Gap junction composed of connexin43 modulates 5-fluorouracil, oxaliplatin and irinotecan resistance on colorectal cancers

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Abstract. Chemotherapy is one of the most commonly used therapeutic strategies for metastatic colon cancer. However, the development of resistance to chemotherapeutic agents limits their application in clinical use. The underlying mechanisms of this resistance development require further elucidation. The current study investigated the effects of connexin43 (Cx43) gap junctions on 5-fluorouracil (5-FU), oxaliplatin and irinotecan in colon cancer cells. Three different methods were used to manipulate Cx43 gap junction function: i) Cell culture at different densities; ii) pretreatment with a Cx43 specific inhibitor or enhancer; and iii) Cx43 gene knock-down. Results indicated that the cell toxicity of 5-FU, oxaliplatin and irinotecan was cell density-dependent, which was mediated by gap junctions. Downregulation of Cx43 gap junction functioning attenuated 5-FU, oxaliplatin and irinotecan toxicity in colon cancer cells, which was increased in cells treated with a Cx43 gap junction function enhancer. Thus, the results of the present study suggest that resistance to 5-FU, oxaliplatin and irinotecan in colon cancer cells was relative to Cx43 expression loss as cancer developed, which may indicate a novel basis for therapeutic strategy development to combat drug resistance in numerous cell types, in addition to colon cancer cells.

Introduction

Colorectal cancer (CRC) is the third most common cancer in humans and has a high mortality rate, it is an important public health problem worldwide (1). Various types of therapeutic strategies have been used for the treatment of colon cancer, including radiotherapy, chemotherapy, targeted therapy, and immune therapy, however, the five-year survival rate of metastatic colon cancer remains <10% (2). Chemotherapy is one of the most commonly used therapeutic strategies for metastatic colon cancer, agents used include 5-fluorouracil (5-FU), oxaliplatin and irinotecan, however, development of resistance markedly limits their application in clinical use (3-5). For example, 5-FU, which has been used for many years, has a single agent effective rate of 24% (2). Although oxaliplatin improves the response rate of patients in advanced colon cancer, >40% patients develop serious resistance (6). Resistance to irinotecan, as a first-line therapy for metastatic colon cancer in combination with other antitumor agents, has also been reported in recent years (7). The development of resistance has been hypothesized to occur via multiple different mechanisms, a number of which are supported with clinical data. However, the development of resistance requires further elucidation.

Gap junctions composed of connexin directly connect the cytoplasm of neighboring cells, thereby mediating direct intercellular movement of cytoplasmic signaling molecules (8). Signaling molecules with weight <1 kDa can be transferred via these channels, including cyclic adenosine monophosphate, cyclic guanosine monophosphate, calcium and glutathione. Almost every cellular and tissue level process can be influenced by this type of direct communication pathway, such as differentiation, migration, and apoptosis (9). Thus, gap junctions are known to have important roles in cancer biology and drug resistance (10,11). Cx43 (molecular weight, 43 kDa) is regarded as one of the most important known connexins, and it is often associated with drug resistance. For example, alteration of Cx43 contributes to temozolomide resistance in glioblastoma multiforme (10). Yu *et al* (12) demonstrated that Cx43 reversed the resistance of A549 lung adenocarcinoma cells to cisplatin by inhibiting epithelial-mesenchymal transition. It has not, to the best of our knowledge, yet been reported whether resistance to 5-FU, oxaliplatin and irinotecan in colon cancer is associated with Cx43. Thus, the current study investigated effects of the Cx43 gap junction on these commonly used chemotherapeutic agents in colon cancer cells to indicate a novel basis for therapeutic strategy development for combating drug resistance.

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Materials and methods

Cell line and cell culture. The RKO human colon cancer cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Eagle's minimum essential medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin (both from Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator with 90% humidity.

Colony-forming assay. Colony-forming assays at high and low cell density was used to detect the toxicity dependent on gap junctions. In culture at a high cell density, cells were seeded at 30,000 cells/cm^2 to ensure that cultures were 70-100% confluent at the time of therapeutic agent exposure. At this density, each cell was in contact with approximately four to five others cells and there was substantial opportunity for gap junction formation. Cells were treated with 5-FU (0-250 μM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), oxaliplatin (0-125 μM; Sigma-Aldrich; Merck Millipore), or irinotecan (0-12.5 μM; Sigma-Aldrich; Merck Millipore) for 24 h, and subsequently washed with Eagle's minimum essential medium, harvested by trypsinization (Invitrogen; Thermo Fisher Scientific, Inc.), counted, diluted, and seeded into six-well dishes at 100 cells/cm^2. Colony formation was assessed by staining with crystal violet (Sigma-Aldrich; Merck Millipore) and assessed at 7 days. Colonies containing >50 cells were scored under an Eclipse E800 light microscope (Nikon Corporation, Tokyo, Japan). At low cell density culture, cells were seeded into six-well plates at 100 cells/cm^2 directly and treated with the same 5-FU, oxaliplatin or irinotecan concentrations for 24 h following attachment. Subsequently, the colony formation was assessed as described above for the high density cell culture (13).

Gap26, retinoic acid (RA) treatment, and survival assays. RKO cells were pretreated with connexin channel inhibitors 300 μM Gap26 (Sigma-Aldrich; Merck Millipore), a connexin mimetic peptide, for 1 h, and 10 μM retinoic acid (Sigma-Aldrich; Merck Millipore), a Cx43 expression enhancer, for 1 h prior to a parachute dye-coupling assay or survival assays. Dimethyl sulfoxide (DMSO) was used as a solvent for Gap26 and RA (Sigma-Aldrich; Merck Millipore). Cell growth was determined in 24-well plates with the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Tokyo, Japan), which was conducted according to the manufacturer's protocols.

Parachute dye-coupling assay. A parachute dye-coupling assay was used to detect gap junction function. The donor and receiver cells were grown to confluence. Donor cells were labeled with 5 μM CM-DiI (Invitrogen; Thermo Fisher Scientific, Inc.), which did not spread to coupled cells, and 5 μM calcein-acetoxymethyl ester (Invitrogen; Thermo Fisher Scientific, Inc.), which was converted into the gap junction-permeable dye calcein in an intracellular process. Subsequently, at a 1:150 donor/receiver ratio, donor cells were seeded onto the receiver cells. Donor cells and receiver cells formed gap junctions. After 4 h, gap junction function was examined with the fluorescence microscope (Eclipse E800). The mean number of receiver cells containing dye per donor cell was counted and normalized to that of control cultures (14).

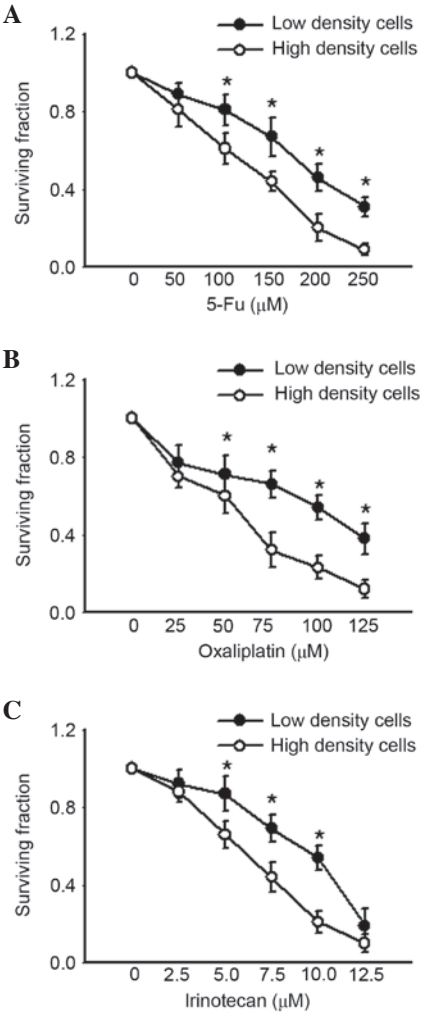

Figure 1. Inhibition of clonogenic survival of RKO cells exposed to 5-FU (0-250 μM, 24 h), oxaliplatin (0-125 μM, 24 h), or irinotecan (0-12.5 μM, 24 h) is cell density-dependent. The clonogenic survival of cells exposed to a range of concentrations of (A) 5-FU, (B) oxaliplatin, or (C) irinotecan at low cell density or at high cell density. n=5-7, *P<0.05 vs. the high density cell group. Data are presented as the mean ± standard error. 5-FU, 5-fluorouracil.

Western blotting. Cells were washed three times with wash buffer [0.01 mol/l phosphate-buffered saline, 0.138 mol/l NaCl, 0.02% NaN3 (pH 7.4)] and then incubated with 0.05 ml/cm^2 lysis buffer (Nanjing Keygen Biotech Co., Ltd., Nanjing, China) for 2 h at 4˚C. The bicinchoninic acid (BCA) method using the BCA Protein Assay kit (Nanjing Keygen Biotech Co., Ltd.) was used to measure protein concentrations. Cell lysates (30 μg) were separated by SDS-PAGE on 10% Tris-glycine mini-gels (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 5% nonfat dry milk (Sigma-Aldrich; Merck Millipore) was used to block the membranes at room temperature for 30 min. Subsequently,

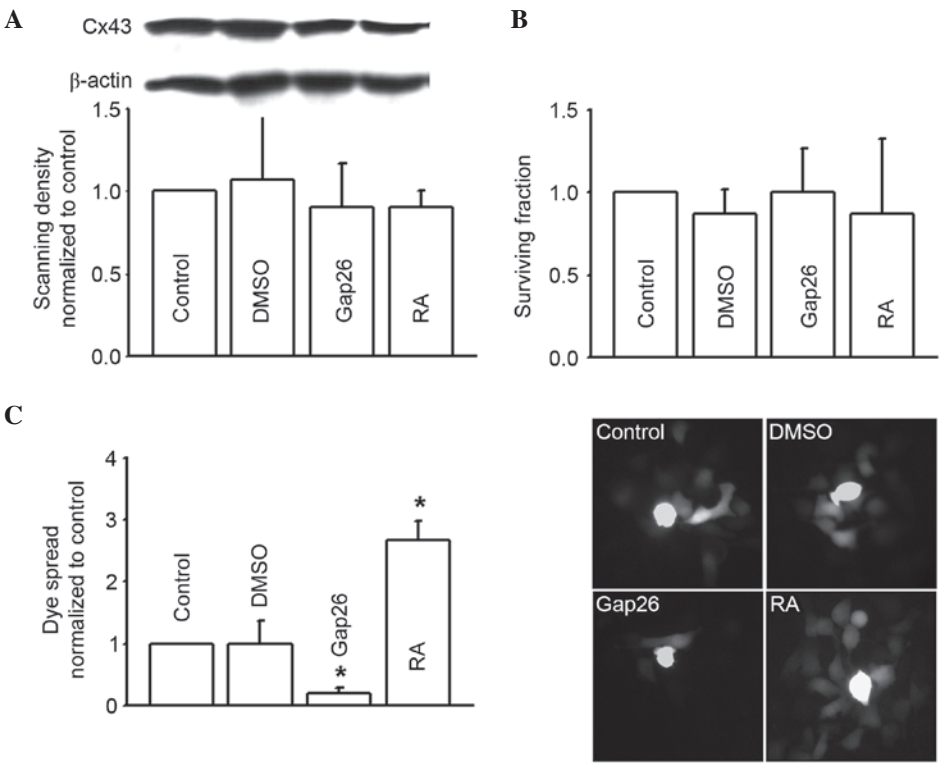

Figure 2. Effects of connexin43 (Cx43) inhibitor Gap26 and enhancer RA on RKO cells. (A) Gap26 and RA had no effects on Cx43 expression in RKO cells (n=3). (B) Concentrations of Gap26 or RA used in the present study had no cell toxicity in RKO cells (n=3). (C) Gap26 decreased dye coupling of RKO cells, which was enhanced by RA (n=5). The vehicle control for Gap26 and RA were DMSO, which had no significant effects on the abovementioned parameters. *P<0.05 vs. the control group. Data are presented as the mean ± standard error. Cx43, connexin43; RA, retinoic acid; DMSO, dimethyl sulfoxide.

the membranes were immunoblotted using mouse monoclonal anti-Cx43 antibody (1:4,000; Sigma-Aldrich; Merck Millipore; cat. no. C8093) and mouse monoclonal anti-β-actin antibody (1:10,000; Sigma-Aldrich; Merck Millipore; cat. no. A1978) overnight at 4˚C. Following a number of washes with Tris-buffered saline and Tween-20 (0.05%), the membranes were incubated for 1 h at room temperature with polyclonal goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4,000; Sigma-Aldrich; Merck Millipore; cat. no. M6898). The protein bands were detected with an enhanced chemiluminescence system (KGP1125; Nanjing KeyGen Biotech. Co., Ltd.). Protein band sizes were estimated using Alpha View software (version 2.2.14407; ProteinSimple, San Jose, CA, USA).

Cx43 knock-down with small interfering RNA (siRNA) transfection. Two specific siRNAs were used to target the Cx43 gene and reduce expression, the sequences were as follows: GCTGGT TACTGGTGACAGA for siRNA1-Cx43; and CCGCAATTA CAACAAGCAA for siRNA2-Cx43. A non-specific siRNA-NC was used as a negative control. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect siRNA according to the manufacturer's protocols (15).

Statistical analysis. Statistical analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Multiple comparisons among groups were analyzed using one-way analysis of variance, followed by Tukey's post hoc comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Association between toxicity and concentration of 5-FU, oxaliplatin and irinotecan depends on cell density. The RKO human colon cancer cell line was cultured under two different conditions, at low density and high density cell culture. At low density cell culture the cells were well dispersed as single cells as the cell density was 100 cells/cm^2 and, thus, no gap junctions were formed. At high density cell culture, cells were 70-100% confluent at the time of therapeutic agent exposure, which allowed formation of gap junctions. Fig. 1 presents the survival of cultures exposed to 5-FU, oxaliplatin and irinotecan for 24 h under low- or high-density conditions. All three chemotherapeutic agents decreased clonogenic survival of cells at low and high density in a concentration-dependent manner. However, as the concentrations of the agents increased, cell survival was significantly lower in high density cell culture compared with low density (P<0.05). These results indicated that the cell toxicity of 5-FU, oxaliplatin and irinotecan was density-dependent, and greater in high density cell culture where gap junctions were formed.

Cell toxicity of 5-FU, oxaliplatin and irinotecan was mediated by gap junctions. Loss of gap junctions is widely considered to be associated with tumorigenic phenotypes, however, the RKO human colon cancer cell line used in the present study highly expressed Cx43 (Fig. 2A). The current study aimed to investigate the function of the Cx43 gap junction on the toxicity of commonly used chemotherapeutic agents

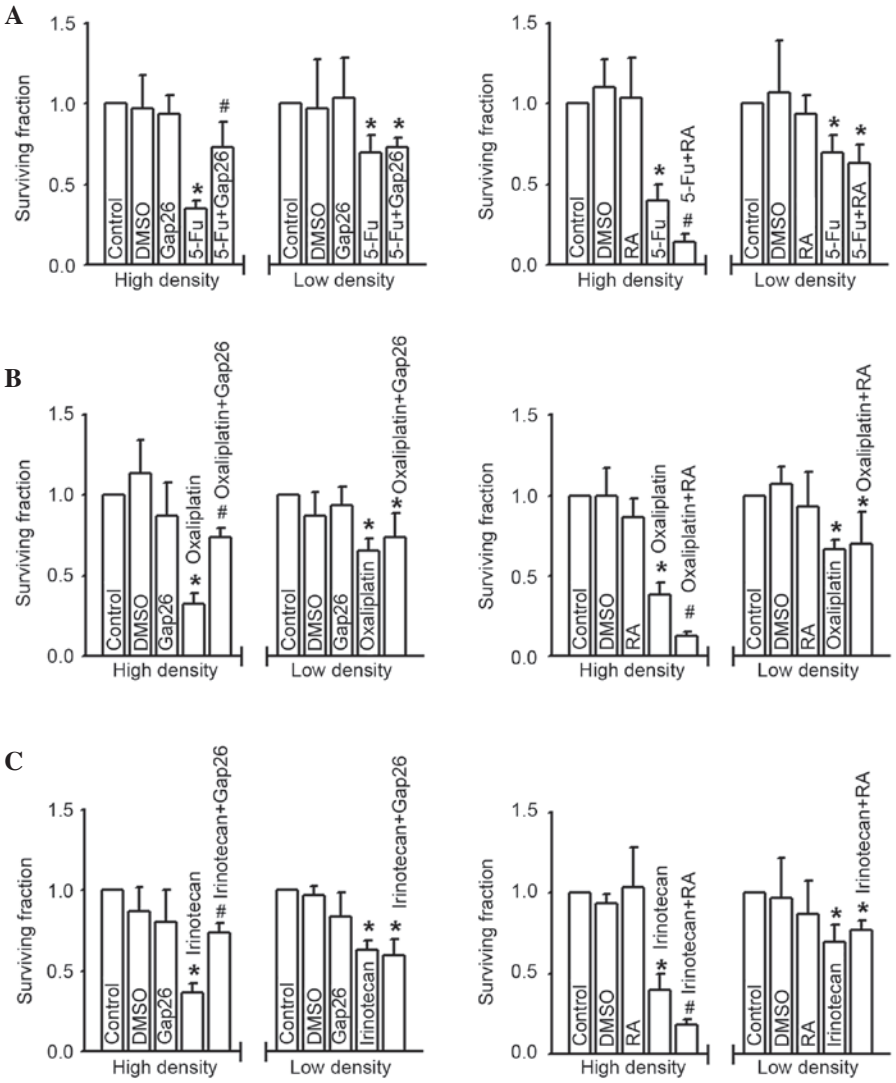

Figure 3. Cell toxicity of 5-FU, oxaliplatin and irinotecan was mediated by gap junctions. (A) Clonogenic survival of RKO cells exposed to 5-FU (200 μM, 24 h) at low and high cell density and co-incubated with or without Gap26 and RA (n=5). *P<0.05 vs. the control group; #P<0.05 vs. the 5-FU group at high density cell culture. (B) Clonogenic survival of RKO cells exposed to oxaliplatin (100 μM, 24 h) at low and high cell density and co-incubated with or without Gap26 and RA (n=5). *P<0.05 vs. the control group; #P<0.05 vs. the oxaliplatin group at high density cell culture. (C) Clonogenic survival of RKO cells exposed to irinotecan (10 μM, 24 h) at low and high cell density and coincubated with or without Gap26 and RA (n=5). *P<0.05 vs. the control group; #P<0.05 vs. the irinotecan group at high density cell culture. Data are presented as the mean ± standard error. The vehicle control of Gap26 and RA was DMSO, which had no significant effect on the abovementioned parameters. 5-FU, 5-fluorouracil; RA, retinoic acid; DMSO, dimethyl sulfoxide.

targeting colon cancer cells. Thus, roles of the gap junction composed of Cx43 on 5-FU, oxaliplatin and irinotecan toxicity in RKO cell line were determined. Pharmacological inhibitors or enhancers were used to alter the function of gap junctions composed of Cx43. Results demonstrated that dye coupling was significantly reduced by the inhibitor Gap26, but significantly increased by the enhancer RA (P<0.05). Neither Gap26 or RA alone, or the solvent DMSO, had any toxicity on the RKO cells or altered Cx43 expression (Fig. 2). Gap26 and RA were demonstrated to alter the function of Cx43 gap junctions.

Fig. 3 presents the effects of 5-FU, oxaliplatin and irinotecan toxicity on RKO cells at low and high density cell culture. Survival fraction (detected by colony-forming assay) was significantly downregulated following treatment of RKO cells with 5-FU (200 μM), oxaliplatin (100 μM), or irinotecan (10 μM) for 24 h at high cell density (gap junctions formed; P<0.05), suggesting the cells were more sensitive to the three commonly used chemotherapy agents. Notably, the cytotoxicity of 5-FU, oxaliplatin and irinotecan was attenuated following Gap26 pretreatment at the concentration verified to inhibit gap junction function in these cells (P<0.05), but exacerbated following RA treatment in RKO cells cultured in high-density (P<0.05) (Fig. 3). By contrast, at low density cell culture (gap junction not formed), the cytotoxicity of 5-FU, oxaliplatin and irinotecan was not significantly different irrespective of Gap26 or RA pretreatment compared with control groups (Fig. 3). The results demonstrate the effects of altering gap junction function on 5-FU, oxaliplatin and irinotecan toxicity only occurs following high density cell culture, which supports the hypothesis that gap junctions are important in the efficacy of chemotherapeutic agents.

Cx43 gene knock-down inhibited Cx43 gap junction function and attenuated RKO cell toxicity of 5-FU, oxaliplatin and irinotecan. In order to confirm the effects of Cx43 gap

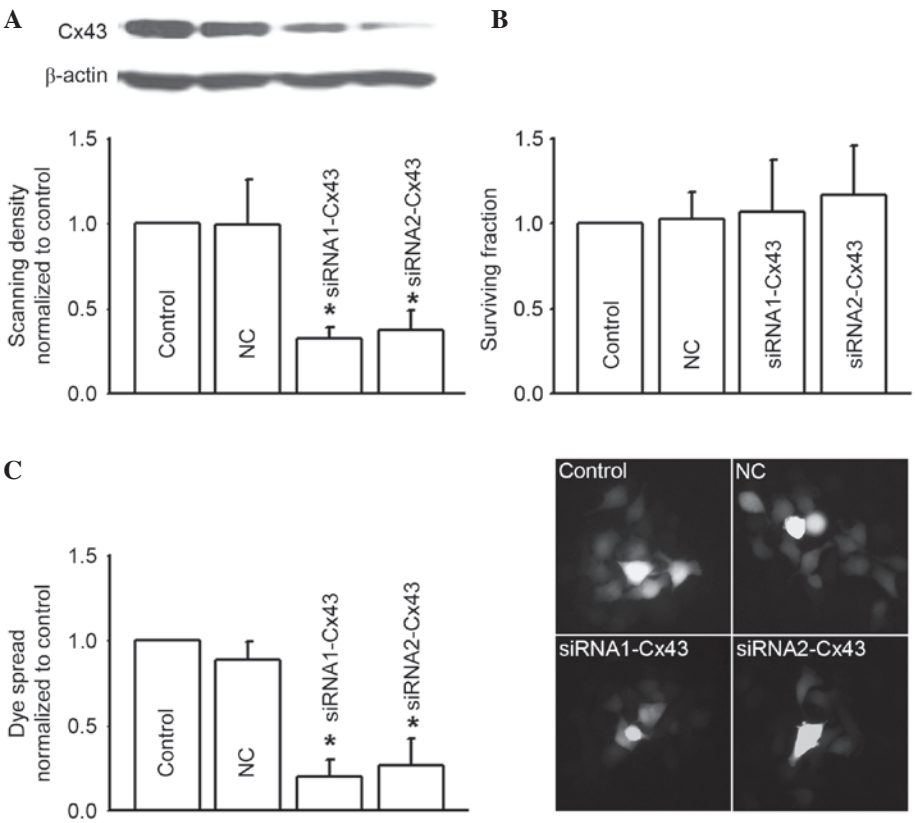

Figure 4. Specific siRNA attenuated gap junction function composed of Cx43. (A) Two different specific siRNAs (siRNA1-Cx43, siRNA2-Cx43) decreased Cx43 expression (n=5). *P<0.05 vs. the control group. (B) The two different specific siRNAs had no cell toxicity on RKO cells (n=3). (C) The two different specific siRNAs attenuated dye coupling of RKO cells (n=5). *P<0.05 vs. the control group. Data are presented as the mean ± standard error. NC had no significant effects on the abovementioned parameters. siRNA, small interfering RNA; Cx43, connexin43; NC, negative control.

junction function on the cytotoxicity of 5-FU, oxaliplatin and irinotecan, two different Cx43 siRNAs (siRNA1-Cx43 and siRNA2-Cx43) were synthesized to specifically knockdown Cx43 expression (Fig. 4A). Cx43 knockdown alone did not influence RKO survival fraction (Fig. 4B). However, as Cx43 expression was depressed, dye coupling (gap junction function) was also significantly decreased (P<0.05; Fig. 4C).

In Fig. 5, the CCK-8 assay was used to investigate the cytotoxicity of 5-FU, oxaliplatin and irinotecan on RKO following depression of Cx43 expression levels with Cx43 siRNAs. Results indicated that Cx43 knock-down attenuated the cytotoxicity induced by 5-FU, oxaliplatin and irinotecan in RKO cells, significantly increasing the survival fraction to different degrees (P<0.05; Fig. 5A to C). These results indicate that gap junctions composed of Cx43 are important in 5-FU, oxaliplatin and irinotecan-induced cytotoxicity of RKO cells.

Discussion

The present study investigated the effect of Cx43 gap junctions on the toxicity of 5-FU, oxaliplatin and irinotecan. Cx43 is involved in resistance to these commonly used chemotherapeutic agents targeting colon cancer cells. The present study demonstrated that these antitumor therapeutic agents worked in a cell density-dependent manner. At high-density cell culture (gap junctions formed), alteration of gap junction function using different methods, such as the inhibitor Gap26 or the enhancer RA, or Cx43 knock-down, affected 5-FU, oxaliplatin and irinotecan-induced cytotoxicity. The cytotoxicity was attenuated subsequent to depression of Cx43 gap junction functioning, but exacerbated as Cx43 gap junction function increased. However, this result was absent in low-density cell culture, which lacked gap junction formation. This conclusion was consistent with previous studies (12,13), and provided more information regarding the importance of gap junctions composed of Cx43 in 5-FU, oxaliplatin and irinotecan-induced cytotoxicity in RKO cells.

Gap junctions enable the direct transfer of small molecules or electrical charge between neighboring cells, which contributes to different physiological and pathological effects, including cell growth, differentiation, damage, and response to trauma (16). Molecular signals resulting in the amplification of cytotoxicity or apoptosis are termed 'death signals,' which are predominantly regulated by gap junctions between neighboring cells, particularly in cancer cells (17). Toxic products generated in one cell can enter another via gap junctions and subsequently enhance the likelihood of cell death, which in turn generates its own toxic products as part of a positive feedback mechanism (9). Toxic products, or 'death signals' not only damage the neighboring cells directly, but also trigger activation of different signal pathways, resulting in cytotoxicity or apoptosis indirectly (13). This type of 'death signal' transfer between the neighboring cells via gap junctions amplifies the cytotoxicity induced by antitumor therapeutic agents, which is termed the 'bystander effect' (18-20). The

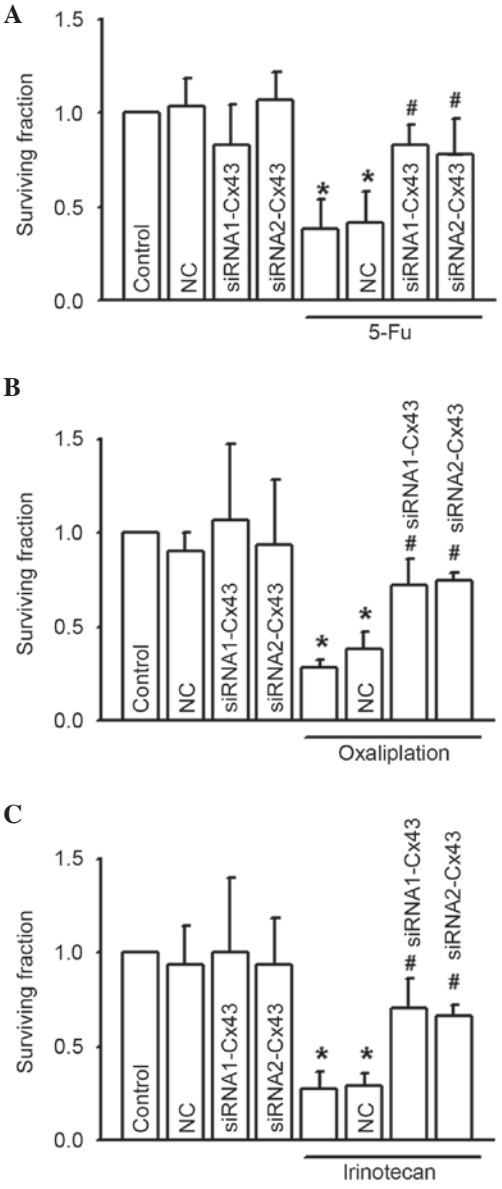

Figure 5. RKO cell growth was increased by specific siRNA when exposed to (A) 5-FU (200 μM, 24 h), (B) oxaliplatin (100 μM, 24 h), or (C) irinotecan (10 μM, 24 h), n=5-7. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the 5-FU, oxaliplatin and irinotecan groups. Data are presented as the mean ± standard error. NC and these two siRNAs alone had no significant effect on the above-mentioned parameters. 5-FU, 5-fluorouracil; NC, negative control; siRNA, small interfering RNA.

identity of the 'death signals' has not yet been identified. Although the possibility of calcium, reactive oxygen species or cell metabolites have been recently discussed, more proof is required (8).

Gap junction deficiency is widely considered to be associated with tumorigenic phenotypes (21-23), however, exceptions remain, including the RKO human colon cancer cell line used in present study. Cx43 is highly expressed in RKO cells. As previously reported, gap junctions are important in different stages of cancer progression, including invasion, extravasation, and metastasis in various types of cancer cell, such as HeLa cells, breast carcinoma, melanoma, lung carcinoma and glioma (24-27). The results of the present study demonstrated the effects of altering gap junction function on colon cancer chemotherapy that had not, to the best of our knowledge, been previously reported, that enhancing the function of the Cx43 gap junction increased 5-FU, oxaliplatin and irinotecan-induced cytotoxicity. By contrast, the cytotoxicity could be reduced by inhibition of gap junction function.

CRC has a high mortality rate and is considered to be the third most common cancer in humans (28). In metastatic colon cancer, the five-year survival rate is <10% (2). 5-FU, oxaliplatin and irinotecan are the most commonly used chemotherapeutic agents in CRC. However, their response rates are low, even in combination with other chemotherapeutic agents. As previously reported, 5-FU as a single agent has an effective rate of 24%, and ~31% at higher doses, however, dosage increase results in serious side-effects and the development of drug resistance limits its dosage and therapeutic effect (2). Oxaliplatin is a third generation platinum-based antineoplastic agent, used as a chemotherapeutic agent in advanced colon cancer treatment. Although oxaliplatin application has notably improved response rates and progression-free survival in advanced colon cancer, ~40% of patients develop resistance (29). Irinotecan is also used as a first-line therapy for metastatic colon cancer in combination with other antitumor agents. Unfortunately, resistance to irinotecan has been observed in the clinic (30). Thus, it is clear resistance to chemotherapeutic agents for colon cancers is a great challenge in clinical use.

Toxic effects of gap junction-mediated intercellular transfer had been investigated in a number of different systems (31-33). Notably, it has been observed that gap junctions composed of Cx43 act synergistically with 5-FU, oxaliplatin and irinotecan to result in cytotoxicity. It has also been reported that resistance to chemotherapeutic agents is associated with loss of gap junctions in specific stages of cancer progression. He *et al* (9) and Wang *et al* (13) demonstrated that gap junction recovery or enhancement improved resistance of cisplatin. Toxic metabolites, such as 5-fluorouracil converted from 5-FU by cytosine deaminase, can transfer between neighboring cells via gap junctions to amplify the cytotoxicity (34). This is consistent with the results of the present study, which indicate that enhancing the function of Cx43 gap junctions increased 5-FU, oxaliplatin and irinotecan-induced cytotoxicity, which may be reduced by inhibition of gap junction functioning, suggesting that gap junction reduction may be an important mechanism underlying the development of resistance. Thus, recovery of connexin expression or enhancing gap junction functioning may be useful strategies to attenuate resistance or increase the efficacy of anticancer chemotherapeutic agents.

Gap junction loss was widely accepted to be associated with tumorigenic phenotypes. As tumors progress, gap junction functioning and connexin expression levels decrease, as observed in HeLa cells, bladder cancer cells, lung carcinoma, breast carcinoma, glioma or other colon cancer cell lines. All of these tumors were not sensitive to chemotherapy agents, which always results in a high rate of mortality (35-39). If connexin expression could be recovered and gap junction function enhanced, the responses of these tumors to chemotherapeutic agents may be improved significantly.

In conclusion, the current study indicated that the cytotoxicity of chemotherapeutic agents was attenuated with gap junction inhibition, however was strengthened with gap junction enhancement. This conclusion provided a novel basis for therapeutic strategy development to combat drug resistance in numerous cell types, not only for colon cancer cells, however additionally for other types of cancer.

Acknowledgements

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