysis was done to measure expression of subunits. B, correlation of expression of each ATP synthase complex protein subunit with each other. Absorbances obtained from A were used in statistical analysis. 5-FU sensitivity was positively correlated with ATP synthase d-subunit protein expression (C) and ATP synthase activity (D). E, no correlation between ATP synthase activity and either mitochondria membrane potential or intracellular ATP content. Columns, mean of three independent experiments; bars, SD.
These findings provide strong evidence that down-regulation of ATP synthase can be a single molecular event to modulate the response to 5-FU in human colorectal cancer cells.

In summary, the present study reports the down-regulation of ATP synthase in human colon cancer cells with induced 5-FU resistance. The mechanism(s) explaining how decreased ATP synthase, and thus decreased intracellular ATP, leads to intracellular events responsible for increasing cell proliferation in the presence of 5-FU remains to be determined. However, our findings show that ATP synthase down-regulation may not only be a bioenergetic signature of colorectal carcinomas but may also lead to cellular events responsible for 5-FU resistance.

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Figure 6. Suppressed ATP synthase d-subunit expression by siRNA transfection (A) and its effect on 5-FU sensitivity (B). SNU-81 cells were transfected with either 5 μmol/L of siRNA specific to ATP synthase d subunit, 5 μmol/L of nonsilencing control siRNA, or PBS buffer only. Twenty-four hours after transfection, cells were treated with various concentrations of 5-FU (0.1, 1.0, 10, or 100 μg/mL). Every 24 hours after transfection, cell lysates were prepared from each transfected cells and expression of ATP synthase d subunit was determined by Western blot analysis. Suppressed expression of ATP synthase d subunit was monitored 24 hours after transfection of ATP synthase d-subunit siRNA, and this suppression was continued up to 72 hours regardless of the treatment of 5-FU. A, an example of Western blot analysis obtained from cells treated with 100 μg/mL 5-FU at 24 hours after transfection. Cell viability was determined by MTT assay at 72 hours after transfection (48 hours after 5-FU treatment; B). Transfection with ATP synthase d-subunit siRNA not only led to suppression of ATP synthase d-subunit expression (A) but also allowed SNU-81 cells higher viability in the presence of 5-FU compared with controls (B). Columns, mean of three independent experiments; bars, SD.

References


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