Connexin-26 Is a Key Factor Mediating Gemcitabine Bystander Effect

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Abstract

Gemcitabine is a nucleoside analogue with anticancer activity. Inside the cell, it is sequentially phosphorylated to generate the active drug. Phosphorylated nucleoside analogues have been shown to traffic through gap junctions. We investigated the participation of gap junctional intercellular communication (GJIC) as a possible mechanism spreading gemcitabine cytotoxicity in pancreatic tumors. Immunohistochemical analysis of pancreatic cancer biopsies revealed increased connexin 26 (Cx26) content but loss of connexins 32 (Cx32) and 43 (Cx43) expression. Cx26 abundance in neoplastic areas was confirmed by Cx26 mRNA in situ hybridization. Heterogeneity on the expression levels and the localization of Cx26, Cx32, and Cx43 were identified in pancreatic cancer cells and found to be associated with the extent of GJIC, and correlated with gemcitabine bystander cytotoxic effect. The abundance of Cx26 at the contact points in tumoral regions prompted us to study the involvement of Cx26 in the GJIC of gemcitabine toxic metabolites and their influence on the antitumoral effects of gemcitabine. Knockdown of Cx26 led to decreased GJIC and reduced gemcitabine bystander killing whereas overexpression of Cx26 triggered increased GJIC and enhanced the gemcitabine cytotoxic bystander effect. Gemcitabine treatment of mice bearing tumors, with a high GJIC capacity, resulted in a significant delay in tumor progression. Interestingly, gemcitabine administration in mice bearing tumors that overexpress Cx26 triggered a dramatic tumor regression of 50% from the initial volume. This study shows that Cx26 participates in the gap junction–mediated bystander cytotoxic effect of gemcitabine and provides evidence that upregulation of Cx26 improves gemcitabine anticancer efficacy. Mol Cancer Ther; 10(3); 505–17. ©2011 AACR.

Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; dFdCyd) is a deoxycytidine analogue currently used as the standard chemotherapeutic agent for the treatment of pancreatic cancer. However, the antimtoral activity of gemcitabine varies from patient to patient and the development of drug resistance remains an important cause of low response rates and lack of efficacy in relapsed tumors (1–3).

Gemcitabine is transported into the cells by nucleoside transporters, where it is phosphorylated to its monophosphate by deoxycytidine kinase and subsequently to its active metabolites gemcitabine di- and triphosphate (dFdCDP and dFdCTP, respectively). In this sense, gemcitabine is a prodrug which requires cellular uptake and intracellular activation (4).

Ganciclovir (GCV) is another nucleoside analogue that needs to be phosphorylated to exert its cytotoxic effects. It has been widely shown that the herpes simplex virus thymidine kinase (TK) is highly efficient in triggering the first phosphorylation event to generate GCV-monophosphate, a key step in the anticancer effect of the TK/GCV prodrug-activated therapy (5). This TK/GCV system is probably the best documented example of a therapy that benefits from having a bystander effect mediated by the traffic of phosphate derivatives through gap junctions (6). In this line, it is known that TK/GCV has limited toxicity in gap junctional intercellular communication (GJIC)-deficient tumor cells with a low bystander effect whereas enhanced antitumor activity of the TK/GCV therapy can be achieved by favoring communication in poor communicating cells (7–9).

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Structure similarity between GCV and gemcitabine prompted us to hypothesize that gemcitabine might exert cytotoxicity in good communicating cells through a gap junction–mediated bystander effect. In that direction a recent study has shown that in fact gemcitabine cytotoxicity can be partially blocked in vitro in glioblastoma and osteosarcoma cells by a gap junction inhibitor (10).

Gap junctions connect the cytoplasms of apposed cells via an intercellular conduit formed by the docking of 2 hexameric hemichannels called connexons, where each hexamer is formed by the oligomerization of 6 connexins (11). Till now, only a few more than 20 different connexins have been identified in humans, found in a variety of tissues. Altered expression of connexins, such as reduced expression or aberrant localization, has been reported in many tumor tissues (12–14). However, accumulating evidences indicate that connexin 26 (Cx26), a connexin family member, is overexpressed in carcinomas of head and neck, colon, prostate, and pancreas (12, 15).

Alterations in GJIC impair the normal traffic of endogenous ions and signaling molecules as well as exogenous metabolites with a molecular weight smaller than 1 kDa. However, functional rescue of GJIC likely restores associated defects. In this sense, we have shown that reintroduction of Cx26 in pancreatic tumor cells results in increased gap junction plaque formation and this associates to improved TK/GCV bystander cytotoxic effect (7).

In this study, we have analyzed the involvement of Cx26 in gemcitabine bystander effect and whether by facilitating gap junction communication, through Cx26 overexpression, gemcitabine antitumor efficacy could be increased.

Materials and Methods

**Immunohistochemical assessment**

Five normal pancreas and 5 pancreatic ductal adenocarcinomas (PDAC) were studied. Surgical specimens were obtained as formalin-fixed paraffin-embedded tissues as previously indicated (16). Sections (5–micrometer thick) were submitted to standard deparaffin processing and treated with 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval. Sections were then incubated with streptavidin–peroxidase complex and diaminobenzidine as the substrate, according to the manufacturer’s instructions (LSAB+ Detection System; Dako). Sense or antisense riboprobe against Cx26 were generated by a 394 bp 3′UTR fragment of the Cx26 subcloned into the pGEM-TEasy vector (Promega). Digoxigenin-labeled probes were prepared by T7 or SP6 RNA polymerase, following the DIG RNA Labeling kit instructions (Roche). Five-micrometer thick sections of paraffin-embedded human pancreatic tissue were hybridized with 800 ng/mL of probe at 45°C for 16 hours in a standard hybridization buffer with 50% formamide. Alkaline phosphatase staining was done with the DIG Nucleic Acid Detection Kit following the manufacturer’s instructions (Roche). The specificity of hybridization was ascertained, under the same conditions, by a Cx26 probe that had the same length, guanosine-cytosine content, and the specific activity of the antisense probe. Five normal and PDAC specimens were examined.

**Cell culture**

The human pancreatic tumor cell lines MIA PaCa-2, Panc-1, and BxPC-3 and the neuroblastoma cell line N2A were obtained from the American Type Culture Collection (ATCC). Human pancreatic ductal epithelial (HPDE) cells were cultured and maintained as reported (17). NP-18 cells were derived from human pancreatic adenocarcinoma biopsies perpetuated as xenografts in nude mice (18). Cell lines obtained from ATCC were immediately expanded and frozen. Every 2 months, cells were plated again from a frozen vial of the original batch. HPDE and NP-18 cells were treated similarly to ATTC cells but were not authenticated by the authors. Inter-species contamination was tested by PCR routinely.

MIA PaCa-2, Panc-1, and BxPC-3 cells were maintained in Dulbecco’s Modified Eagle’s Medium whereas NP-18 cells were maintained in RPMI 1640 medium. Both mediums were supplemented with 10% FBS, and antibiotics (GIBCO-BRL).

NP-18, BxPC-3, and Panc-1 cells expressing short hairpin RNA (shRNA) against Cx26, or a shRNA with no target gene, were generated by lentiviral transduction with 100 to 200 µL of the viral stock shCx26 or shCt for 2 hours at 2,500 rpm plus overnight without centrifugation at 37°C. Twenty-four hours after transduction, cells were selected in 0.5 µg/mL puromycin (Sigma) for 3 days. The resistant clones were pooled and used for subsequent experiments. Lentiviral particles were generated by calcium-phosphate DNA precipitation transfection of vectors pSIV-G, pCMVΔ8.91, and pLKO.1-shRNACx26 (MISSION SHGLYC-TRCN0000059893; Sigma) or pLKO.1-shControl (MISSION SHC002, Sigma) into HEK293T cells (ATCC). At 24 and 48 hours, the viral supernatant was collected, concentrated by ultracentrifugation in a 4% sucrose buffer, and used for transduction.

Stable transfectants of NP-18 cells overexpressing Cx26 were previously generated as described in ref. 7. MIA PaCa-2 and BxPC-3 cells overexpressing Cx26 were obtained after retroviral transduction with pLXSN/Cx26. pLXSN/Cx26 retroviral particles were generated in HEK293T cells via an intercellular conduit formed by the docking of 2 hexameric hemichannels called connexons, where each hexamer is formed by the oligomerization of 6 connexins (11).
by calcium-phosphate DNA precipitation transfection of the retroviral vector pLXSN/Cx26 into the packaging cell line Phoenix Amphi (ATCC) and 48 hours later, viral supernatant was collected, filtered, and used for transduction. Cells were transduced with 2 mL of pLXSN/Cx26 virus-containing media in the presence of 8 µg/mL polybrene (Sigma) for 16 hours at 37°C. Forty-eight hours after the third transduction, cells were selected in 800 µg/mL G418 (Sigma) for 7 days. Resistant clones were pooled and used for subsequent experiments.

**Western blot analysis**

Cells were lysed in a buffer [10 mmol/L Na2HPO4 (pH 7.2), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS] containing 1% Complete Mini protease inhibitors (Roche Diagnostics GMBH) and phosphatase inhibitors (20 mmol/L Na4P2O7 and 100 mmol/L NaF). Sixty micrograms of total protein were resolved by electrophoresis on 12% or 8% SDS-PAGE gels (for Cx26 and Cx32 or Cx43, respectively) and transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were immunoblotted with rabbit anti-Cx26 (71-0500; Zymed Laboratories), mouse anti-Cx32 (Ci344; Sigma) or mouse anti-Cx43 (MAB3068; Chemicon International) overnight at 4°C. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or rabbit anti-mouse IgG antibodies (DakoCyto-Mation). Antibody labeling was detected by the Enhanced ChemoLuciminescent Method (Amersham Biosciences Inc.).

**Immunofluorescence**

Confluent cells grown on glass coverslips were fixed for 5 minutes at -20°C in acetone. Fixed cells were blocked with 5% nonfat milk in PBS 2 hours at room temperature, then incubated for 2 hours at room temperature with mouse anti-Cx26 (33-5800; Zymed Laboratories), mouse anti-Cx32 (C6344; Sigma) or mouse anti-Cx43 (MAB3068; Chemicon International) overnight at 4°C. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or rabbit anti-mouse IgG antibodies (DakoCyto-Mation). Antibody labeling was detected by the Enhanced ChemoLuciminescent Method (Amersham Biosciences Inc.).

**Gap junction function**

GJIC assays were carried out when cultures reached 90% to 100% confluence following a modified protocol of the method described by Kapoor and colleagues (19). Donor and recipient cell populations were differentially labeled for 30 minutes at 37°C with the gap junction-permeable dye calcine AM (50 nmol/L; Molecular Probe) and the lipophilic dye DiI (9 µmol/L; Molecular Probes) in complete medium, respectively. Donor cells were trypsinized and mixed with acceptor cells at different ratios for the indicated times (2-5 hours) at 37°C as stated in the figure legends. Cells were then harvested, resuspended in PBS, and subjected to FACS analysis. Cells were analyzed for the presence of calcine (green fluorescence), Dil (red fluorescence), or both by flow-cytometric analysis (Becton Dickinson).

**Bystander effect cytotoxic studies**

Different number of cells were seeded in 96-well plates to obtain the desired percentages in a final mixed population of 5,000 cells. When cells were attached, cultures were exposed to gemcitabine for 24 hours. Gemcitabine treatment doses were chosen to assure the mortality of at least 80% of the treated cells (60 nmol/L for NP-18 cell line; 100 nmol/L for BxPC3, PANC-1, and MIA PaCa-2 cells). After treatment, the number of untreated cells needed to obtain the different percentages (10%–75%) were seeded above treated cells and cocultured for 72 hours. Then, viability was assessed by MTT (Sigma). The percentage of viability was referred to untreated cells cocultured under the same conditions.

**Tumor growth studies**

Tumor xenografts were developed by subcutaneous injection of 7 × 106 NP-18 and NP-18 Cx26 cells into each posterior flank of BALB/c nude mice (Charles River France). Tumor volume was measured 3 times a week and was calculated according to the equation: V (mm³) = π/6 × L × W², where L and W are length and width of the tumor, respectively. Once tumors reached 50 to 70 mm³, mice were randomized (n = 8–10 per group), and 160 mg/kg of gemcitabine was administered intraperitoneally twice weekly, for 5 weeks. Two tumors of each treatment group were harvested 48 hours after the last treatment and fixed with paraformaldehyde or cryopreserved with Tissue-Tek O.C.T. (Sakura).

All animal procedures met the guidelines of European Community Directive 86/609/EEC, and were previously approved by the Local Ethical Committee.

**Histologic analysis of xenografts**

Tumors were excised and fixed overnight with 4% paraformaldehyde in PBS and submitted to standard paraffin processing. Five-micrometer sections were analyzed for standard histology, by staining with hematoxylin and eosin. Photomicrographs were obtained by a Leica DMR microscope.

**TUNEL assay**

TUNEL analysis was done by terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique by an in situ Cell Death Detection Kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Five-micrometer thick OCT-embedded sections were permeabilized and incubated with fluorescein-labeled deoxynucleotides and TdT at 37°C for 1 hour. Sections were mounted in Vectashield mounting medium (Vector Laboratories) with DAPI to counterstain.
the nuclei. Photomicrographs were obtained by a Leica DMR microscope.

**Statistical analysis**

Statistical analysis was done by SPSS software (SYSTAT Software Inc.). In the *in vitro* studies, the Mann–Whitney nonparametric test (2-tailed) was applied and $P < 0.05$ was taken as the level of significance. For the *in vivo* experiments, the General Linear Model for Repeated Measures test was used to estimate the effects of treatment on tumor growth (20). Comparisons between the different groups were established and $P < 0.05$ was considered statistically significant.

**Results**

**Connexin expression in pancreatic ductal adenocarcinomas**

In the pancreas, 4 major connexins, Cx26, Cx32, Cx43, and Cx36, participate in cell to cell communication via gap junctions channels for the control of insulin and amylase secretion (21). Cx26, Cx32, and Cx43 have been

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Figure 1. Expression of Cx26, Cx32, and Cx43 in normal and tumor tissue. A, immunohistochemical staining of Cx43, Cx32, and Cx26; scale bar, 50 μm. Insets show higher magnification images; scale bar, 25 μm. B, in situ hybridization showing Cx26 mRNA in tumor and nontumor samples; scale bar, 50 μm. Insets show higher magnification images; scale bar, 25 μm.
Figure 2. Expression of Cx26, Cx32, and Cx43 in BxPC-3, MIA PaCa-2, NP-18, and PANC-1 pancreatic cancer cell lines and GJIC analysis. A, Western blot expression analysis of Cx26, Cx32, and Cx43 from the indicated pancreatic cell lines and from HPDE cells. Actin and tubulin were used as internal loading control. B, immunofluorescence of Cx26, Cx32, and Cx43 stained with specific antibodies (green) and counterstained with DAPI (blue). White arrows point to gap junction plaques. Magnification, ×400. C, dye transfer flow cytometry assay. Donor cells were labeled with calcein AM and cocultured with acceptor cells, labeled with Dil, at a ratio of 1:4. After 2 hours at 37°C, cells were quantified for the presence of calcein, Dil, or both. Double-labeled cells are the communicating cells in this assay. The total number of counted events was 10,000. Representative dot plots. Donor cells, green; acceptor cells, red; communicating cells, blue; and clumps, purple. Quantification of communicating cells from 3 independent experiments ± SEM.
widely shown to be deregulated in a variety of tumors; therefore, we were interested in examining the expression of Cx26, Cx32, and Cx43 in PDAC and normal pancreatic tissues by immunohistochemical analysis (Fig. 1A). We detected a diffuse pattern of Cx26 expression with some punctuate areas in the epithelia of normal pancreatic ducts and acinar cells. Strong Cx26 expression was found in PanIN areas of PDAC with membranous and cytoplasmic staining. Strong immunoreactivity against Cx32 was detected in acinar and ductal cells of normal pancreas, but no Cx32 immunostaining was observed in any of the PDAC samples analyzed. Cx43 immunohistochemistry in normal pancreas revealed predominant nuclear expression of the protein whereas a complete loss of Cx43 was found in PDAC tissues.

Cx26 RNA in situ hybridization showed strong signal in PDAC but almost negligible in normal pancreas (Fig 1B).

These results confirm previous data showing upregulation of Cx26 protein in pancreatic carcinoma (15).

Furthermore, and in agreement with microarray analysis, the in situ hybridization studies show that this upregulation occurs at the RNA level. This might be specific of Cx26 because Cx32 and Cx43 expression were lost in PDAC tissue.

**Connexin expression and gap junction communication analysis in pancreatic cancer cell lines**

Expression of Cx26, Cx32, and Cx43 were then studied in the pancreatic cancer cell lines BxPC-3, MiaPaCa-2, NP-18, and PANC-1 and in HPDE cells derived from normal ductal epithelium. Western blot analysis revealed that all 4 tumor cell lines expressed Cx26; Cx32 was detected in MiaPaCa-2, NP-18, and PANC-1 but not in BxPC-3; and only BxPC-3 and NP-18 cells expressed Cx43. Different Cx43 phosphorylated isoforms were detected in NP-18 cells but only one of the major isoforms was present in BxPC-3 cells (Fig 2A). HPDE cells showed Cx26 and Cx43 expression but no Cx32. Immunofluorescent

![Figure 3. Bystander effect of gemcitabine (GE) in pancreatic cancer cell lines. Different ratios of GE-treated and -untreated cells were cocultured to determine the bystander effect. Cell viability (black) was determined 72 hours after GE treatment. In NP-18 (A) and PANC-1 (B) cells, 10%, 20%, and 30% of treated cells were cocultured and in BxPC-3 (C) and MiaPaCa-2 (D), 25%, 50%, and 75% of treated cells were cocultured. Bars represent the mean from 5 independent experiments ± SEM.](https://example.com/fig3.png)
to an increase in cell viability ranging from 8% to 15% at the different analyzed ratios (Fig. 4C). No changes on the bystander effect were observed in the NP-18 shCx26 cocultures (data not shown). The apparent discrepancy of the bystander effect when compared with shCt cells, leading to an increase in cell viability ranging from 8% to 15% at the different analyzed ratios (Fig. 4C). No changes on the GE bystander effect were observed in the NP-18 shCx26 cultures (data not shown). The apparent discrepancy of having a reduction in the GJIC of NP-18 shCx26 cells and the lack of GE bystander effect could be explained by the sensitivity of the different methods. It could be speculated that the Cx26 knockdown achieved in the transduction studies was insufficient to measure potential changes in GE bystander effect. On the contrary, we cannot discard the presence of a compensatory effect by Cx32 or Cx43 channels.

To further study the participation of Cx26 in the GJIC, NP-18, BxPC-3, and MIA PaCa-2 cells overexpressing Cx26 were generated by transduction with pLCx26 retrovirus (Fig. 5A). GJIC was assessed in the newly generated

**Bystander effect of gemcitabine in pancreatic tumor cells**

To explore the possibility that gemcitabine metabolites could traffic to neighboring cells through gap junctions, we set up an assay to assess the bystander effect of gemcitabine in all the mentioned pancreatic tumor cells. Gemcitabine sensitivity was measured by the MTT assay in cocultures in which only a percentage of cells (ranging from 10% to 75%) were treated with gemcitabine for 24 hours and then mixed with parental cells. As shown in Fig. 3, a strong bystander effect was observed in NP-18 cells. Indeed, 45% and 70% of the NP-18 cultures were killed when 20% and 30% of cells, respectively, were treated with gemcitabine (Fig. 3A). In PANC-1 cells, 30% and 38% cell death were counted in 20% and 30% of gemcitabine treated cultures (Fig. 3B). In BxPC-3 cultures, cell death rates of 32% and 52% were detected when 25% and 50% of the cultured cells were treated (Fig. 3C) No bystander effect was observed in MIA PaCa-2 cocultures with a percentage of cell death less than the percentage of treated cells at all the different ratios (Fig. 3D).

The experiments described earlier point toward a gemcitabine bystander effect in pancreatic tumor cells through gap junctions. Further supporting these data was the absence of a bystander effect in cocultures of NP-18, a pancreatic cancer cell line with high GJIC capacity, and the reference cell line N2A that do not form gap junctions (Supplementary Fig. S1).

**Dependence of GE bystander effect on Cx26 expression levels**

To further evaluate the involvement of connexins in the gap junction-mediated bystander effect of gemcitabine, we centered our studies on Cx26 because this connexin was found to be present in all the pancreatic cancer cell lines analyzed and highly expressed in cell–cell contacts of PanIN regions from tumor biopsies. Moreover, we have previously reported that Cx26 participates in the bystander effect of the TK/GCV system (7).

Knockdown of Cx26 in NP-18, BxPC-3, and PANC-1 cells was carried out by lentiviral transduction of shRNAs against Cx26 mRNA (shC26) or an shRNA against a nontarget sequence (shCt). Analysis of the protein levels showed downregulation of Cx26 content in the shCx26-transduced cultures (Fig. 4A). GJIC competence in shCx26 lines and shCt was evaluated by the dye-transfer assay. In these studies donor–acceptor ratios and coculture times were of 1:4 and 2 hours for NP-18, 1:4 and 3 hours 30minutes for BxPC-3, and 1:4 and 5 hours for PANC-1–derived cells. Data showed a statistically significant reduction in the percentage of coupled cells in the 3 cell lines with reduced Cx26 expression, suggesting the involvement of Cx26 in the GJIC of pancreatic cancer cells (Fig. 4B). We next studied whether the reduction in GJIC would have any impact on the gemcitabine bystander effect by doing coculture experiments similar to those described earlier in the text. BxPC-3 shC26 and PANC-1 shC26 pancreatic cancer cells showed a reduction in GE bystander effect when compared with shCt cells, leading to an increase in cell viability ranging from 8% to 15% at the different analyzed ratios (Fig. 4C). No changes on the GE bystander effect were observed in the NP-18 shCx26 cultures (data not shown). The apparent discrepancy of having a reduction in the GJIC of NP-18 shCx26 cells and the lack of GE bystander effect could be explained by the sensitivity of the different methods. It could be speculated that the Cx26 knockdown achieved in the transduction studies was insufficient to measure potential changes in GE bystander effect. On the contrary, we cannot discard the presence of a compensatory effect by Cx32 or Cx43 channels.
Cx26 lines and compared with that of the basal parental cells. The percentage of coupled cells was higher in the 3 cell lines overexpressing Cx26 when compared with their parental cells, although the increased efficiency varied in the different cell lines (Fig. 5B). NP-18 Cx26 cells showed a statistically significant increase of 1.2-fold in cell–cell communication when cocultured at a 1:5 ratio for 2 hours. BxPC-3 Cx26 and MIA PaCa-2 Cx26 cells presented a 2- and 3-fold increase in GJIC over the parental cells when cocultured at a 1:1 ratio for 3 hours 30 minutes and 5 hours, respectively. Interestingly, in all the Cx26-overexpressing cell lines a significant increase in the gemcitabine bystander effect was observed (Fig. 5C). The maximum effect was achieved in the NP-18 cell line where the treatment of only 10% of NP-18 cells led to 15% of the culture being killed whereas the gemcitabine treatment of 10% of NP-18 Cx26 cells killed 50% of the cells in the culture.

All these results indicate that Cx26 is actively participating in the GJIC of the pancreatic cancer cells enhancing cell communication. This enhancement improves the intercellular exchange of gemcitabine toxic metabolites resulting in higher tumor cell cytotoxicity.

**Antitumor effects of gemcitabine on NP-18 and NP-18 Cx26 tumors**

To evaluate the effect of Cx26 on gemcitabine antitumor activity, we assessed the tumorigenicity of NP-18 and NP-18 Cx26 in nude mice and did a follow-up of tumor growth on gemcitabine administration in preestablished NP-18 and NP-18 Cx26 xenografts. There were notable differences in the incidence of tumor formation. Although 95% of NP-18 tumors were formed 25 days after tumor cell inoculation, only 65% of NP-18 Cx26 tumors were detected. Moreover, at the end of the experiment, NP-18 Cx26 xenografts were smaller than the corresponding NP-18 (250 mm³ versus 400 mm³), indicating a clear suppressive effect of Cx26 overexpression on tumor growth.

Gemcitabine treatment was administered intraperitoneally twice a week for 5 consecutive weeks in a group of mice bearing NP-18 or NP-18 Cx26 tumors. Gemcitabine treatment showed a significant antitumor response in both types of tumors. In NP-18 tumors, a near-stabilization of the tumor growth was shown during all the treatment period (Fig. 6A). Importantly, in NP-18 Cx26 xenografts, the effect observed was much more dramatic,
reaching a 50% reduction of the initial tumor mass at the end of the treatment period (Fig 6A).

Histologic analysis of the tumors before treatment showed no major differences between both tumor types. However, a reduced number of tumor cells were seen in the tumor sections after gemcitabine treatment (Fig. 6B).

Extensive tumor cell apoptosis was observed in all gemcitabine-treated tumors although it was higher in NP-18 Cx26–treated xenografts, in good correlation with the observed growth reduction (Fig. 6C).

Taken together, these results indicate that gemcitabine antitumoral response can be potentiated by Cx26 overexpression, probably by facilitating the spread of the toxic metabolites in the tumor mass through a gap junction–mediated bystander effect.

Discussion

Gemcitabine chemotherapy is currently considered to be the standard therapy for pancreatic cancer. However, clinical reports indicate limited effects in treated patients. Alterations in the uptake, or impairment at several enzymes in the metabolic pathway of gemcitabine such as equilibrative transports, cytidine deaminase, or deoxycytidine kinase have been reported as causes for this incomplete response in preclinical models and clinical samples (22–24). Another important contributing factor
Figure 6. Tumor growth and gemcitabine (GE) sensitivity of subcutaneous NP-18 and NP-18 Cx26 tumors. Established tumors were treated after reaching 70 mm³. GE (160 mg/kg) or saline were intraperitoneally administered twice a week for 5 consecutive weeks. Arrows indicate the start and the end of treatment. A, NP-18 group injected with saline (●) and GE (▲). NP-18 Cx26 group injected with saline (●) and GE (▲). Data are expressed as percentage of growth of each group (n = 8–10). Bars, mean ± SEM. *, P < 0.05; ***, P < 0.005. Analysis of tumor sections obtained 48 hours after the last GE dose. B, histologic analysis of tissue preparations by hematoxylin and eosin staining: NP-18 control (a) and GE-treated tumors (b); NP-18 Cx26 control (c) and GE-treated tumors (d). Magnification, ×200. Black arrows indicate tumor cells. White arrows point to stromal cells. C, TUNEL detection of cell death by TdT-terminal staining of OCT-embedded sections. TUNEL-positive cells were detected with a FITC-conjugated antibody (a, c, e, g) and tissue samples were counterstained with DAPI (b, d, f, h). Representative sections from NP-18 control (a and b) and GE-treated tumors (c and d); NP-18 Cx26 control (e and f) and GE-treated tumors (g and h). Magnification, ×200. Quantification of TUNEL-positive cells.
might arise from a poor distribution of gemcitabine in the tumor mass that will limit gemcitabine activity in areas close to tumor vessels or in the peritoneal surfaces when administered intraperitoneally (25).

A well-known phenomenon of neighboring cells in a tissue is that they establish gap junction channels to allow for the direct transfer of small molecules, thus facilitating cell–cell communication by the spreading of metabolites in the tissue. This has also been shown to be a mechanism governing the transfer of exogenous small molecules such as GCV toxic metabolites in the suicide gene therapy TK/GCV (6). The cytotoxicity coming from this bystander effect has been shown to have a great influence on the tumor response to the TK/GCV therapy. In this study, we point out that gemcitabine possesses a cytotoxic bystander toxic effect that is also mediated by GJIC in which Cx26 is a key contributing factor. Moreover, we show that by upregulating Cx26, in pancreatic tumor cells, gemcitabine cytotoxic effect can be increased and can trigger improved antitumor responses.

The experimental evidence that gemcitabine metabolites can traffic through gap junctions was shown in the bystander culture studies carried out in 4 cell lines with different degree of GJIC communication. On the basis of the dye transfer assay, cells that present high communication such as NP-18 showed an important bystander cytotoxic effect. Indeed, a 45% of cytotoxicity was obtained, when only 20% of the cells were treated with gemcitabine. Whereas in cell lines with intermediate communication ability such as BxPC-3 or PANC-1 cells a 32% and 30% of cytotoxicity was respectively obtained, when only 20% of the cells were treated with gemcitabine. Recently, in cell lines with intermediate communication ability such as HxPC-3 or PANC-1 cells, a 32% and 30% of cytotoxicity was achieved, respectively, in MIA PaCa-2 cells this effect was not observed. Expression analysis of 3 major connexins found in pancreatic tissue revealed that NP-18 expressed and correlated with elevated mRNA levels, probably indicating enhanced transcription or increased mRNA stability. Several studies have shown that connexins are abnormally localized in cancer cells and accumulate in the cytoplasm. Accumulation of Cx26 in the cytoplasm has been associated with lung metastasis in colorectal cancer (12) and with poor prognosis in lung squamous cell carcinoma and breast carcinoma (26, 27). Many studies point a role of Cx26 in tumorigenesis that might be independent of GJIC. Supporting this model was the correlation found with Cx26 expression and insulin-like growth factor I receptor in active proliferation and inhibition of apoptosis (28). However, GJIC-mediated effects cannot be discarded, as gap junction formation can also be formed with heterologous cells. In this line, Cx26 has been shown to form gap junctions between melanoma cancer cells and vascular endothelial cells, suggesting that transmission of certain molecules derived from cancer cells might facilitate vascular invasion (29). Such observation is of interest to our work because it could be speculated that similarly to endogenous molecules, the traffic of gemcitabine toxic metabolites could take place thorough heterologous gap junctions, thus facilitating gemcitabine response at distant metastasis. In fact our results show that in pancreatic tumor cells Cx26 overexpression improves GJIC leading to increased gemcitabine bystander effect. Importantly, all 3 Cx26-overexpressing cells showed a stronger bystander effect than their corresponding parental cell lines, this effect being more dramatic in NP-18 cells. The enhanced bystander effect observed in MIA PaCa-2 cells will be in line with recent observations indicating that a very limited number of gap junctions can be highly functional and sensitize for a TK/GCV therapy (30). Furthermore, our data from in vivo assays show that gemcitabine treatment stopped tumor progression in highly communicating NP-18 tumors. Of major significance is the fact that in NP-18 Cx26 xenografts, gemcitabine treatment provoked a reduction in tumor growth which could be at least partially explained by a better distribution of the cytotoxic gemcitabine metabolites into the tumor mass. Other factors contributing to the increased gemcitabine response in NP-18 Cx26 xenografts could rely on the gap junction–mediated cell to cell transfer of other signaling molecules causing apoptosis or necrosis. The transfer of molecules trough gap junctions between cells is also a function of the kind of connexin that composed the channel. Permeability/selectivity to exogenous or endogenous solutes has been found to be specific. In our models, we observed that overexpression of Cx26 increased gemcitabine bystander effect. In such situation, an increase in Cx26 channels might occur but because all cell lines expressed other connexins, we cannot discard that heteromeric channels composed of more than one connexin were established, contributing to the transfer of gemcitabine metabolites. Phosphorylated gemcitabine compounds will be negatively charged, suggesting that the connexin composition of the channel should be permeable to them. Cx26 channels have been reported to be permeable to anionic molecules (31, 32). Moreover, the recently resolved crystal structure of the pore architecture of...
Cx26 gap junctions revealed a group of positively charged residues concentrated around the entrance what would favor the accumulation of negatively charged molecules that could be pulled into the cells (33, 34). This is of relevance because it has been shown that the negatively polarity of Cx32 can be a contributing factor to the limited permselectivity of phosphorylated adenosine nucleosides. The addition of phosphate to adenosine shifts its relative permeability from channels formed by Cx32 to channels of Cx43 (35). Although additional studies are needed to specifically elucidate the permselectivity of gap junctions channels to gemcitabine metabolites, available data suggest that homomeric Cx26 or specific Cx combinations could be compatible.

The overall data suggest that the upregulation of at least Cx26 can improve gemcitabine therapy in vivo. In line with these data there have been reported observations showing that the histone deacetylases inhibitor 4-phenylbutyrate, an agent known to increase gap junction communication capacity, enhances gemcitabine-mediated apoptosis in pancreatic tumor cells (36). In this sense, another pharmacologic agent that is known to enhance GJIC and has proved benefits to gemcitabine treatment is tamoxifen. In a phase II study conducted to assess the benefit of the gemcitabine and tamoxifen in the treatment of pancreatic cancer, a partial response was achieved in 11% of patients whereas 48% experienced stable disease, lasting at least 8 weeks (37). Although the aim of this clinical trial was to combine chemotherapy with endocrine therapy, it cannot be discarded that some of the clinical benefits described might have arisen from the GJIC modulation of tamoxifen, and consequently, an increase of the bystander effect of gemcitabine.

In conclusion, our study found that Cx26 is a relevant connexin participating in the gap junction-mediated bystander effect of gemcitabine and that the increase in GJIC-mediated by Cx26 enhances the antitumoral effect of gemcitabine chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest were disclosed.

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