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Enhanced Effect of Connexin 43 on Cisplatin-Induced Cytotoxicity in Mesothelioma Cells

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Abstract. The expression levels of connexin (Cx) proteins, which are gap junction (GJ) components, are often decreased in many cancers, and restoring their levels has been shown to have antitumor effects. Previously, dysfunctional gap junctional intercellular communication (GJIC) has been observed in several malignant mesotheliomas (MMs), and among the many Cx proteins, Cx43 is prominently expressed in nontumorigenic mesothelial tissues. Therefore, we investigated whether Cx43 upregulation has an antitumor effect on an MM cell line (H28 cell), especially with regard to drug resistance. After treatment with the chemotherapeutic agent cisplatin (CDDP), MM cell viability significantly decreased, and apoptosis induction was observed in Cx43-transfected clones. A specific GJIC inhibitor could not abrogate this effect. On the other hand, the Src protein is known to phosphorylate Cx43, which results in GJIC inhibition. This suggests that Src activity might also be regulated by the hyperexpression of Cx43. In fact, the Src protein level was decreased in Cx43-transfected clones. Moreover, Src inhibition reinforced CDDP cytotoxicity in parental H28 cells. These data suggest that Cx43 could improve the resistance to CDDP in a GJIC-independent manner, which may be partly mediated by the suppression of Src activity.

Keywords: connexin, mesothelioma, chemotherapy, cisplatin, drug resistance

Introduction

Malignant mesothelioma (MM) is an aggressive and devastating malignancy of the pleura and peritoneum, well known in occupational medicine and legal communities (1). Although it has been the subject of intense clinical and laboratory research, the case-fatality rate for MM remains high, and conventional treatments remain inadequate. Systemic chemotherapy is important since it is a whole-body treatment and is useful for patients who cannot be treated with surgery. Cisplatin (CDDP) has been used in clinical MM therapy, and its chemotherapeutic effect as a single agent as well as in combination with other drugs such as gemcitabine has been examined (2 – 4). Recently, pemetrexed, a novel multitargeted antifolate, has shown modest activity when used as a single agent and in combination with CDDP in MM patients (5). However, although pemetrexed is promising, it has yet to be standardized because of the severity of its side effects and the presence of nonresponders.

Generally, the maintenance of cellular homeostasis is the key mechanism in the control of cellular life cycle, from cell growth to cell death (6). Direct intercellular communication is mainly mediated by gap junctions (GJs), via GJ intercellular communication (GJIC). It is generally accepted that tumor promotion could be due to a lack of cell–cell communication. GJ activity and expression of GJ component protein, connexin (Cx), are reportedly decreased in developing tumors. Moreover, many transfection experiments using Cx cDNAs suggest

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that Cx is a tumor suppressor in cells originating from tissues in which they are normally expressed (7). In line with this, we have recently reported that Cx32 has a tumor-suppressive effect in renal cancer, acting against growth and migration (8 – 10), improving drug sensitivity (11), and inhibiting metastasis (12, 13).

With regard to MM, Linnanimaa et al. have reported that GJIC is reduced in MM cells as compared with that in parental mesothelial cells (14). Cx43 has been shown to act as a major GJ protein in normal human mesothelial cells (15). Furthermore, although both the mRNA and protein expression of Cx43 was detected in six out of seven MM cell lines, these cells did not have the capacity for GJIC (14). These reports suggest that Cx43 and GJIC, originally expressed and functional, are gradually suppressed by various mutations, activated oncogenes, or by the deactivation or loss of tumor-suppressor genes during carcinogenesis. Among the many regulatory mechanisms of GJIC, phosphorylation has been most extensively studied. Most Cx's are phosphoproteins (16); however, Cx43 accounts for the majority of the available literature because of its widespread expression. The proto-oncogene product Src is one of the proteins that can directly phosphorylate Cx43 (17). Including MM, the Src family of nonreceptor tyrosine kinases are overexpressed and/or aberrantly activated in various human tumors, and moreover, their activity is correlated to tumor progression and metastatic potential (18). Indeed, a previous report revealed that total c-Src is highly expressed in some MM cells (19). On the other hand, it has also been shown that Cx43 regulates Src kinase through interaction of the Cx43 carboxy-terminal region with the kinase, irrespective of the GJIC function (20).

In this context for improving clinical usage, the present study was undertaken to investigate whether Cx43 has an antitumor effect on MM by using a forced-expression clone of Cx43; in addition, another aim of this study was to determine whether there was a combination effect produced by Cx43 with CDDP, that is, GJIC-dependent or -independent.

Materials and Methods

Reagents

All cultures and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated.

Cell culture, construct creation, and transfection

H28, a representative human MM cell line, was obtained from ATCC and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.5 units /ml penicillin, 1 μg/ml streptomycin, 0.01 M HEPES buffer solution, 1 mM sodium pyruvate, and 4.5 g/l glucose at 37°C in an atmosphere with 5% CO_2. All chemicals were purchased from Invitrogen (Carlsbad, CA, USA), except for FBS (MP Biomedicals, Aurora, OH, USA). A human Cx43 cDNA insert containing the entire coding region was subcloned into the expression vector pcDNA™ 3.1 D/V5-His-TOPO® (Invitrogen) according to the manufacturer's instructions, and the sequence of the vector-Cx43 construct was confirmed by DNA sequencing. Parental cells were transfected with 1 μg of Cx43 cDNA as well as an empty vector, which was used as the control, by using FuGENE® HD Transfection Reagent (Roche Applied Science-Japan, Tokyo). After 72 h, the cells were selected in a culture medium containing 0.7 mg/ml G418. Over 3 weeks, the G418 concentration was gradually decreased, and finally, the medium was replaced with normal culture medium. In order to avoid clonal variations due to selection, all surviving clones were combined and used to estimate the tumor-suppressive effects of Cx43. Cx43-transfected H28 cells (H28-T) and mock-transfected H28 cells (H28-W) were then established.

Isolation of total RNA and real-time RT-PCR

Total RNA was isolated using a ChargeSwitch® Total RNA Cell kit (Invitrogen), and cDNA was synthesized as previously described (21). Real-time reverse transcribed (RT)-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Ltd., Tokyo) and SYBR® Premix Ex Taq™ (TaKaRa Bio Inc., Shiga), according to the manufacturer's instructions. The following primers were used: RPL32 [accession number (NM_000994)] sense (nucleotides-CATCTCCTTCTCGGCATCA) and antisense (nucleotides-AACCCTGTTGTCAATGCCTC); Cx43 [accession number (NM_000165)] sense (nucleotides-ACTCAACTGCTGGAGGGAAG) and antisense (nucleotides-GCACATGAGAGATTGGGAAA).

Assessment of anchorage-dependent and anchorage-independent cell growth

Anchorage-dependent growth rate was measured by determining the cell count. Cells were seeded in a 96-well plate (5.0×10^4 cells/well) and cultured. At each time point, the cells were trypsinized and counted. Anchorage-independent growth ability was determined by assessing colony formation. Briefly, a 0.6% agarose gel (bottom layer) was plated in a 6-well plate. Then, a 0.4% agarose gel (top layer) containing the cell culture (1,000 cells) was placed over the bottom layer. To prevent drying of the medium, culture media was added once a week. After 18 days, the number of cell colonies (each consisting of more than 20 cells) formed in the

three-dimensional gel were counted.

Immunoblot analysis

A total of 1.2×10^6 H28, H28-W, and H28-T cells were seeded in 60-mm dishes and cultured for 24 h. Cells were collected by scraping and then dissolved in ice-cold lysis buffer [10% glycerol, 10% β-mercaptoethanol, 0.5 mM phenylmethane sulfonyl fluoride solution, 2% SDS (Wako Pure Chemical Industry, Osaka), 1 mM sodium orthovanadate, 50 mM Tris-HCl (pH 6.5), and protease inhibitor cocktail]. Each sample, including 20 μg of protein, was electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. The membranes were then subjected to immunoblotting with polyclonal antibody for Cx43 (Zymed Laboratories, San Francisco, CA, USA) at a dilution of 1:500; then they were incubated with antiSrc (clone GD11; Upstate Biotechnology, Lake Placid, NY, USA), dilution 1:250; antiphospho-Src family (Tyr416; Cell Signaling Technology, Danvers, MA, USA), dilution 1:125; and finally, with anti β-actin antibodies, dilution 1:2000. Detection was accomplished using ECL™ Western Blotting Detection Reagents (Amersham Biosciences Corp, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver system (Atto, Tokyo). Protein bands were determined using densitometric analysis (Scion Image). Each protein density value was normalized to β-actin.

CDDP-induced cytotoxicity

CDDP is one of the chemotherapeutic agents used for treating MM patients and was, therefore, used here to investigate drug resistance. A total of 1.0×10^4 cells were seeded in a 96-well plate. After 24-h incubation, 5.0 – 100 μM CDDP (Wako Pure Chemical Industry) was added to each well, followed by culturing for 24 h. Finally, to assess the sensitivity of the cells to CDDP, cell viability was assessed using the Proliferation Reagent WST-1 (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Control cells were treated with 0.1% (v/v) dimethyl sulfoxide (vehicle for CDDP).

Cell cycle analysis

The cells were plated (2.0×10^5 cells per 60 mm dish) and cultured in normal medium for 24 h. The cells were then cultured in FBS-free medium to synchronize all cells in the G1 phase. After 16-h incubation, the cells were treated with 25 μM CDDP for each indicated time. The cells were then fixed in 70% ethanol for 30 min at 4°C and incubated for 30 min at room temperature in PBS containing 10 μg/ml propidium iodide and 100 U RNase A. The cell suspension was filtered by a nylon mesh filter, and the filtrate was analyzed using a FACS Calibur analyzer (Becton Dickinson Japan, Tokyo).

GJIC inhibition

To determine the influence of GJIC on CDDP-induced cytotoxicity, the cells were incubated with a GJ blocker, 18β-glycyrrhetinic acid (GA). After 4 h of culturing with GA (0.5 μM), CDDP (5 μM) treatment followed, and cell viability was then determined using WST-1 Reagent.

Short-interfering RNA (siRNA) treatment

In order to further clarify the inhibitory effect of Cx43 on CDDP-induced cytotoxicity, Cx43 expression was silenced by using siRNA targeting the Cx43 gene; the siRNAs used were Hs_GJA1_5 HP Validated siRNA and a negative control siRNA (AllStars Negative Control siRNA). Both siRNAs were obtained from Qiagen (Tokyo). Target sequences are not disclosed. For transfection, cells were seeded in a 12-well plate (1.5×10^5 cells/well) and transfected using FuGene HD Transfection Reagent (Roche Applied Science-Japan), according to the manufacturer's protocol. Cells were transfected for 24 h with a final concentration of 10 nM siRNA, subsequently trypsinized, and then used for real-time RT-PCR, WST-1 assay, and western blotting.

Scrape loading and dye transfer assay

The methodology followed was the same as that previously reported (22). Briefly, cells were seeded on a 35-mm glass base dish and cultured until they reached a semiconfluent state. GJ activity was determined by measuring the extent to which a low molecular weight fluorescent dye, Lucifer yellow, was transferred. Before adding Lucifer yellow, the cells were rinsed with PBS. Then, 0.05% Lucifer yellow dissolved in PBS (1 ml) was added to the cells before scrape loading at room temperature. The dye solution was left on the cells for 3 min, after which it was discarded and the dishes washed with PBS to remove detached cells and to eliminate background fluorescence. Finally, the cells were fixed with 4% neutral buffered formalin and examined under a fluorescence phase microscope (FV500; Olympus, Tokyo). To determine GJIC, the fluorescence spread of the cells was compared with that of the original scrape load.

Statistical analyses

The data were analyzed with one-way analysis of variance, followed by the Tukey-Kramer test. A P value of less than 0.05 was considered significant.

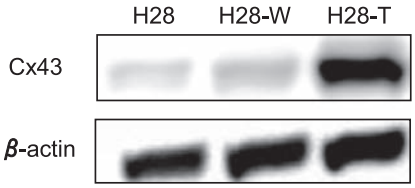


Fig. 1. Cx43 protein expression detected by western blotting. *β*-Actin was used as an internal standard.

Results

Restoration of Cx43 protein expression

Cx43 protein expression was detected in H28-T cells, although no expression was observed in parental H28 or H28-W cells (Fig. 1). Cx43 phosphorylation has been reported to result in distinct Cx43 forms, in the range of 41 – 43 kDa (22, 23). However, the Cx43 band from the H28-T cells was of a single molecular size. Immunohistochemical analysis showed that Cx43 was expressed as a dotted line on the plasma membranes of H28-T cells (data not shown), indicating that Cx43 induced GJ formation in H28 cells.

Suppressive effect of Cx43 against cell growth

The growth rate of H28-T cells was significantly slower than that of H28 and H28-W cells when the cells were attached to culture dishes (Fig. 2A). Moreover, the number of H28-T cells at 48 h was fewer than that at 24 h. It is suggested that some of the H28-T cells that were seeded into the plate wells could not survive and were discarded before a cell count was conducted. Similarly, a slow growth of H28-T cells was observed when the cells were seeded in the three-dimensional gel. However, the difference in growth rates was not significant (Fig. 2B).

Effect of Cx43 on CDDP-induced cytotoxicity

It was confirmed that H28 cells were resistant to CDDP; approximately 80% of cells survived after exposure to high concentrations of CDDP (Fig. 3A). H28-W cells showed a tendency similar to that of parental H28 cells. In contrast, H28-T cells were damaged when they were exposed to lower concentrations of CDDP, and only approximately half of the control cells survived after being treated with 50 μM of CDDP (Fig. 3A). To confirm the contribution of Cx43 to the enhancement of CDDP-induced damage, we knocked-down Cx43 expression by using specific siRNA. The knockdown efficacy is shown in Fig. 3B. Because of knockdown, sensitivity to CDDP was attenuated in H28-T cells treated with the Cx43-specific siRNA (Fig. 3C); this result supported the involvement of Cx43 in CDDP-induced damage.

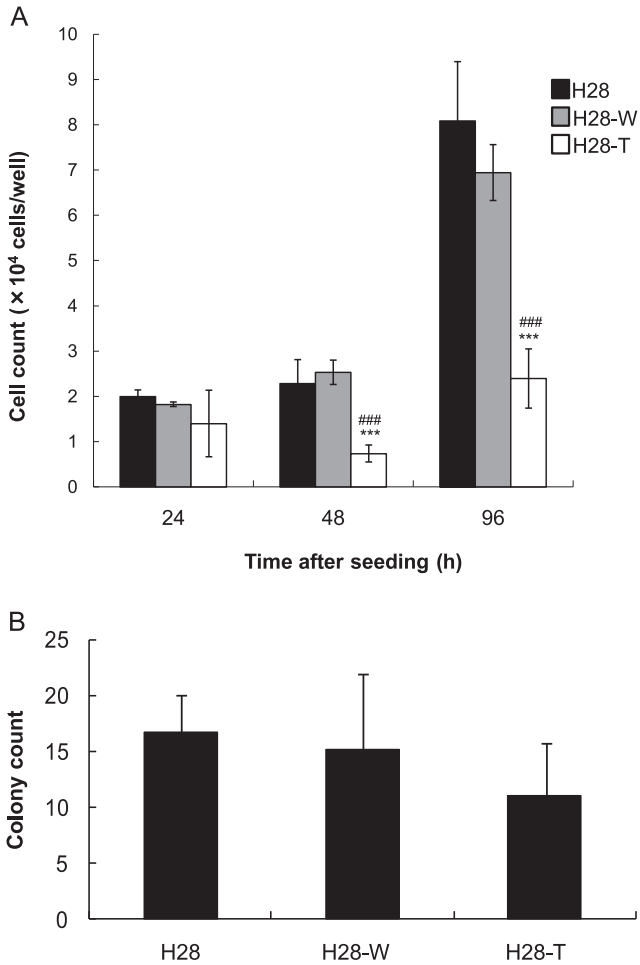


Fig. 2. Suppressive effect of Cx43 against cell growth. A: Cell growth rates measured by determining cell counts. After seeding, cells were trypsinized and counted at each time point. Data are expressed as the mean ± S.D. (n = 4). $^{###}P<0.001$: Significant difference from H28 cells, $***P<0.001$: Significant difference from H28-W cells, using the Tukey-Kramer test. B: Anchorage-independent cell growth measured using colony counts. Cells were seeded in a three-dimensional agarose gel in 6-well plates. Colony numbers in each well were counted 18 days after seeding. Data are expressed as the mean ± S.D. (n = 4).

Effect of GJ blockade experiments on CDDP-induced cytotoxicity

Scrape loading revealed a notable Lucifer yellow fluorescence gradient from the edge of the scraped area for H28-T cells but only at the periphery of the scraped areas for H28 and H28-W cells (Fig. 4). This indicated restoration of gap junctional function, that is, GJIC, in H28-T cells. Furthermore, to investigate the effect of GJIC, cells were incubated with GA before CDDP treatment; this incubation resulted in inhibition of the Lucifer yellow gradient (Fig. 5A). However, such

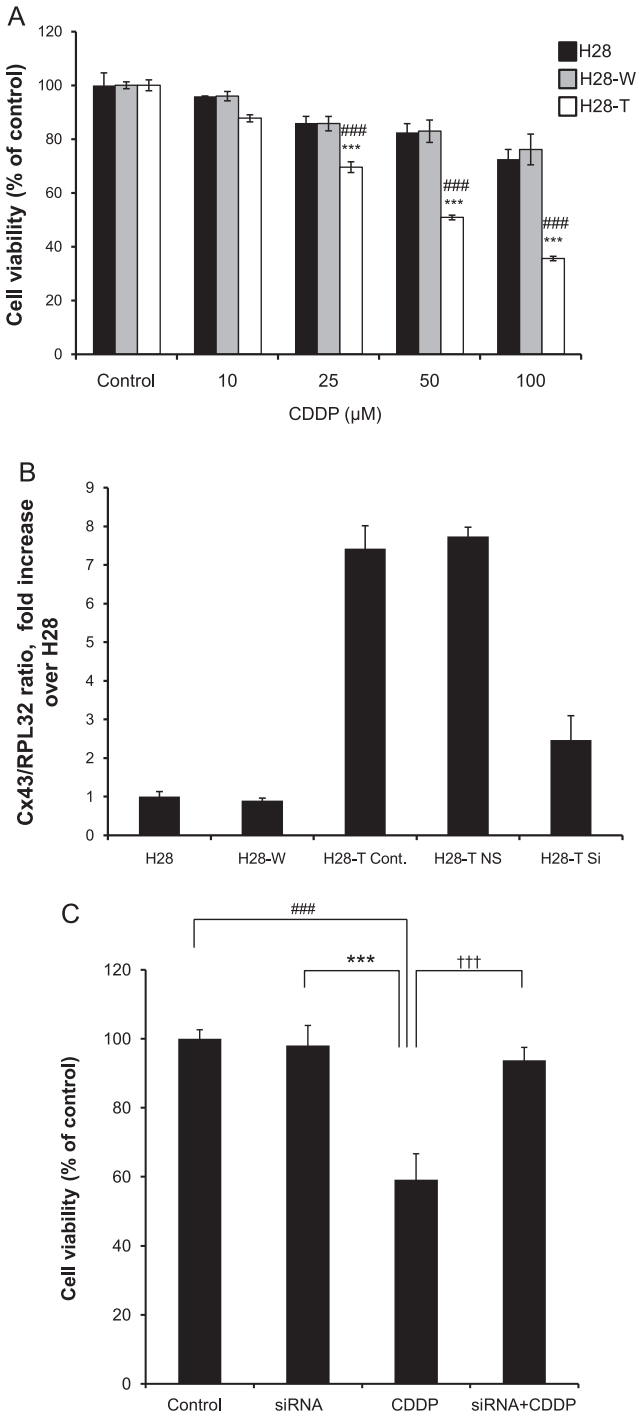


Fig. 3. Effect of Cx43 on CDDP-induced cytotoxicity. A: CDDP cytotoxic effect on H28, H28-W, and H28-T cells measured by a WST-1 assay. Cells were treated with CDDP for 24 h. Each bar represents the mean cell viability normalized to the control in each group, and vertical lines indicate S.D. (n = 6). ***P<0.001: Significant difference from H28, $^{###}P$<0.001: Significant difference from H28-W, using the Tukey-Kramer test. B: Cx43 mRNA expression determined by real-time RT-PCR after treatment with or without 10 nM siRNA. Each value represents the mean of four samples and is expressed as the fold-increase as compared to the expression in H28 control cells. RPL32 was used as an internal standard. H28-T cont.: H28-T control cell, H28-T NS: H28-T cell treated with negative control siRNA, H28-T Si: H28-T cell treated with validated Cx43-specific siRNA. C: Results of the WST-1 assay showing the changes in the CDDP (25 μM) cytotoxic effect on H28-T cells after a 4-h treatment with Cx43-specific siRNA. Data are expressed as the mean cell viability normalized to the control, and vertical lines indicate S.D. (n = 4). Control: treated with negative control siRNA, siRNA: treated with Cx43-specific siRNA, CDDP: treated with CDDP, siRNA + CDDP: treated first with siRNA followed by treatment with CDDP. $^{###}P$<0.001: Significant difference between Control and CDDP, ***P<0.001: Significant difference between siRNA and CDDP, and $^{†††}P$<0.001: Significant difference between CDDP and siRNA + CDDP, using the Tukey–Kramer test.

inhibition of GJIC had almost no effect on cytotoxicity (Fig. 5B).

Estimation of cell-cycle distribution and apoptosis

In both H28 and H28-T cells, an increase in the G_1-phase population was observed after CDDP treatment for more than 24 h (Fig. 6A). On the other hand, the S-phase population in H28-T cells was higher than that in H28 cells, both in the control and CDDP-treated groups (Fig. 6A). Next, the apoptotic cell population was estimated from the sub-G_1 peak. A higher sub-G_1 population was observed in H28-T cells than in H28 cells (Fig. 6B).

Effect of Cx43 on apoptosis factors

Proteins of the Bcl-2 family play an important role in the regulation of programmed cell death. Overexpression of Bcl-2, an anti-apoptotic factor, is associated with resistance to various cytotoxic agents, while the Bax protein is a pro-apoptotic member of the Bcl-2 family. In this study, no differences in the levels of Bcl-2 among H28, H28-W, and H28-T cells were noted. On the other hand, Bax expression was higher in H28-T cells than in parental H28 and H28-W cells (Fig. 7). Thus, the Bcl-2 family balance appeared to lean toward enhancing apoptosis in H28-T cells.

Effect of Cx43 on Src protein level and activity

Src activity is regulated by tyrosine phosphorylation. Phosphorylation of Tyr416 in the activation loop of the kinase domain upregulates enzyme activity. We assayed for Src levels and activation using antibodies that recognized total-Src and phospho-Src protein (an activated form, phosphorylated at Tyr416). Tyr416 antibody detects endogenous levels of the Src-family proteins, when phosphorylated at Tyr416. As shown in Fig. 8A, both phospho-Src and total-Src were decreased in H28-T cells, although there were no significant differences among cells. Then, we investigated whether the inhibi-

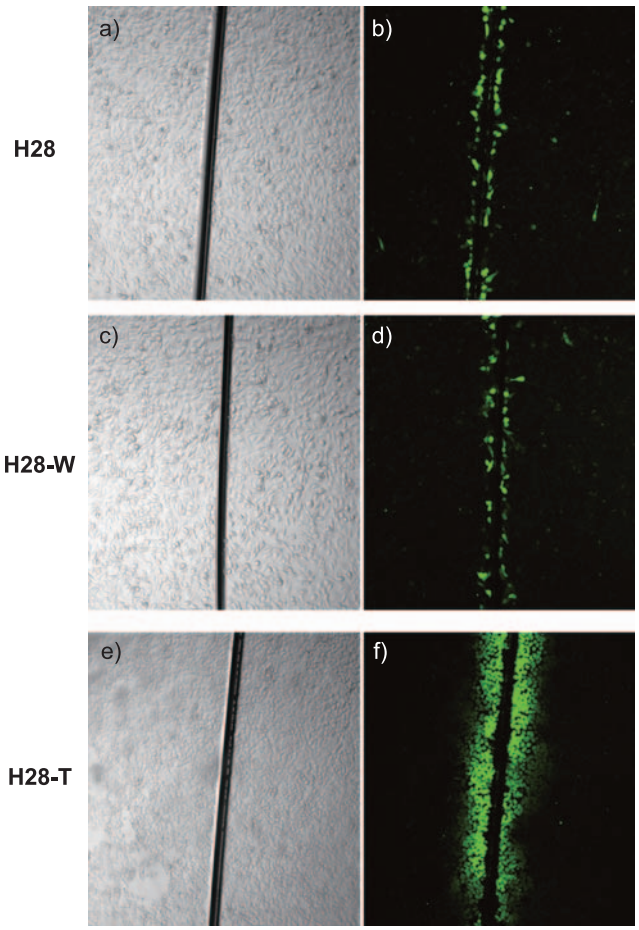


Fig. 4. Results of a scrape-loading assay showing the gap junctional intercellular communication (GJIC) in H28, H28-W, and H28-T cells cultured until confluence. GJIC was assessed using Lucifer yellow dye transfer as described in the main text. Left: phase-contrast pictures, right: dye transfer from the scrape lines. Magnification: ×100

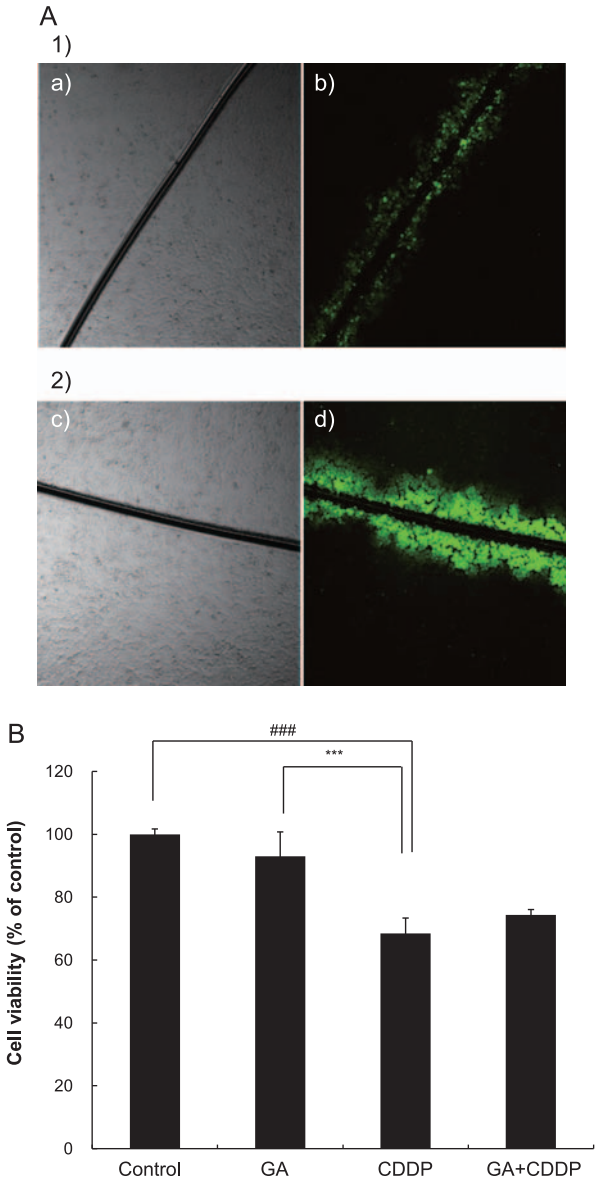
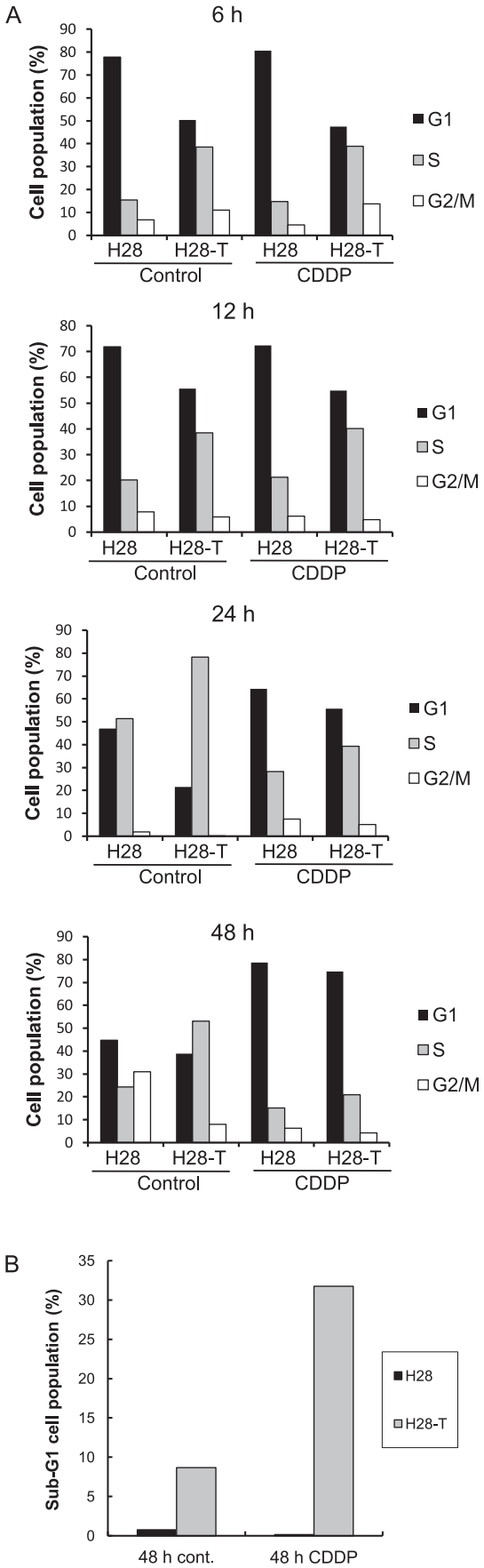


Fig. 5. Effect of GJ blockade on CDDP-induced cytotoxicity. A: GJ inhibitor (GA) downregulates GJIC. Cells were treated 1) with or 2) without GA for 4 h, and a scrape-loading assay was performed. a) and c) Phase-contrast image; b) and d) dye transfer of Lucifer yellow from the scrape lines. Magnification: ×100. B: Effect of GA on the CDDP cytotoxic effect measured by WST-1 assay. H28-T cells were treated with or without GA for 4 h, followed by CDDP treatment for 48 h. Data are expressed as the mean cell viability normalized to the control, and vertical lines indicate S.D. (n = 4). Control: Cells treated with only DMSO, GA: treated with GA, CDDP: cells treated with CDDP, GA + CDDP: cells treated with GA followed with CDDP. $^{###}P<0.001$: Significant difference between control and CDDP, $***P<0.001$: significant difference between GA and CDDP, using the Tukey–Kramer test.

tion of Src activity affects the cytotoxicity of CDDP in H28-W cells by using a specific Src kinase inhibitor, SU6656. We found that when the cells were treated with both CDDP and SU6656, cell viability significantly decreased as compared to that when the cells were treated with CDDP or SU6656 alone (Fig. 8B).

Discussion

The aim of this study was to determine whether Cx43 has an antitumor effect against MM cells. First, we evaluated the effect of Cx43 by using Cx43-transfected cells. As expected, when attached, the growth rate of H28-T cells was significantly slower than that of parental H28 and mock-transfected H28-W cells. Since there were fewer cells at 48 h than at 24 h after seeding, it seemed difficult for H28-T cells to survive immediately after passage. Therefore, we hypothesized that H28-T cells were intolerant in the nonattached condition. However, there was almost no difference between cells in terms of anchorage independency. This finding indicates that the proliferation ability of a single



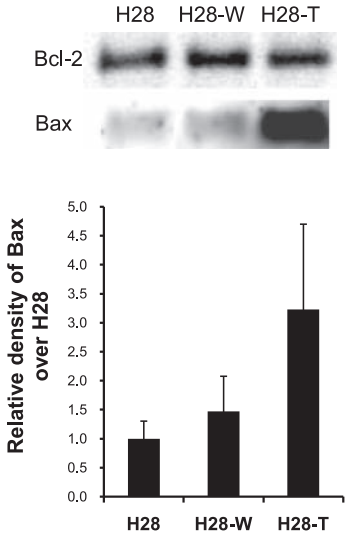


Fig. 7. Upper photos indicate Bcl-2–family protein expression detected by western blotting. β-Actin was used as the internal standard. Each photo is representative of two independent experiments. The lower graph indicates the densitometric data for the pro-apoptotic protein Bax (fold-increase over H28), n = 4.

cell, which is needed for survival in a nonmatrix environment, is almost the same between the different cell types used in this study. There was also no difference in migration ability (data not shown). These results suggest that restoring Cx43 expression alone cannot prevent tumor expansion or metastasis.

Next, we examined the combined effect of Cx43 and chemotherapeutic agents. We previously reported that the Cx gene (the molecule being Cx32) enhances the sensitivity of chemotherapeutic agents for renal cancer (11) and lung cancer cells (24). CDDP is one of the most effective chemotherapeutic agents for treating many malignancies, including MM; however, intrinsic or acquired resistance to CDDP often reduces this agent's efficacy. Fortunately, the result of a cell viability test showed that Cx43 significantly enhances CDDP cytotoxicity. We also observed that inhibition of GJ-dependent function with a specific inhibitor, GA, did not abrogate CDDP-induced cytotoxicity. These results suggest that Cx43-mediated enhancement of cytotoxicity

Fig. 6. Estimation of cell-cycle distribution and apoptosis. A: Changes in cell-cycle distribution in H28 and H28-T cells after stimulation with CDDP (25 μM). Cells were treated with CDDP for the indicated times, and 20,000 cells of each type were analyzed by flow cytometry. Control cells were treated with 0.1% (v/v) DMSO. B: Effect on the induction of apoptosis detected as the pre-G_0-G_1 position (sub-G_1 population) during the cell cycle of H28 and H28-T cells treated with CDDP for 48 h. Flow cytometry was used to analyze 20,000 cells of each type.

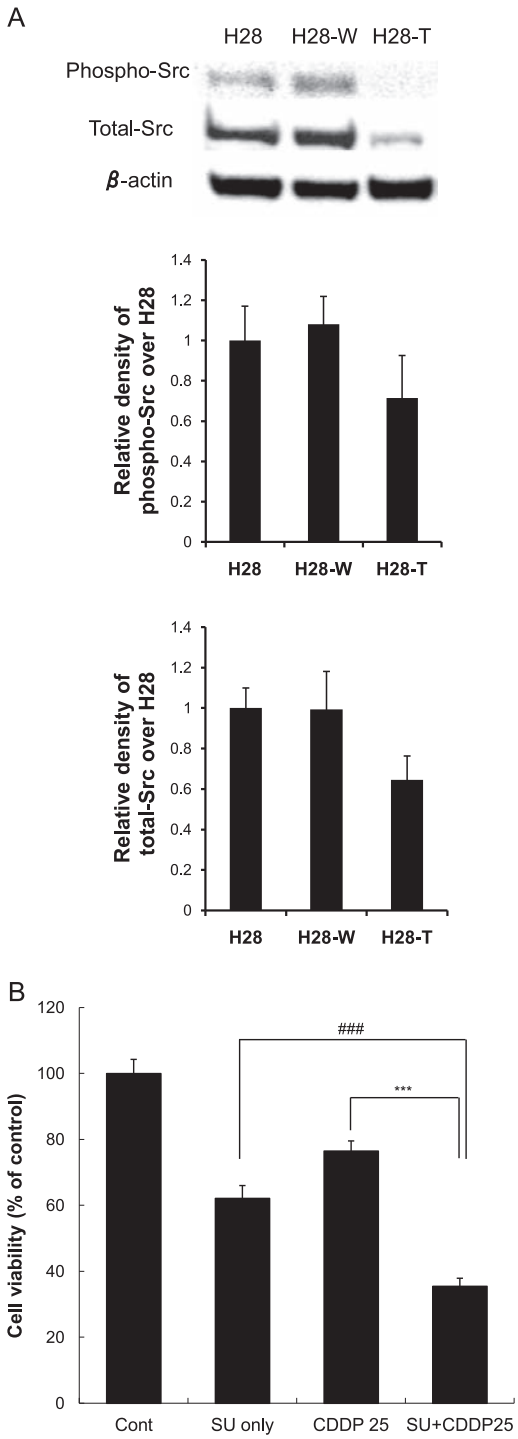


Fig. 8. Effect of Cx43 on Src protein level and activity. A: Upper images indicate Src protein expression detected by western blotting. Total-Src was detected using anti-Src, a clone GD11 antibody, and phospho-Src (active form) was detected by phospho-Src family (Tyr416) antibody. β-Actin was used as the internal standard. Each image is representative of six independent experiments. The middle graph indicates the densitometric data for phospho-Src, and the lower graph indicates the densitometric for total-Src, n = 6. B: Results of a WST-1 assay showing the effect of SU6656 on the CDDP cytotoxic effect. Cells were treated with CDDP (25 μM) or SU6656 (10 μM) or both for 24 h. Data are expressed as the mean cell viability normalized to the control, and vertical lines indicate S.E. (n = 4). Cont: Cells treated with only DMSO, SU only: cells treated with SU6656, CDDP 25: cells treated with CDDP, SU + CDDP 25: cells treated with both SU6656 and CDDP. $^{###}P<0.001$: Significant difference between SU and SU + CDDP 25, $^{***}P<0.001$: significant difference between CDDP 25 and SU + CDDP 25, using the Tukey–Kramer test.

is independent of GJ function.

Subsequently, we investigated whether Cx43 influences cell-cycle distribution. It has been reported that DNA modification by CDDP activates the G_1 checkpoint (25). This checkpoint exists to halt cell-cycle progression in the event of DNA damage to allow time for repair before initiating DNA replication (26). However, even those cells that retain the G_1 checkpoint eventually die when they are exposed to persistent treatment. Therefore, it is suggested that G_1 arrest represents a critical determinant of CDDP cytotoxicity (25). In the present study, G_1 arrest was observed in both H28 and H28-T cells. Therefore, the G_1 checkpoint does not appear to have broken down in these cells. As expected, an obvious induction of apoptosis was observed in H28-T cells. This observation might be explained by the additive effect of Cx43 on cell-cycle distribution. As shown in Fig. 6A, Cx43 causes an increase in the S-phase cell population, indicating that Cx43 induces S-phase arrest. Therefore, two surveillance mechanisms possibly work independently in the G_1 and S checkpoints, making it easy to suppress progression of the cell cycle and induce apoptosis in H28-T cells.

Next, we examined whether Cx43 affects Src, which is involved in multiple signaling cascades in cancer cells. Our results showed that not only phospho-Src level but also total-Src protein level was decreased in H28-T cells, suggesting that Cx43 somehow suppressed the Src protein expression, which in turn suppressed the total amount of its activated form. Interestingly, a previous report showed that some selective inhibitors of Src kinases are specific inhibitors of cell-cycle progression into the mid-S phase (27). Therefore, Cx43 may inhibit Src activation, which is reflected as S-phase arrest, resulting in the suppression of H28 cell proliferation.

It has also been indicated that simultaneous inhibition of Src and its downstream factor, signal transducer and activator (Stat) 3, results in synergistic death of MM cells (19, 28). Moreover, CDDP inactivates Stat 3 by modulating Janus kinase (JAK) 2 through dephosphorylation of JAK/Stat in cancer cells (29). In summary, inhibition of Src by Cx43 and inactivation of Stat 3 by

CDDP could induce synergistic death of MM cells.

Under the in vivo condition, Src upregulates vascular endothelial growth factor (VEGF), the most important factor in angiogenesis. Tumors must undergo angiogenesis for survival and for metastatic spread in a limited physiological environment (30). Indeed, a previous report showed that a novel Src inhibitor, M475271, significantly inhibited VEGF-induced HUVEC proliferation, migration (31), and angiogenesis (32). It is, therefore, possible that Cx43 has a more suppressive role in growth and metastasis in vivo, where angiogenesis always contributes to tumor survival.

On the other hand, the Bcl-2 family proteins are involved in most of the apoptosis pathways, so they are attractive targets for cancer therapy (33). A previous study reported little or no expression of Bcl-2 but high expression of Bcl-xL and a pro-apoptotic protein, Bax, in MM histological sections and cells (34). Our results showed a different trend: Bcl-2 but not Bcl-xL was detected (data not shown). However, the important finding was the balance between pro- and anti-apoptotic factors. Any approach that changes the balance in favor of apoptosis may have a therapeutic benefit. Our results suggest that Cx43 influences the balance between pro- and anti-apoptotic factors in the direction of apoptosis, possibly contributing to the improved sensitivity of cancer cells to CDDP. However, some studies have reported low Bcl-2 and high Bax expression in MM samples. A previous study helped the current understanding of apoptosis regulation by raising the following possibilities: one is Bax mutation, which makes it nonfunctional and blocks its pro-apoptotic effect (35); and the other is breaking out of antagonists to Bax as previously suggested (36). To clarify this issue, a functional assay of Bax in H28-T cells that also compares Bax function in H28-T cells with that in parental H28 cells is required.

In conclusion, we found that Cx43 could improve the chemoresistance of H28 cells to CDDP in a GJIC-independent manner. This finding will help in overcoming resistance to current chemotherapy for MM.

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