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Original Research

Connexin 43 enhances oxaliplatin cytotoxicity in colorectal cancer cell lines

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Abstract: Oxaliplatin, a platinum-based chemotherapeutic agent, is an important first-line drug in the treatment of colorectal cancers, but drug resistance causes treatment failure. It has been reported that gap junctional communication can enhance the cytotoxicity of platinum drugs. The gap junction formed of connexin proteins provides a direct pathway for electrical and metabolic cell-cell interaction. The voltage-dependent gating of gap junction allows small hydrophilic molecules and ions to permeate to adjacent cells. Connexin 43 is a diagnostic marker for cancer therapy and the predominant connexin isoform in many cell types. The purpose of this study was to investigate the role of connexin 43 in oxaliplatin activity by using colorectal cancer cell lines. LoVo and HCT116 cell lines were used for analysis. Connexin 43 expression was confirmed by western blot and immunocytochemistry. MTT, western blot, "Parachute" dye-coupling assays and reactive oxygen species measurement were used to detect cytotoxicity and the inhibition of connexin 43 expression induced by oxaliplatin. Results showed that connexin 43 enhanced oxaliplatin cytotoxicity through gap junctional communication function and high concentration of oxaliplatin inhibited connexin 43 expression to counteract its cytotoxicity. This study suggested that connexin 43 could be considered a molecular target of oxaliplatin activity in colorectal cancer.

Key words: Oxaliplatin; Cytotoxicity; Connexin 43; Gap junctional communication; Colorectal cancer.

Introduction

Colorectal cancer is one of the most common cancer worldwide (1). Oxaliplatin is presently used as a first-line platinum drug in the treatment of colorectal cancer (2). However, intrinsic or acquired tumor resistance to oxaliplatin contributes to treatment failure, which necessitates the identification of additional chemotherapeutic drugs for combination therapy (3). Another approach is to identify tumor biomarkers that can enhance the cytotoxicity of chemotherapeutic drugs (4).

Gap junctional communication (GJC) which can connect the cytoplasm of two adjacent cells , is a water-filled pore composed of connexin molecules. When cells expressing connexins contacted each other, they promptly formed gap junction with cell-cell coupling characterized by asymmetric junctional conductance dependence on transjunctional voltage (5). The voltage-dependent gating of GJC allows small hydrophilic molecules and ions to permeate to adjacent cells, such as potassium, calcium, sodium, ADP/ATP, cAMP/cGMP and inositol 1,4,5-triphosphate (6).The direct exchange of these metabolites and messengers results in the metabolic and electric coupling of cells. Like many integral membrane proteins, connexins are synthesized in association with the endoplasmic reticulum and traffic through the Golgi apparatus to plasma membrane. Connexins located on plasma membrane contribute to normal GJC function, which generates gradients of growth control signals allowing cells to control their own population density. Many studies showed that loss of GJC is an important event in carcinogenesis and results in loss of growth control (6).

Down regulation or dislocation of many connexins contributes to GJC function, such as connexin 26, 32 and 43(7). Among them, connexin 43 (Cx43) is the most ubiquitously expressed member of the connexin family (8) and the predominant connexin isoform in many cell types (9,10). Previous studies have shown that up regulation of GJC function enhances the cytotoxicity of cisplatin (7) and oxaliplatin (11). It has been reported that Cx43 can act as a diagnostic marker in chemotherapy (12) and enhance chemotherapy-induced apoptosis (13). Loss of Cx43 expression has been also found in some colorectal cancer cell lines and is associated with reduced patient survival (14). However, the effects of GJC function or Cx43 expression on the cytotoxicity of oxaliplatin in colorectal cancer have not been elucidated.

The mitochondrion is the primary target for platinum drugs induced oxidative stress, so oxidative stress is an important mechanism involved in platinum drugs cytoxicity (15). Oxidative stress conditions can upset regular cellular biological functions to trigger cell death. GJC transfers small molecules from cell to cell directly because it can connect the cytoplasm of two adjacent cells. Some of these molecules could be involved in platinum drugs induced oxidative stress (16, 17). Our previous study also showed that GJC can up regulate reactive oxygen species (ROS) level in cisplatin treated cells(4). For these reasons, we hypothesis that Cx43 expression may enhance oxaliplatin cytotoxicity by up regulating ROS level in colorectal cancer.

In the present study, we examined the effects of Cx43 expression on the cytotoxicity of oxaliplatin in colorectal cancer cell lines. The cytotoxicity of oxaliplatin in cells with different Cx43 expression levels were assessed to determine the relationship between the

cytotoxicity of oxaliplatin and Cx43 expression. Whether oxaliplatin can inhibit Cx43 expression and neutralize its cytotoxic efficacy was also analyzed.

Materials and Methods

Reagents and antibodies

RPMI-1640 medium, trypsin, and antibiotics were purchased from Gibco (Grand Island, NY, USA), and fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Antibodies against connexin 43, caspase-3, caspase-8, caspase-9, poly (ADP-ribose) polymerase (PARP), β-actin, and IgG-peroxidase and FITC-conjugated antibody, DAPI, methyl thiazolyl tetrazolium (MTT), neomycin (G418), oxaliplatin, oleamide, and retinoic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). pTARGET mammalian expression vector was from Promega (Madison, WI, U.S.A). Lipofectamine reagent, CM-Dil, and Calcein-AM were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Electrochemical luminescence (ECL) plus, bicinchoninic acid (BCA) reagents, and RIPA lysis buffer were from CWBIO (Beijing, China). Reactive Oxygen Species Assay Kit and normal goat serum were purchased from the Beyotime Institute of Bio-technology (Shanghai, China).

Cell lines

Human colorectal carcinoma cell lines LoVo and HCT116 were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ℃ under 5% CO_2.

Vector construction and transfection

The cDNA of the human Cx43 coding region was amplified by polymerase chain reaction. The sequence of the forward primer was 5′-cacaattgagtggaatcttgatg-3′, and the reverse primer was 5′-caacatgggtgactggagc-3′. Polymerase chain reaction products were purified and inserted in the pTARGET vector. Expression vectors were transfected into LoVo and HCT116 cells using Lipofectamine Reagent. After transfection, the cultures were selected with 400 μg/mL G418. Cells of subclones were diluted and seeded to obtain further clones.

Western blot analysis

Cells were harvested and lysed with RIPA lysis buffer. Protein content was quantitated using BCA reagent. Crude cytosolic fractions were separated on a 12% SDS gel (20 μg/lane) and examined by western blot analysis. The signals were visualized by ECL plus and the blots were exposed to X-ray film.

"Parachute" dye-coupling assay

Functional GJC was examined as described by Wang et al. (4). Wild type (WT) and transfected cells were seeded in 6-well plates and grown to 80-100% culture density where GJC formation was possible. Two fluorescent dyes, CM-Dil and Calcein-AM, were used to measure GJC function. CM-Dil is a membrane dye that cannot spread to coupled cells. Calcein-AM can be converted intracellularly to calcein, which is a GJC-permeable dye. Donor cells in one well were stained with 10 μg/mL Calcein-AM and 5 μg/mL CM-Dil for 30 min at 37°C. After removing unincorporated dye, donor cells were trypsinized and seeded onto a monolayer of receiver cells grown in another well. The ratio of donor to receiver cells was 1:150. GJCs between donor and receiver cells were formed for 4 h at 37°C and were measured by a fluorescence microscope (Olympus CKX41). CM-Dil emitted red fluorescence and was used to identify donor cells. Green fluorescence of calcein-AM was used to calculate the average number of illuminant receiver cells around each donor cell. This number was used as a measure of GJC function.

Immunocytochemistry analyses of Cx43

Immunofluorescence staining was adapted to detect Cx43 expression. Cells were washed gently with PBS, fixed in 10% formalin at room temperature for 20 min, treated with 0.5% Triton X-100 for 5 min at 4 ℃, and blocked with 5% normal goat serum overnight at 4 ℃. The slides were then washed with PBS and incubated with anti-Cx43 antibody for 1 h at 37 ℃. Subsequently, the slides were incubated with FITC-conjugated antibody for 1 h at 37 ℃ and washed with PBS. The coverslips were sealed and the cells were photographed immediately using an Olympus fluorescence microscope (Olympus, Japan).

MTT assay

Cell growth inhibition was measured by MTT assay as described previously (18). Cells were seeded in 96-well plates and were treated with 0-10 μM oxaliplatin for 48 h. MTT was added to each well for 4 h. After the supernatant was removed, DMSO was added to dissolve crystals. After 10 min of slow vibration, absorbance was measured at 550 nm. Untreated cells were used as negative control. Measurements were performed in triplicate.

For the high-density condition, cells were seeded at 30,000 cells/cm^2 so that cultures were 70 to 100% confluent at the time of drug exposure. For the low-density condition, cells were seeded at 100 cells/cm^2 and treated with oxaliplatin after attachment.

Measurement of intracellular reactive oxygen species

The ROSlevel of cells after 2-h oxaliplatin treatment was quantified by the Reactive Oxygen Species Assay Kit. DCFH-DA has no fluorescence signal and can be oxidized by ROS in viable cells to 2',7'-dichlorofluorescein, which is highly fluorescent at 530 nm. Cells were treated with 5 μM oxaliplatin for 2 h and washed three times with serum-free medium. DCFH-DA (10μM) was then added and incubated in a 37°C incubator for 30 min. After three washes with PBS to remove excess DCFH-DA, the fluorescence intensity was measured using a microplate reader (485 nm excitation and 535 nm emission). Rosup, a positive control for DCFH-DA oxidation, was used at a final concentration of 0.1 mg/ml. Measurements were performed in triplicate.

Results

Confirmation of transfection and GJC function

Previous studies have shown that the expression of Cx43 decreases in some colorectal cancer cell lines (12). Therefore, we selected LoVo and HCT116 colorectal cell lines for analyses in this study. It has been reported that LoVo cells have a basal level of Cx43 expression but HCT116 cells do not express Cx43 (12). We evaluated Cx43 expression in WT and Cx43-transfected cells by western blotting and immunocytochemistry (Fig. 1). LoVo-WT cells showed a detectable level of endogenous expression of Cx43 in cytoplasm while HCT116-WT cells showed almost no endogenous Cx43 expression (Fig 1A-B). Both transfected cell lines showed high levels of Cx43 expression in cell membrane, which contributes to GJC function as shown in Fig. 1C-F. The "Parachute" dye-coupling assay revealed a positive correlation between GJC function and Cx43 expression (Fig. 2). In both LoVo and HCT116 cells, Cx43 transfection leads to higher levels of GJC function than seen in their WT counterparts.

Cx43 enhances apoptosis in oxaliplatin-treated cells

To determine whether Cx43 expression enhances the cytotoxic effect of oxaliplatin, we measured apoptosis in LoVo and HCT116 cell lines by MTT assay. It should be noted that the cells seeded at low culture density do not come in contact with each other to form GJC. As shown in Fig. 3A-B, the effect of Cx43 expression and culture density on surviving fraction of cells was analyzed. Cx43 expression decreased the surviving fraction in the two cell lines at high culture density when the cells were treated with lower concentration of oxaliplatin (≤5μM), but the effect was not robust at higher concentration of oxaliplatin (10 μM). Cx43 expression had no apparent effect on the surviving fraction at low culture density. The surviving fraction of WT LoVo cells was affected by the culture density when the cells were treated with lower concentration of oxaliplatin (≤5 μM), but that of WT HCT116 cells was not affected by the culture density. In addition, when the cells at high culture density were treated with 1.25 μM oxaliplatin, Cx43 expression showed no effect on LoVo cells but decreased the survival fraction in HCT116 cells. This effect of 1.25 μM oxaliplatin was not pronounced because changes in surviving fraction were very small at low concentration of oxaliplatin and experimental errors could lead to failure in detection of small differences in the surviving fraction between "Cx43" and "WT" group.

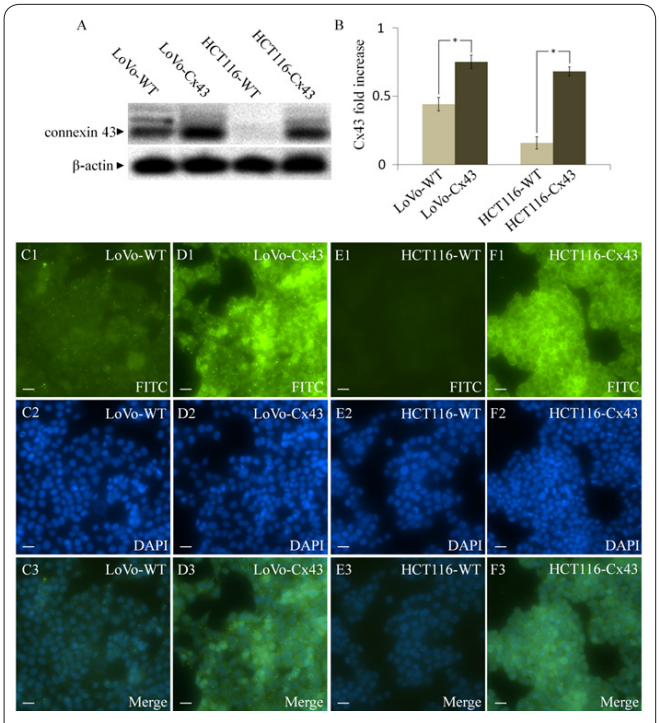

Figure 1. Cx43 expression in wild type and transfected cells. (A) Western blot analysis of LoVo and HCT116 cell lines transfected with Cx43 expression vector. β-Actin served as a loading control; (B) Quantification of bands observed in panel A was performed using Quantity One software (Bio-Rad Laboratories). Cx43 expression level was normalized to β-actin expression level. (C-F) Fluorescent micrographs of cells immunostained with connexin 43 antibody followed by FITC-conjugated antibody; (C) Wild type LoVo cells; (D) Transfected LoVo cells; (E) Wild type HCT116 cells; (F) Transfected HCT116 cells. The scale bars represent 20 μm.

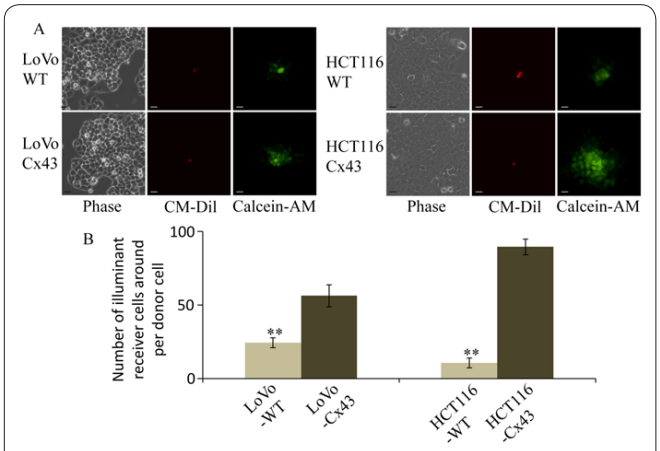

Figure 2. "Parachute" dye-coupling assay. (A) The distance travelled by the dye through GJC was measured in LoVo and HCT116 cell lines by "Parachute" dye coupling assays. WT: wild type cells; Cx43: Cx43 expression vectors transfected cells. The dye transfer distance was longer in cells overexpressing Cx43 compared with WT cells. The scale bars represent 20 μm. (B) Columns show the mean from five independent experiments and standard deviations are shown as error bars. $^{**}P < 0.05$, significantly different from the Cx43 group of the same cell line.

To further understand the molecular mechanisms of oxaliplatin treatment-induced apoptosis, activation of different caspases essential for apoptotic pathways and PARP, a cellular substrate of caspases, was analyzed by western blotting (Fig. 3C). Oxaliplatin induced cleavage of PARP, caspase-9, caspase-8, and caspase-3 after 24-h treatment at high culture density, and Cx43 expression enhanced this effect of oxaliplatin.

Oxaliplatin-induced ROS generation was enhanced by Cx43 expression

The generation of ROS is one of the critical events in platinum-induced cell death (19). To determine whether Cx43 expression can modulate oxaliplatin-induced ROS generation, we quantified the intracellular levels of ROS in the cells following 2-h treatment with oxaliplatin at high culture density. As shown in Fig. 4, there were marked increases in the generation of ROS in both cell lines treated with oxaliplatin. Furthermore, Cx43-

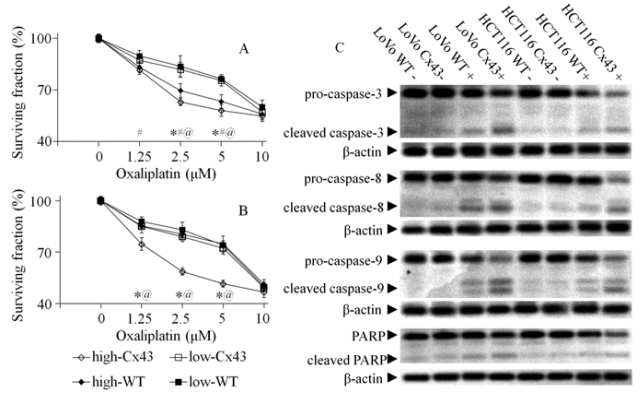

Figure 3. Effect of connexin-43 on apoptosis in oxaliplatin-treated cells. (A) and (B): Cell survival assay in (A)LoVo and (B)HCT116 cells at high or low culture densities. Cells were treated with indicated concentrations of oxaliplatin in 10% FBS-containing medium for 48 h. Data represents means ± SD of three separate experiments. "High-Cx43" or "low-Cx43" represents transfected cells that were cultured at high or low density, respectively. "High-WT" or "low-WT" represents wild type cells that were cultured at high or low density, respectively. The statistical significance of differences in surviving fraction between "high-WT" and "high-Cx43" (*P<0.05), "low-WT" and "low-Cx43", "high-WT" and "low-WT" ($^{\#}P$<0.05), "high-Cx43" and "low-Cx43" ($^{@}P$<0.05), were calculated by Student's t-test. (C) Western blot analysis of PARP, caspase-9, caspase-8 and caspase-3 levels after cells were incubated with oxaliplatin (5 μM) for 24 h. β-Actin was used as a loading control. "LoVo" and "HCT116" represents cancer cell lines; "WT" and "Cx43" represent wild type and transfected cells respectively; "+" represents oxaliplatin treatment; "-" represents no treatment.

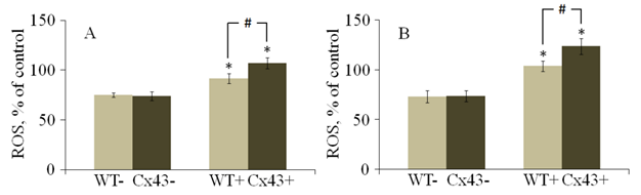

Figure 4. The generation of ROS in LoVo (A) and HCT116 (B) cells was induced by oxaliplatin. The results were normalized by positive control (Rosup). "WT" represents wild type cells; "Cx43" represents transfected cells; "+" represents oxaliplatin treatment; "-" represents no treatment. *P<0.05 (the differences in ROS generation between "-" and "+"); #P<0.05 (the differences in ROS generation between "WT" and "Cx43").

transfected cells had higher ROS level than WT cells after oxaliplatin treatment.

Oxaliplatin inhibits connexin 43 expression and GJC function

Effect of oxaliplatin on Cx43 expression of the transfected cells at high culture density was determined by western blotting (Fig. 5A). As shown in Fig. 5B, expression of Cx43 declined in oxaliplatin concentration-dependent manner. Further, GJC function was inhibited by high concentration of oxaliplatin (≥ 2.5 μM) as shown in Fig. 5C.

Discussion

The present study was designed to investigate the effect of Cx43 expression on cytotoxicity of oxaliplatin

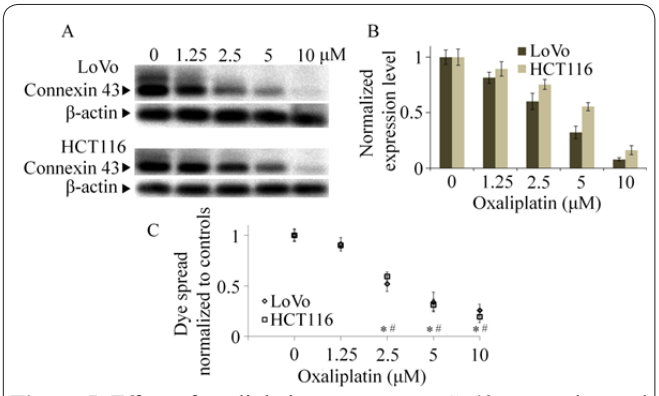

Figure 5. Effect of oxaliplatin treatment on Cx43 expression and GJC function. (A) Western blot probed with anti-connexin 43 antibody showing the effect of oxaliplatin on Cx43 expression at 48 h of culture. (B) Bar graphs were derived from densitometric scanning of the blots. Data are expressed as means ± SE from three experiments. The differences in expression between untreated and treated cells were significant in all concentrations of oxaliplatin (P<0.05). (C) Effects of oxaliplatin on dye coupling through gap junctions. Cells without treatment were used as control group. The number of dye coupling cells was normalized by control. Data points are means ± SE from five experiments. The differences in dye spread between untreated and treated cells were significant when oxaliplatin concentration was ≥ 2.5 μM (LoVo: *P<0.05, HCT116: # P<0.05).

in colorectal cancer cell lines. Cx43 expression and GJC function were both up regulated in LoVo and HCT116 cell lines by transfection of Cx43 expression vector. The results showed that Cx43 can enhance the cytotoxicity of oxaliplatin by up regulating GJC function but high concentration of oxaliplatin can inhibit GJC function by down regulating Cx43 expression.

It was reported that loss or down regulation of Cx43 expression in colorectal cancer is common (14). It has been confirmed that LoVo has a baseline level of Cx43 expression but HCT116 does not express Cx43 (14). Therefore, these two cell lines were used for investigation in our work. Cx43 expression and GJC function were both increased by transfection, which is a commonly used method (20) to investigate the effects of Cx43.

Previous reports have shown that cytotoxicity of platinum-based agents can be enhanced by Cx43 expression (21) and that these agents can inhibit Cx43 expression or GJC function, which counteracts their cytotoxic efficacy (22). Our work confirmed this effect in colorectal cancer cell lines. Cx43-transfected cell lines have higher GJC function than their WT counterparts do. The surviving fraction of transfected cell lines was affected by culture density when oxaliplatin concentration was low; the surviving fraction was not affected by culture density when oxaliplatin concentration was high. The surviving fraction of WT HCT116 cell line has no relationship with culture density unlike LoVo cell line in which surviving fraction is affected by culture density. This difference is likely due to a measurable baseline expression of Cx43 in WT LoVo cells. Combining these results with the finding that oxaliplatin inhibits Cx43 expression and GJC function, we conclude that GJC function contributes to oxaliplatin-induced cytotoxicity. Further, this inhibition of Cx43 expression and GJC function by high concentration of oxaliplatin likely ne-

gates its cytotoxicity in colorectal cell lines, as has been suggested in a previous report (22). On the other hand, transfected cells were more sensitive to oxaliplatin than their WT counterparts were when they were cultured in high density conditions, but transfected cells had similar sensitivity as their WT counterparts when they were cultured in low density conditions. These results suggest that Cx43 enhances the cytotoxicity of oxaliplatin via GJC. Similar observations have been made in other studies on chemotherapeutic drugs such as cisplatin (23) or paclitaxel (24).

Besides MTT assay, measurements of caspases and ROS were also used to determine cytotoxicity of oxaliplatin. Caspases are a family of cysteine proteases and crucial mediators of programmed cell death (25). Cleavage of caspases can be induced by platinum drugs (26). Our caspase cleavage assay also suggested that Cx43 enhances oxaliplatin-induced caspase-dependent apoptosis. ROS are the byproducts of the normal metabolism of oxygen, but under oxidative stress conditions, excessive ROS can damage cellular proteins. Oxidative stress is one of the most important mechanisms involved in cytotoxicity of platinum drugs (27,28). Similar to results of caspase cleavage assay, the findings of our ROS assay suggested that Cx43 expression can enhance the oxidative stress induced by oxaliplatin.

Connexins are targets for cancer chemoprevention and chemotherapy, but loss of GJC is commonly observed in human cancer (6). Some compounds can enhance the cytotoxicity of platinum agents by up regulating GJC function, for example, total flavonoids of litsea coreana (29) and retinoid acid (30). Our studies have shown that GJC function can also enhance oxaliplatin cytotoxicity in colorectal cancer cell lines. Our findings suggest that upregulation of GJC function can be used to treat colorectal cancer and that oxaliplatin has a counter-therapeutic effect by inhibiting GJC function and Cx43 expression. This effect has been reported in other cell lines (22) and we provide evidence that it also occurs in colorectal cancer cell lines. Small hydrophilic molecules and ions less than 1-2 kDa in size can permeate to neighboring cells through the voltage-dependent gating of GJC (31). Oxaliplatin and its cytoplasmic aquated species have molecular mass of about 400 Da, so it is likely that they can permeate GJC. In light of this, inhibition of GJC would decrease the cytotoxic action of oxaliplatin. In addition, inhibition of GJC by oxaliplatin could be a potential mechanism of resistance to its therapeutic effects.

Drug resistance usually results in chemotherapy failure in colorectal cancer treated with oxaliplatin. In this report, the effect of Cx43 expression on oxaliplatin cytotoxicity was investigated by using WT and Cx43-transfected colorectal cancer cell lines. Results showed that Cx43 enhances oxaliplatin cytotoxicity through GJC function and high concentration oxaliplatin inhibits Cx43 expression to counteract its cytotoxicity. Our studies have identified Cx43 as a diagnostic marker for oxaliplatin treatment in colorectal cancer.

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