Baicalein increases the cytotoxicity of cisplatin by enhancing gap junction intercellular communication

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Abstract. Drug resistance limits the clinical application of cisplatin, a widely used chemotherapeutic agent. Gap junction (GJ) is a channel that enhances cytotoxicity of certain chemotherapeutic agents. Baicalein is well known for its antitumor activity. This study investigated the effect of baicalein on cisplatin cytotoxicity and the relationship between this effect and the modulation of the GJ function in connexin 26 (Cx26)-transfected HeLa cells. The sulforhodamine B (SRB) assay was used to examine the effect of baicalein on cell viability. A ‘parachute’ assay was used to investigate the effect of baicalein on GJ function. The effects of baicalein on cisplatin cytotoxicity and GJ function were assayed by standard colony-forming assays. The expression of Cx26 was monitored by western blotting. It was observed that exposure of Cx26-transfected cells to cisplatin reduced the number of colonies formed in low-density cultures (no GJ formation) and in high-density cultures (GJ formation), but the toxic effect was greater when cells were seeded at a high density. In the absence of connexin expression or with blockage of connexin channels however, cell density had no effect on cisplatin toxicity. Baicalein significantly enhanced cisplatin cytotoxicity, but this effect required the presence of functional GJs between the cells. In conclusion, the dependence of cisplatin toxicity on cell density is mediated by GJs. Baicalein increases cisplatin cytotoxicity through enhancing GJ intercellular communication.

Introduction

Cisplatin is a chemotherapeutic agent. It was approved by the Food and Drug Administration (FDA) in 1978 and has been widely used in the treatment of tumors, such as testicular, ovarian, head and neck, bladder, and small cell lung cancer.

Two disadvantages limit its clinical application: severe adverse drug reactions and drug resistance (1,2). Severe adverse drug reactions limit the clinical dose applied to patients, which in turn attenuates its effects. Drug resistance, including natural and acquired drug resistance, leads to failure of the chemotherapeutic treatment. It is thus important to improve the sensitivity of malignant tumors to chemotherapeutic agents, in order to reduce drug dosage and improve the efficacy of treatment.

Gap junction (GJ), a special channel composed of proteins known as connexins (Cx), connects adjacent cells, allowing for direct exchange of small hydrophilic molecules and ions less than 1-2 kDa in size, including metabolites and messengers such as sodium, potassium, calcium, cAMP/cGMP, ADP/ATP, and thereby resulting in the metabolic and electric coupling of cells (3). GJ can promote apoptosis induced by several chemical agents in tumor cells (4,5). Our previous study showed that the enhancement of cisplatin and etoposide cytotoxicity is dependent on the GJ intercellular communication (GJIC) (6). It was hypothesized that GJs may transmit ‘death signals’, i.e., the induced apoptotic or necrotic processes from one cell to neighboring cells, which is known as the ‘bystander effect’. In light of this, intercellular amplification of the death signals by enhancement of GJ formation or function would increase the cytotoxic action of cisplatin, and thus, cisplatin sensitization.

There are a few approaches to increase GJIC in tumor cells, including the use of certain plant-derived flavonoids (7,8). Baicalein is a flavonoid known to display antitumor effects (9-13). However, it remains unknown whether baicalein can improve GJ function and cisplatin cytotoxicity. This study aimed to investigate the effect of baicalein on the cytotoxicity of cisplatin and the relationship between this effect and the modulation of GJ function by baicalein in HeLa cells expressing Cx26. We found that baicalein significantly enhances cell coupling and cisplatin cytotoxicity only in the presence of functional GJs composed of Cx26. These results indicated that baicalein can increase the cytotoxicity of cisplatin through enhancement of GJ function.

Materials and methods

Drugs, antibodies and reagents. Baicalein was purchased from the China National Institutes for Food and Drug Control,
per donor cell was used as a measure of the GJIC. The average number of receiver cells containing calcein (magnification, x400) to count receiver cells containing calcein per donor cell. Images were captured of ~20 cells per well (magnification, a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Cells were rinsed and assessed for colony formation as described above. Cells in this condition were unable to form GJs, since there is no possibility to contact each other at such low density. To avoid discrepancy in results caused by the fact that cells are at different cell cycle phases, we incubated the cells in low-density condition, cells were seeded onto 6-well plates at a 500 cells/well density. After 4 h adherence, cells were exposed to cisplatin and then replenished with fresh medium. They were rinsed and assessed for colony formation as described above. Cells in this condition were unable to form GJs, since there is no possibility to contact each other at such low density.

Cell lines and culture. The HeLa cell line expressing Cx26 was previously described and characterized (14). In this cell line, obtained via transfection, Cx26 expression is under the control of a single bidirectional tetracycline-inducible promoter. Cx26 has a thrombin-cleavable C-terminal epitope tag (3.2 kDa) that includes an HA epitope. Cx26-expressing HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% newborn bovine serum, 100 µg/ml G418 sulfate, and 200 µg/ml hygromycin B at 37°C in a 5% CO₂, humidified incubator. Connexin expression was induced by incubation with 1 µg/ml doxycycline for 48 h prior to all experiments.

Sulforhodamine B (SRB) assay. The SRB colorimetric assay was used to measure the toxic effect of baicalein on cell viability (15). Cells were seeded onto 96-well plates for 24 h, followed by incubation with different concentrations of baicalein for 24 h, and were cultured for an additional 12 h. The culture medium was then removed and the cells were fixed with 50 µl of ice-cold 50% trichloroacetic acid solution at 4°C for 60 min, rinsed five times with tap water and dried at room temperature overnight. Next, 100 µl of 0.4% SRB solution was added to each well and incubated at room temperature for 30 min. Unbound dye was removed by washing five times with 1% acetic acid solution and drying at room temperature. A total of 10 mM Tris base solution (pH 10.5) was used to dissolve the protein-bound dye, and the plate was placed on a plate shaker for 15 min. The optical density (OD) at 570 nm was measured using a 96-well MRX plate reader (Dynex Technologies, Chantilly, VA, USA).

Gap junction dye-coupling (parachute) assay. Functional GJs were examined as described by Goldberg et al (16). Cells were grown to confluence in 12-well plates. Donor cells from one well were incubated with growth medium supplemented with a freshly made solution of 5 µmol/l calcein AM for 30 min at 37°C. Calcein AM is intracellularly converted into the GJ-permeable dye calcein. After three consecutive washes with phosphate-buffered saline (PBS) to remove excess dye, the donor cells were trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. They were allowed to form GJs for 4 h at 37°C and then examined under a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Images were captured of ~20 cells per well (magnification, x400) to count receiver cells containing calcein per donor cell. The average number of receiver cells containing calcein per donor cell was used as a measure of the GJIC.

Western blotting. Following three washes with ice-cold PBS, cells were lysed using lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 mM β-glycerophosphate, 1:1,000 protease inhibitors). The cell lysate was sonicated and centrifuged at 14,167 x g for 30 min at 4°C. The DC protein assay kit was used to determine the protein concentration (Bio-Rad, Hercules, CA, USA). A total of 20 µg of protein from each sample was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose blotting membranes, followed by immunoblotting. Monoclonal antibodies against HA IgG (1:1,000) or β-actin (1:10,000) were used. Immunopositive bands were visualized using the Amersham ECL™ Plus Western Blotting Detection kit (GE Healthcare).

Colony-forming assay. All experiments of cell exposure to cisplatin were performed in the dark at 37°C. Baicalein was dissolved in DMSO. When combined treatment with cisplatin and baicalein was performed, baicalein was added to the cells 3 h prior to cisplatin addition. The GJ inhibitor oleamide was added to the cell culture medium at a 50 µM concentration 3 h prior to the exposure to cisplatin.

Cisplatin toxicity was assessed by a standard colony forming assay as described by Jenser et al (5) with a few modifications. Briefly, cells were cultured at low (100 cells/cm²) or high density (30,000 cells/cm²), corresponding to conditions permissive of gap junction formation or not, respectively. For the high-density condition, cells were grown to confluence prior to cisplatin exposure. Following treatment with cisplatin for 1 h, cells were washed with PBS, harvested by trypsinization, counted, and seeded onto 6-well dishes at a 500 cells/well density. The cells were incubated for an additional 7-day period, and next fixed and stained with 1% crystal violet in ethanol. Colonies containing ≥50 cells were counted. Colony formation rates were normalized to the efficiency of colony formation of cells not treated with drugs (control). For the low-density condition, cells were seeded onto 6-well plates at a 100 cells/ml density. After 4 h adherence, cells were exposed to cisplatin and then replenished with fresh medium. They were rinsed and assessed for colony formation as described above. Cells in this condition were unable to form GJs, since there is no possibility to contact each other at such low density.

To avoid discrepancy in results caused by the fact that cells are at different cell cycle phases, we incubated the cells in serum-free medium for 24 h prior to the addition of cisplatin, to ensure cell synchronicity at the G1 phase.

Statistical analysis. Data from the experiments of different treatments were analyzed by unpaired Student's t-tests using the SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA). Data are presented as mean ± SEM. P<0.05 was considered to indicate statistically significant differences.

Results

Cisplatin cytotoxicity is cell density-dependent. GJ channels are formed by the end-to-end docking of two hemichannels in adjacent cells; GJIC occurs only when cells contact...
each other. As an initial experiment to determine the effect of GJIC on cisplatin toxicity, Cx26-expressing HeLa cells were cultured under conditions where GJ formation was possible (high density; 30,000 cells/cm²) or not (low density; 100 cells/cm²). In both conditions, cisplatin caused cell death in a concentration-dependent manner. The toxic effect of cisplatin is greater in these cells compared to those cultured at low density. Points, mean values from 6 independent experiments; bars, SEM; *P<0.05 compared to the control (non-treated cells); **P<0.05 compared to the low-density group.

Effects of cell density are due to GJIC. The formation of GJs is only one of the numerous differences between cells cultured at low and high density. To determine whether the effects of high cell density were due to GJIC, GJ coupling was examined in the cultures under different conditions of connexin expression and chemical inhibition.

First, connexin expression was studied in Cx26-expressing HeLa cells. Connexin expression was induced by incubation with 1 µg/ml doxycycline for 48 h (see Materials and methods). Fig. 2A and B show the expression of connexin and GJ dye coupling 48 h following exposure to doxycycline. The cells treated with doxycycline (expressing connexin, GJs formed) showed a markedly higher sensitivity to cisplatin at high compared to low density growth conditions. The surviving fraction decreased from 0.78±0.04 to 0.53±0.05, respectively. Notably, the inhibition of cell survival by cisplatin was not affected by addition of doxycycline in low density cultures (Fig. 3).
To further assess the role of GJIC on cell density-dependent cisplatin sensitivity, oleamide, a GJ channel inhibitor (18), was used to inhibit GJIC in HeLa cells, and its effect was assessed by the GJ dye-coupling assay (Fig. 4A and B). In low-density cultures (without GJIC), treatment with oleamide did not affect cisplatin toxicity. However, in high-density cultures (with GJIC), cisplatin toxicity was attenuated upon inhibition of GJIC by oleamide, manifested as a significant increase of the cell surviving fraction from 0.57±0.04 to 0.73±0.06 (Fig. 5). Thus, inhibition of GJIC during the 1 h of exposure to cisplatin reversed the effect of high density cell culture on cisplatin toxicity.

These results indicated that GJIC enhances cisplatin toxicity and fully accounts for the observed density-dependent effect of cisplatin toxicity.

Effects of baicalein on cell viability. The formation of GJs composed of connexin 26 (Cx26) affected the cytotoxicity of cisplatin. This observation was consistent with our hypothesis that enhancement of the GJ function would increase the cytotoxic action of cisplatin, and thus increase cisplatin sensitization. Would baicalein affect the GJ function and cisplatin cytotoxicity? To determine this, we first examined the cytotoxic effect of baicalein using the SRB assay.

As shown in Fig. 6, baicalein had no effect on cell viability until the concentration reached 100 µM. Thus, the concentrations of baicalein used in the following experiments (≤0.1 µM) had no cytotoxic effect on HeLa cells.

Effects of baicalein on GJ function. The effect of baicalein on dye coupling among cultured cells was assayed by the parachute assay. Donor cells were labeled with the junction-permeable dye calcein and then seeded onto unlabeled receiver cells with different concentrations of baicalein for 4 h. GJ intercellular communication was expressed as the number of receiver cells containing calcein from each donor cell, normalized to that of controls (non-treated cells). As shown in Fig. 7A and B, baicalein markedly increased the dye spread from donor cells to receiver cells in a dose-dependent manner. Fig. 8A and B show that the dye spread through GJs treated with 0.1 µM baicalein was markedly decreased from 4 to 24 h in Cx26-transfected HeLa cells. These results indicated that baicalein can enhance GJIC in Cx26-expressing HeLa cells.
for 3 h, followed by exposure to 0.5 µM cisplatin + baicalein for 1 h. The clonogenic survival of HeLa cells was assessed 7 days following exposure to cisplatin and baicalein. Baicalein enhanced cisplatin cytotoxicity in high-density cultures but had no effect in low-density cultures; the surviving fraction substantially decreased from 0.57±0.04 in low-density cultures to 0.34±0.04 in high-density cultures. This effect of baicalein was only observed in conditions permissive of GJ formation.

To further assess the role of GJIC in the improvement of cisplatin sensitivity induced by baicalein, oleamide was used to inhibit GJIC in HeLa cells (Fig. 9A and B). Consistent with our previous results, treatment with oleamide and baicalein did not affect cisplatin toxicity in low-density cell cultures (without GJIC). However, the improvement of cisplatin toxicity induced by baicalein was attenuated upon inhibition of GJIC by oleamide in high-density cultures (with GJIC). An increase of the surviving fraction, from 0.34±0.04 to 0.58±0.07, was observed (Fig. 10). These results showed that oleamide can inhibit the improvement of the dye spread through GJs induced...
Effects of baicalein on connexin expression. To determine whether baicalein affects the expression of connexin, the level of Cx26 was assessed by western blotting in cells induced by doxycycline and exposed to baicalein. Fig. 11 shows that treatment with baicalein (0.0125-0.1 µM) for 4 h did not affect Cx26 expression. Fig. 12 shows that treatment with baicalein (0.1 µM) for 4 to 24 h did not change Cx26 expression. These results suggested that the mechanism of baicalein-induced enhancement of GJIC does not involve an increase in the expression of connexin.

Discussion

Cx26 is widely and highly expressed in numerous tissues such as liver, mammary gland, skin and appendages, mucous membranes, pancreas, intestine, endometrium, lung and brain (19). GJs composed by Cx26 play an important role in the ‘bystander effect’. HeLa cells transfected with the herpes simplex virus thymidine kinase gene (HSV-tk) were more susceptible to death when expressing Cx26 and forming GJs. The bystander effect disappeared when GJIC was inhibited (20). This phenomenon was also observed in a bladder cancer cell line transfected with HSV-tk. Cx26 expression and the induction of functional GJs facilitated HSV-tk/ganciclovir (GCV) gene therapy through the bystander effect (21).

Importantly, we found that in high-density cultures, where there was substantial intercellular contact, baicalein enhanced the function of GJ and the cytotoxicity of cisplatin, with the cell surviving fraction decreasing from 0.57±0.04 to 0.34±0.04. This decrease was not observed in low-density cultures, which lacked intercellular contacts. The results of the experiments with the GJ inhibitor oleamide further supported the link between GJIC and the enhancement of cisplatin cytotoxicity. In high-density cultures, inhibition of GJIC via oleamide attenuated the improvement of cisplatin cytotoxicity induced by baicalein, with the surviving fraction significantly increasing from 0.34±0.04 to 0.58±0.07. By contrast, in low-density cultures, oleamide did not significantly alter the effects of baicalein. Our study provided the first evidence that the enhancement of cisplatin cytotoxicity by baicalein may be achieved through the enhancement of the GJ function in HeLa cells.

It is very common in clinical tumor therapy to combine chemotherapeutic agents to obtain additive or synergistic effects. Cisplatin is one of the most widely used cancer chemotherapeutic agents. Baicalein was also shown in recent studies to exert beneficial effects in clinic tumor therapy (9-11). While the underlying mechanism was not clarified, it was hypothesized that baicalein inhibits multi-drug resistance gene expression and decreases the level of the permeability glycoprotein (P-gp), thus increasing the intracellular concentration of chemotherapeutic agents (23). Our study found that the Cx26 channel may also mediate the improvement of cisplatin cytotoxicity induced by baicalein. We also found that in the presence of GJ, baicalein (0.1 µM) is not toxic to HeLa cells, but increases the cytotoxicity of cisplatin by ~23%, suggesting that when baicalein is combined with cisplatin, the dosage of the latter can be reduced without compromising the efficiency of its tumoricidal effect.

As for the mechanism of enhancement of GJIC induced by baicalein, our results showed that baicalein did not affect the expression of Cx26 at any tested concentration (0.0125-0.1 µM) or duration of treatment (4-24 h). Therefore, baicalein improved GJIC without changing the expression of Cx26. There are numerous factors modulating the activity of GJ channels. Cx26 is a connexin, and connexins have long been reported to be regulated by phosphorylation at serine and threonine residues (24,25). However, Cx26 is not a phosphoprotein (26,27). It is thus unlikely that baicalein can modulate the activity of Cx26-composed GJs by phosphorylation. A number of studies showed that flavonoids mostly influence GFJ function by changing the expression of connexin and its phosphorylation status (7,8,28,29). It was reported that two flavonoids, apigenin and

![Figure 12. Effect of treatment with 0.1 µM baicalein on connexin 26 (Cx26) expression in Cx26-expressing HeLa cells during the course of 4-24 h. Cells were induced with doxycycline and then treated with 0.1 µM baicalein for 4 to 24 h. (A) Cx26 expression assessed by western blotting, with β-actin used as the loading control. (B) Results from densitometric scanning of the western blots. Columns, scanning densities from 3 independent experiments; bars, SEM; P<0.05 compared to the control (non-treated cells).](image)
tangeretin, can counteract tumor promoter-induced inhibition of intercellular communication of rat liver epithelial cells without changing connexin 43 and in its phosphorylation state (7). Our results are similar to results of this study, but the mechanism by which baicalein enhances GJ function in HeLa cells was not elucidated. Nevertheless, our results demonstrated that baicalein enhances GJ function without changing connexin expression. Moreover, the present results also suggest that baicalein may be developed as a non-toxic chemo-adjuvant and could be used to increase the efficacy of existing anticancer chemotherapies by enhancing the GJ functionality.

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