Combination of non-viral connexin 43 gene therapy and docetaxel inhibits the growth of human prostate cancer in mice

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Abstract. Docetaxel (DTX) is used for the treatment of advanced hormone refractory prostate cancer. Connexin 43 (Cx43) is a tumor suppressor gene, and transfection of the Cx43 gene increases sensitivity to several chemotherapeutic agents. The objective of this study was to evaluate the effectiveness of combination therapy of Cx43-expressing plasmid DNA (pCMV-Cx43) and DTX both in vitro and in vivo using a non-viral vector in human prostate cancer PC-3 cells. Transfection of pCMV-Cx43 into the cells neither inhibited tumor growth nor increased gap junctional intercellular communication; however, combination therapy of pCMV-Cx43 and DTX significantly inhibited cell growth. Forced expression of Cx43 in the cells induced apoptotic cells by down-regulation of Bcl-2 expression and significantly more up-regulation of caspase-3 activity than either treatment alone. The combination of repeated intratumoral injection of pCMV-Cx43 (10 μg/tumor) with non-viral vector and a single intravenous injection of DTX (15 mg/kg) was compared with a repeated injection of Cx43 alone and a single injection of DTX alone on PC-3 tumor xenografts. Significant antitumoral effects were observed in mice receiving combined treatment, compared with DTX alone. The data presented here provide a rational strategy for treating patients with advanced hormone refractory prostate cancer.

Introduction
Prostate cancer is a significant problem and is reported to be the leading cancer diagnosed in man (1). Cytotoxic chemotherapy has shown significant palliative benefit in the treatment of androgen-independent prostate cancer, but with no survival advantage demonstrated to date (2). Current chemotherapy is limited by drug tolerance and the ultimate emergence of resistant disease (3). Novel approaches incorporating potentially more active and less toxic agents that may overcome drug resistance mechanisms need to be investigated. Increased understanding of the tumor biology of prostate cancer offers promise of novel treatments for this disease.

Docetaxel (DTX), a member of the taxane family, is semisynthesized from an inactive taxoid precursor extracted from the needles of the European yew, Taxus baccata. DTX has shown clinical activity in a wide spectrum of solid tumors including breast, lung, ovarian, and prostate cancers (4,5). The known basic cellular target of DTX is the microtubule. Furthermore, DTX down-regulates genes for cell proliferation, mitotic spindle formation, transcription factor, and oncogenesis, and up-regulates genes related to the induction of apoptosis and cell cycle arrest in prostate cancer PC-3 and LNCaP cells (6,7).

Connexins (Cxs) are a family of transmembrane proteins that enable gap junctional intercellular communication (GJIC) (8). GJIC is one mechanism of growth control that involves cell-cell contact (9). In general, cancer cells exhibit altered Cxs expression, with a profile that is often significantly reduced or undetectable. Connexin 43 (Cx43) and connexin 32 (Cx32) expressions were reduced in prostate tumor biopsy in contrast to normal prostate epithelial cells (10-13). Since Cx43 is a tumor-suppressor gene, Cx43 gene therapy was reported (14-16). Transfection of Cx43 in human mammary carcinoma MDA-MB-435 cells (17), human glioblastoma U251 and T98G cells (16), lung cancer PG cells (18) and prostate cancer LNCaP cells (19) significantly reduces cell growth in vitro and/or in vivo. Transfection of Cx43 in human glioblastoma U251 cells (20) and ovarian carcinoma SKOV-3 cells (21) increased sensitivity to several chemotherapeutic agents. However, the effect of transfection of the Cx genes combined with DTX on prostate tumor PC-3 cells has not been reported to our knowledge.

In this study, we investigated whether the transfection of plasmid DNA (pCMV-Cx43) coding for the Cx43 gene by non-viral vector combined with DTX increased the inhibition of PC-3 cell growth. A novel combination of pCMV-Cx43 and DTX induced significantly greater growth inhibition in PC-3 cells and tumor xenografts than DTX alone. This combination increased apoptosis via the down-regulation of Bcl-2 expression and up-regulation of caspase-3 activity.

Materials and methods
Cell culture. PC-3 and LNCaP cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University.
PC-3 and LNCaP cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (100 μg/ml) at 37°C in a 5% CO₂ humidified atmosphere.

**Plasmid constructions.** Plasmid pCMV-Cx43 encoding the Cx43 gene under the control of CMV promoter was constructed as previously described (22). Plasmid pCMV-luc encoding the luciferase gene under the control of the CMV promoter was constructed as previously described (23). pGL3-basic (Promega, Madison, WI) was used as a control plasmid. A protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany).

**Sensitivity to DTX assay.** PC-3 and LNCaP cells were seeded separately at a density of 1x10⁶ cells per well in 96-well plates and maintained for 24 h before transfection in RPMI medium supplemented with 10% FBS. Cells at 30% confluence in the well were transfected with 0.2 μg of pCMV-Cx43 or pGL3-basic using lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacture's instructions and incubated for 24 h. The culture medium was then exchanged to medium containing various concentrations of DTX (Taxotere, Sanofi-Aventis, Paris, France) ranging from 0.1 to 1,000 ng/ml and incubated for another 48 h. In co-transfection, cells were transfected with 0.2 μg of pCMV-Cx43 or pGL3-basic using lipofectamine 2000 in medium containing DTX (0.1-1,000 ng/ml) and incubated for 72 h. The cell number was determined with WST-8 assay (Dojindo Laboratories, Kumamoto, Japan).

**Fluorescent dye transfer.** FACS analysis of the GJIC reported by Robe et al (24) was modified. Briefly, cells grown in 35-mm dishes were labeled for 1-h incubation with either 5 μM calcine-AM (acetomethyl ester, Dojindo) or 5 μM DiI (Lambda Probes & Diagnostics, Graz, Austria) in the medium. The two labeled cells were mixed in equal proportions in 35-mm dishes and incubated for 12 h. Subsequently, pCMV-Cx43 or pGL3-basic was transfected into mixed cells in the presence or absence of 50 μM 18-glucyrhethinic acid (18GA, MP Biomedicals, Germany). After 24-h incubation, the cells were trypsinized, washed in phosphate-buffered saline pH 7.4 (PBS), and processed for FACS analysis of calcine-AM and DiI fluorescence with a FACSCalibur flow cytometer as previously reported (22). Data for 10,000 fluorescent events were obtained by calcine-AM fluorescence (530/30 nm) and DiI fluorescence (585/42).

**Cell cycle analysis.** PC-3 cells were seeded at a density of 1x10⁶ cells on 35-mm dishes. Cells were transfected with pCMV-Cx43 or pGL3-basic in the presence or absence of 10 ng/ml DTX in medium. After 24-h incubation, the cells were harvested with EDTA after washing with ice-cold PBS. Detached cells were washed once with ice-cold PBS and gently suspended in PBS-EtOH (70%) and fixed overnight at 4°C. For staining, fixed cells were washed once in PBS and then resuspended in PBS with 50 μg/ml propidium iodide (PI) and 0.5% RNase A. After 30 min at 37°C, cells were processed for FACS analysis of PI fluorescence by a FACSCalibur flow cytometer as described in the above section.

**Western blotting.** PC-3 cells were seeded at a density of 1x10⁶ cells on 35-mm dishes. Cells were transfected with pGL3-basic or pCMV-Cx43, respectively. Twenty-four hours after transfection, the culture medium was replaced with medium containing 10 ng/ml DTX and incubated for 24 h. Cells were suspended in lysis buffer (1% Triton-X 100 and protease inhibitor cocktail set III (Calbiochem, Darmstadt, Germany) in PBS), and then centrifuged at 15,000 rpm for 10 min. The supernatants were resolved on a 15% sodium dodecyl sulphate-polyacrylamide gel by electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (FluoroTrans® W, PALL Gelman Laboratory, Ann Arbor, MI). Expression of Cx43, Bcl-2 and β-actin protein was identified using rabbit anti-Cx43 polyclonal antibody (Sigma, St. Louis, MO), rabbit anti-Bcl-2 polyclonal antibody (Stressgen, Canada) or rabbit anti-β-actin polyclonal antibody (Lab Vision, CA), respectively. Goat anti-rabbit IgG peroxidase conjugate (Santa Cruz Biotechnologie, Inc., Santa Cruz, CA) was used as the secondary antibody. These proteins were detected with peroxidase-induced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce). Optical density of the bands on the film was quantified using ImageQuant TL (Amersham Biosciences, NJ) with correction for the optical density of the corresponding β-actin band.

**Apoptosis analysis.** PC-3 cells were seeded at a density of 1x10⁶ cells on 35-mm dishes. The cells were transfected with pGL3-basic or pCMV-Cx43, respectively. Twenty-four hours after transfection, the culture medium was replaced with medium containing 10 ng/ml DTX and incubated for 24 h. Apoptotic cells were detected with an annexin V-FITC apoptosis detection kit (Sigma) or caspase-3 apoptosis detection kit (Santa Cruz Biotechnologie) according to the manufacturer’s instructions.

**Assessment of PC-3 tumor growth.** For transfection in vitro, we prepared cationic nanoparticle (NP) as previously reported (25). Briefly, NP was formulated using 1 mg/ml cholesteryl-N-hydroxyethylamine (OH-Chol) as a cationic lipid, and 5 mol% Tween-80, and was prepared in 10 ml of water by the modified ethanol injection method (25).

Male BALB/c nu/nu mice (6-8 weeks of age) were purchased from CLEA Japan Inc. (Tokyo, Japan). To generate PC-3 tumor xenografts, 1x10⁶ PC-3 cells suspended in 50 ml of medium containing 60% reconstituted basement membrane (Matrigel: Collaborative Research, Bedford, MA) were inoculated subcutaneously into the flank region of the mice. Tumor volume was calculated using the formula, tumor volume = 0.5ab², where a and b are the larger and smaller diameters, respectively. When the average volume of PC-3 xenograft tumors reached 200 mm³ (day 0), these mice were selected for treatment with DTX alone, pGL3-basic, pCMV-Cx43, pGL3-basic plus DTX, and pCMV-Cx43 plus DTX. For transfection into tumors, the nanoplex was formed by the addition of NP (15.8 μl) to 10 μg of pCMV-Cx43 or pGL3-basic with gentle shaking and standing at room temperature for 10 min. Nanoplexes of 10 μg plasmid per tumor were directly injected into xenografts on days 0 and 1. DTX at a dose of 15 mg/kg was injected i.v. on day 0. Tumor volume was measured on days 0, 3, 6, 9, 11, 13, 15. On day 15, all
mice were sacrificed after anesthetization by i.m. injection of pentobarbital (Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and the tumor weights were measured. The data are shown as the mean ± SE. Animal experiments were conducted with ethical approval from our institutional animal care and use committee.

Statistical analysis. The statistical significance of the data was evaluated with Student's t-test. A P≤0.05 was considered significant.

Results

Effect of Cx43 expression on PC-3 cells. We initially characterized the expression of Cx43 in PC-3 cells. In this study, we used pGL3-basic as a control plasmid. Cx43 expression was observed strongly in pCMV-Cx43-transfected cells, but weakly in pGL3-basic-transfected cells (Fig. 1A). Next, we examined whether the transfection of pCMV-Cx43 induced growth inhibition in the cells. Seventy-two hours after transfection, Cx43 expression did not significantly induce a suppressive effect in PC-3 cells (data not shown).

To investigate whether the expression of Cx43 protein by pCMV-Cx43 caused the formation of gap junctions, we assessed the transfer of calcein-AM, a cytoplasmic dye that crosses gap junctions, in co-culture with calcin-AM-loaded cells and cells marked with DiI, a non-diffusible membrane fluorescent dye, by FACS analysis. As shown in Fig. 1B, GJIC (%) was not significantly increased in pCMV-Cx43-transfected cells compared with pGL3-basic-transfected cells. Moreover, pCMV-Cx43-transfected cells treated with 18GA, GJIC inhibitor did not decrease either the GJIC (%) compared with pGL3-basic- or pCMV-Cx43-transfected cells (Fig. 1B).

In vitro sensitivity of DTX. To evaluate the in vitro growth inhibitory effect of combination therapy of Cx43 and DTX, the WST-8 assay was initially performed. When PC-3 cells were transfected with pGL3-basic or pCMV-Cx43 in the presence of DTX, pCMV-Cx43-transfected cells (IC50 = 1.1 ng/ml) showed 53-fold higher sensitivity to DTX than pGL3-basic-transfected cells (IC50 = 58.7 ng/ml) (Fig. 2A). However, when PC-3 cells were treated with DTX 24 h after the transfection of pGL3-basic or pCMV-Cx43, pCMV-Cx43-transfected cells (IC50 = 1.4 ng/ml) showed 279-fold higher sensitivity to DTX than pGL3-basic-transfected cells (IC50 = 390.0 ng/ml) (Fig. 2B). When PC-3 cells were transfected with pCMV-Cx43 24 h after treatment with DTX, cytotoxicity could not be evaluated since cells were almost dead even at 1 ng/ml of DTX (data not shown). Therefore, in subsequent in vitro experiments, the cells were treated with DTX 24 h after transfection of pCMV-Cx43.

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We next assessed the effect of pCMV-Cx43 and DTX on the cell cycle 24 h after transfection into PC-3 cells by flow cytometric analysis. Transfection of pCMV-Cx43 into the cells did not affect the cell cycle (Fig. 3A and B), but DTX caused an increase in G2/M populations (53.1%) (Fig. 3C). Co-transfection of pCMV-Cx43 with DTX resulted in substantial accumulation in G2 (58.4%) populations (Fig. 3D).

**Effect of Bcl-2 expression and apoptosis activity on PC-3 cells.** Recently, it has been reported that transfection of Cx down-regulated the levels of Bcl-2 (20,26,27). Therefore, to investigate whether transfection with pCMV-Cx43 and/or treatment with DTX affected Bcl-2 expression in PC-3 cells, we examined the levels of protein expression of Bcl-2 in the cells by Western blotting. Either pCMV-Cx43-transfection or DTX treatment down-regulated the levels of Bcl-2 in the cells (Fig. 4). Moreover, pCMV-Cx43-transfected cells treated with DTX exhibited the most down-regulated level of Bcl-2 compared with pGL3-basic-transfected cells (Fig. 4).

Next, we examined the apoptotic effect in cells transfected with Cx43 and/or treated with DTX by annexin V assay. As shown in Fig. 5A, pCMV-Cx43 transfection or treatment with DTX increased apoptosis in the cells compared with pGL3-basic transfection. Moreover, the incidence of apoptosis was highest in pCMV-Cx43-transfected cells treated with DTX.

To investigate the apoptosis mechanism by combination therapy of Cx43 and DTX, we measured caspase-3 activity. As shown in Fig. 5B, caspase-3 activity in pCMV-Cx43-transfected cells, pGL3-basic-transfected cells with DTX and pCMV-Cx43-transfected cells with DTX was 1.6-, 1.4- and 2.0-fold higher than that in pGL3-basic-transfected cells, respectively. Forced expression of Cx43 in the cells induced significantly more up-regulation of caspase-3 activity than either treatment alone. These results suggest that the constitutive expression of Cx43 may play a role in the enhancement of apoptosis by chemotherapeutic agents.

**Synergistic inhibition of the growth of PC-3 tumor xenografts.** The efficacy of combination therapy of Cx43 and DTX in inhibiting the growth of subcutaneous PC-3 tumors was evaluated. We previously reported that NP could efficiently...
deliver DNA into PC-3 xenografts (28). Therefore, we used NP as a DNA transfection vector for in vivo experiment. The anti-tumor effect was evaluated by direct injection of the nanoplex of pCMV-Cx43 or pGL3-basic into the xenografts once a day on two occasions (day 0 and 1) following one i.v. injection of DTX (day 0) according to a previous report on in vivo combination gene therapy with DTX (29). No significant decrease in tumor weight was observed in mice treated with pCMV-Cx43 (Fig. 6B). A growth inhibitory effect was observed in mice treated with DTX alone or pGL3-basic plus DTX compared with control mice (Fig. 6A and B). pGL3-basic plus DTX exhibited a similar tumor suppressive effect with DTX alone, indicating that DNA transfection did not increase tumor growth inhibition. A significant growth inhibitory effect was observed in combination therapy of pCMV-Cx43 and DTX compared with DTX alone (Fig. 6A and B). A comparison of tumor weight and the appearance after excision also demonstrated that tumor growth was attenuated in mice treated with pCMV-Cx43 and DTX (Fig. 6B and C).
Discussion

The limited efficacy of cytotoxic chemotherapy remains a major problem in the treatment of advanced hormone-refractory prostate cancer (30); therefore, novel cancer gene therapy needs to be developed. In this study, we found that Cx43 expression in PC-3 cells significantly enhanced DTX cytotoxicity through down-regulation of Bcl-2 expression and activation of the apoptosis pathway. Furthermore, the combination of non-viral Cx43 gene therapy and DTX significantly suppressed the growth of tumor xenografts compared to DTX alone. This is the first report to highlight that the expression of Cx43 in association with DTX has potential as a tumor growth inhibitor.

Dysregulation of Cx expression is thought to be associated with carcinogenesis; however, there is relatively little information regarding the mechanism of altered Cx expression in prostate cancer. The tumor-suppressing effects of Cx genes largely depend on the Cx species and the cell types used (31). Transduction of Cx32 and Cx43 by retroviral gene transfection into the Cx-deficient prostate cancer cell line LNCaP produced growth inhibition in vitro and in vivo, and cell differentiation associated with gap junction formation (19). Transduction of Cx26 by adenoviral gene transfection into LNCaP and PC-3 cells produced growth inhibition by a GJIC function (26). Regarding Cx43, the expression of Cx43 in PC-3 cells could not form gap junctions (32). In our study, the transfection of pCMV-Cx43 into PC-3 cells exhibited neither the inhibition of cell growth nor increased GJIC (Fig. 1); however, transfection into the cells increased apoptotic cells and caspase-3 activity (Fig. 5). Cx43 expression in PC-3 cells might regulate apoptosis via a GJIC-independent mechanism.

Combination therapy of Cx43 and DTX was significantly more cytotoxic when cells were treated with DTX 24 h after Cx43 transfection compared with treatment with DTX and transfection at the same time. It suggested that Cx43 expression 24 h after transfection affected sensitivity to DTX. We also found that combination therapy 72 h after transfection increased growth inhibition in LNCaP cells (IC50 for DTX = 32 and 4.2 ng/ml in pGL3-basic- and pCMV-Cx43-transfected cells, respectively) (data not shown). Combination therapy using the Cx gene and chemotherapeutic agents for cancer has been reported. Cx43 transfected into human glioblastoma cells (20) associated with gap junction formation (19). Transduction of Cx32 and Cx43 by retroviral gene transfection into LNCaP and PC-3 cells produced growth inhibition by a GJIC function (26). Regarding Cx43, the expression of Cx43 in PC-3 cells could not form gap junctions (32). In our study, the transfection of pCMV-Cx43 into PC-3 cells exhibited neither the inhibition of cell growth nor increased GJIC (Fig. 1); however, transfection into the cells increased apoptotic cells and caspase-3 activity (Fig. 5). Cx43 expression in PC-3 cells might regulate apoptosis via a GJIC-independent mechanism.

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The combination of repeated intratumoral injections of pCMV-Cx43 (10 μg/tumor) with non-viral vector and a single intravenous injection of DTX (15 mg/kg) was compared with a repeated injection of Cx43 alone and a single injection of DTX alone in PC-3 tumor xenografts. Significant antitumor effects were observed in mice receiving combined treatment, compared with DTX alone. The efficacy in vivo might result from direct effects of Cx43 on inducing apoptosis and indirect effects on enhancing the cytotoxicity of DTX by down-regulating Bcl-2. It has been reported that paclitaxel increased the transfection efficiency of cationic lipidosome by inhibiting targeting endosomes to lysosomes (36,37). Therefore, using the combined lipid-mediated transfection of genes with DTX for cancer gene therapy might be a powerful technique due to the effect of enhanced gene expression. Inhibiting Bcl-2 expression by Cx43 in prostate cancer cells, which could restore their sensitivity to chemotherapeutic agents, would be a new therapeutic strategy against prostate cancer.

From a clinical point of view, the doses of DTX used in the combined strategy will be minimal, and will prevent significant toxicity due to DTX. Low doses of DTX can thus be administered in humans for a prolonged period of time, or alternatively, a shorter duration of combined treatment may be administered without loss of effectiveness. Enforced expression of Cx43 increased sensitivity for DTX via the down-regulation of Bcl-2 expression in PC-3 cells. Combining non-viral Cx43 gene therapy with DTX resulted in greater growth suppression of PC-3 in vitro and in vivo. The data presented here provide a rational strategy for treating patients with advanced hormone refractory prostate cancer.

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