Sensitizing Effect of Galectin-7 in Urothelial Cancer to Cisplatin through the Accumulation of Intracellular Reactive Oxygen Species

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Abstract

To improve chemotherapeutic efficacy in urothelial cancer, it is important to identify predictive markers for chemosensitivity as well as possible molecules accelerating cell killing mechanisms. In this study, we assessed the possibility of galectin-7 to accelerate cis-diamminedichloroplatinum (CDDP)–induced cell killing in vitro and also to predict chemosensitivity against CDDP in urothelial cancer patients. The expression of galectin-7 was analyzed in five bladder cancer cell lines with different p53 status after treatment with CDDP. The roles of galectin-7 in chemosensitivity against CDDP were analyzed by transfection of the galectin-7 gene into several of these cell lines. Furthermore, the relationship between the expression of galectin-7 and the response to neoadjuvant chemotherapy was analyzed in 17 human bladder cancer specimens. Exposure to CDDP induced galectin-7 in cell lines with wild-type p53 but not in those with mutated p53. When the galectin-7 gene was transfected into cell lines with mutated p53, the sensitivity to CDDP increased compared with control transfectants. In addition, galectin-7–transfected cells exhibited more accumulation of intracellular reactive oxygen species and activation of c-Jun NH2-terminal kinase (JNK) and Bax than control transfectants. SP600125, an inhibitor of JNK, or antioxidant N-acetyl-L-cysteine inhibited the enhancement of chemosensitivity against CDDP by galectin-7 transfection. In clinical samples, the expression levels of galectin-7 were significantly lower in urothelial carcinomas compared with normal urothelium. When chemosensitivity was tested, its expression levels were higher in the chemosensitive group than in the chemoresistant group. Galectin-7 is a candidate for a predictive marker of chemosensitivity against CDDP, and the targeted expression of galectin-7 might overcome the chemoresistance of urothelial cancer. [Cancer Res 2007;67(3):1212–20]

Introduction

Urothelial cancer is one of the most common genitourinary malignancies, in which 20% to 40% are present with or develop invasive disease (1). Despite radical cystectomy, which is a gold standard for treatment, about half of patients die from this aggressive disease within 5 years (2, 3). To improve the prognosis of invasive bladder cancer, cisplatin-based systemic chemotherapy has been empirically adapted in combination with radical cystectomy, but the overall response rate has been reported to vary between 12% and 73% (4, 5). If empirical chemotherapy was administered for a chemoresistant tumor, some patients suffered severe adverse effects without achieving any benefit. Therefore, the importance of detecting molecular markers for chemosensitivity and modification of chemotherapy to overcome chemoresistance has risen recently for patients with invasive urothelial cancer.

p53 is a tumor suppressor protein and a regulator of the cell cycle and apoptosis. Mutation in the p53 gene is one of the most common genetic events accounting for up to 61% of urothelial cancers (6, 7). Mutated p53 has been considered to be linked to cancer progression, prognosis, and response to chemotherapy (8, 9), although the clinical importance of p53 alterations as a predicting factor for chemosensitivity remains controversial (10, 11). Recently, we reported the importance of the p53-p21–c-Jun NH2-terminal kinase (JNK) pathway in chemosensitivity against cis-diamminedichloroplatinum (CDDP) in urothelial cancers with wild-type (WT) p53. However, in cancer cells with mutated p53, we recognized the necessity of other factors to evoke apoptosis through JNK activation (12). To identify molecules that enhance the p53 response in RT112 urothelial cancer cells, we then screened up-regulated genes in response to CDDP in these cells using microarray analysis and found that galectin-7 mRNA was induced in RT112 cells by CDDP.

Galectin-7 is a 15-kDa protein with a single carbohydrate recognition domain and has been reported to be induced by p53 transfection in a human colon carcinoma cell line DLD-1. Therefore, it is designated as p53-induced gene 1 (PIG1; ref. 13). It also belongs to a family of β-galactoside–binding animal lectins consisting of 15 members (galectins 1–15; ref. 14). The expression of galectin family genes has been reported to be significantly altered in various kinds of cancers, and these alterations are considered to contribute to neoplastic transformation and tumor progression through many biological functions, including cell growth, cell cycle, and apoptosis (15–18). The expression of galectin-7 was identified in normal stratified epithelial cells, including the skin, tongue, esophagus, and Hassal’s corpuscles in the thymus, and interestingly, it was strikingly down-regulated in squamous cell carcinoma cells (19). Functionally, the ectopical expression of galectin-7 in colon carcinoma cells rendered the cells more sensitive to several apoptotic stimuli through the activation of JNK (20, 21). These findings suggested that galectin-7 played important roles in carcinogenesis or chemosensitivity in squamous cell and colorectal carcinomas, but there were no reports about its
expression in urothelial carcinomas. In the present study, we investigated the functional significance and clinical usefulness of galectin-7 in the chemosensitivity of urothelial cancers.

Materials and Methods

Antibodies and reagents. Antibodies were obtained as follows: anti-JNK, phospho-JNK, phospho-c-Jun (Ser73), Bax, and caspase-9 were obtained from Cell Signaling Technology (Beverly, MA). Anti– caspase-3 and caspase-8 were from BD PharMingen (San Diego, CA); anti– poly(ADP-ribose) polymerase (PARP, C2-10) and Bax (6A7) were from Sigma (St. Louis, MO); anti-p53 (Ab-2) was from Calbiochem (San Diego, CA); anti–Bcl-2 and p21 from Cell Signaling Technology (Beverly, MA). Anti–caspase-3 and caspase- inhibitor, and goat serum) was produced as described previously (20). SP600125 was from Abcam (Cambridge, MA). An antibody against galectin-7 (polyclonal goat serum) was produced as described previously (20). SP600125 was purchased from Tocris Cookson Ltd. (Bristol, United Kingdom) as a JNK inhibitor, and N-acetyl-t-cysteine (NAC) was from Calbiochem. CDDP was from Wako (Osaka, Japan).

Cell lines. Five bladder tumor-derived cell lines were used. Four bladder (RT12, TCCsuP, EJ, and J82) cancer cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. RT4 was maintained in McCoy’s 5A medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. cDNA sequencing and genotyping of p53 were done in the manner described previously (22). Briefly, cDNA of p53 synthesized from total RNA of each cell line was amplified by PCR with recombinant Pfu polymerase (Stratagene, La Jolla, CA) and directly sequenced using oligonucleotide primers. Cycle sequencing reactions were carried out using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol.

Bladder cancer tissues and normal urothelium. Clinical tissue samples were obtained from 17 patients suffering from bladder cancer with extravesical invasion (cT3 or greater) who received preoperative systemic chemotherapy and cystectomy between January 2000 and December 2003 at Kyoto University. All samples were obtained by cold cup biopsy before treatment and immediately homogenized with RLT lysis buffer of RNeasy mini kit (Qiagen GmbH, Hilden, Germany) and stored at −80°C until RNA extraction. As preoperative chemotherapy, patients received two cycles of MEC chemotherapy, which included cisplatin, methotrexate, and epidoxorubicin treatments. In general, preoperative chemotherapy was indicated for invasive bladder cancer more than cT3, and the present study included only cases, in which responses to neoadjuvant chemotherapy could be evaluated by radiologic examinations. The patients were classified into two groups, responders and non-responders, based on the reduction ratio of the primary tumor volume after two cycles of chemotherapy: the former group including complete response (CR) and partial response (PR) and the latter including no change and progressive disease (PD), respectively. The clinical and pathologic classifications, both including tumor grade and stage, were assessed according to the WHO system and tumor-node-metastasis classifications, respectively (23).

Human normal urothelium was collected at the time of nephrectomy conducted in diseases other than urothelial carcinomas as described previously (24), and RNA was extracted in the same way as from bladder cancer tissues.

Generation of stable transfectants. pEF1-galectin-7, which encodes galectin-7 cDNA, was constructed as described previously (20). Three bladder cancer cell lines, RT12, TCCsuP, and EJ, were transfected with pEF1-galectin-7 or control vector pEF1-neo using LipofectAMINE 2000 and Plus reagent (Life Technologies, Inc. Grand Island, NY) following the manufacturer’s instructions. Stable transfectants were selected by G418 resistance (Calbiochem). When numerous G418-resistant colonies appeared, the cells in the colonies were mixed and stored in liquid nitrogen until used for studies. The cells were used in experiments within 2 weeks after thawing.

A dominant-negative mutant of p53 (p53DN) containing one missense mutation at codon 135 (TGC → TAC), which was purchased from BD Biosciences (San Diego, CA), was ligated into pCMVneo, a kind gift of Dr. K. Cho (University of Michigan Medical School, Ann Arbor, MI). RT12 cells were transfected with pCMVneo or pCMV-p53DN using Lipofect-AMINE following the manufacturer’s instructions. Stable transfectants were selected by G418 resistance. Overexpression of p53DN was confirmed by sequencing, reverse transcription-PCR (RT-PCR), or Western blot analysis.

Drug treatment. For dose-response and time course studies, cells were grown to 70% to 80% confluency in 100-mm dishes for 48 h and treated with CDDP at the corresponding concentrations of IC50. IC50s of CDDP were 20 and 40 μmol/L in RT4 and RT12, respectively, whereas those of TCCsuP, EJ, and J82 were >100 μmol/L. For analysis of the JNK pathway, cells were treated with 40 μmol/L SP600125 dissolved in DMSO following exposure to CDDP.

RNA isolation and RT-PCR. Total RNA was prepared from culture cells and clinical samples using the RNeasy mini kit. First-strand cDNA was synthesized from 1 to 5 μg total RNA using random primers according to the manufacturer’s protocol (First-Strand cDNA synthesis kit, Amersham Pharmacia Biotech, Piscataway, NJ). Primer sequences used for galectin-7 cDNA amplification were as follows: 5′-ATGGTCCAAGCTTCCCAACAAG-3′ (sense) and 5′-TGGACCGATGATGAGCACCTC-3′ (antisense), resulting in a 282-bp fragment. Primers for amplification of the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 5′-CGAGCCACATCGCTCAGACA-3′ (sense) and 5′-TGGAGCTGTGTCATACTTCTC-3′ (antisense), resulting in a 455-bp fragment. The RT-PCR exponential phase was determined to allow semiquantitative comparisons. Each PCR regime involved 5 min of initial denaturation step at 95°C followed by 33 cycles at 94°C for 1 min and 30 s. PCR products were separated by electrophoresis on 2.5% agarose gels.

Real-time quantitative RT-PCR. The synthesized cDNA was diluted 1:2, and 2 μL of the dilution were used for RT-PCR. Quantitative real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems) was done using the GeneAmp 5700 Sequence Detection System (Applied Biosystems) with the relative standard curve method (25). Primers were the same as mentioned above. The thermal cycling program consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles of 30 s at 64°C, 30 s at 72°C, and 10 s at 78°C. The expression level of each target gene was quantified relative to GAPDH and transformed into log 2 values. All measurements were done in triplicate.

Cell viability assay. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1 × 106 cancer cells with 100 μL suspension were grown in well of 96-well plates. After 24 h of incubation, cells were treated with or without different concentrations of drugs for another 24 or 48 h. Then, 20 μL MTT working solution (5 mg/mL; Sigma) was added to each culture well and incubated for 4 h. The absorbance (A) of each well was measured by a microculture plate reader (ImmunoReader, Japan Intermed Co., Ltd., Tokyo, Japan) at 540 nm. The percentage cell viability = (A of experimental wells / A of control wells) × 100.

Detection of apoptosis and cell cycle distribution. To detect apoptosis, nuclear staining and flow cytometric analysis were done. For nuclear staining, cells were stained with 1 mmol/L Hoechst 33342 solution (Wako) and analyzed with a fluorescence microscope. Apoptotic cells were identified by morphologic changes (condensation and fragmentation of their nuclei). For flow cytometric analysis, cells were harvested 24 h after treatment with CDDP in the presence or absence of NAC or Sp600125 and stained with 7-amino-actinomycin D (7-AAD; BD Biosciences). Flow cytometry was done on a FACScan (Becton Dickinson, San Jose, CA). For staining, harvested cells (1 × 106) were washed with ice-cold PBS and then fixed in 70% ethanol. Fixed cells were treated with DNase-free RNase for 30 min at 37°C washed with PBS, centrifuged, and incubated in PBS containing 7-AAD (5 μmol/L). Cell cycle distribution was analyzed using CellQuest software (Becton Dickinson). Flow cytometric estimation of intracellular redox state. The intracellular redox state was estimated by the levels of intracellular peroxides, which were monitored by flow cytometric analysis with 2′,7′- dichlorofluorescein (DCFH) diacetate (DCFH-DA) as described previously.

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Briefly, $1 \times 10^6$ cells were treated with or without CDDP for the indicated times, and then the culture medium was replaced with freshly prepared medium containing 5 μmol/L DCFH-DA. After 30 min of incubation at 37°C, fluorescence intensity was measured by FACScan using CellQuest software.

**Immunoblotting and immunoprecipitation.** After drug treatment, cells were washed with PBS and lysed in an appropriate volume of ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, containing 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany)]. Cellular lysates were clarified by centrifugation at 13,000 × g for 15 min and the protein concentrations of the lysates were determined by a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Thirty to fifty micrograms of the lysates were boiled for 5 min in SDS sample buffer and separated by SDS-PAGE on a 10% to 15% Tris-HCl mini gel and transferred onto a polyvinylidene difluoride membrane following standard methods. Membranes were probed with appropriate dilutions of primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibodies. After extensive washes, proteins were visualized by a chemiluminescent detection system (GE Healthcare, Buckinghamshire, United Kingdom).

For immunoprecipitation, cells were washed with PBS and lysed in an appropriate volume of ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, containing 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 1 mmol/L PMSF, and protease inhibitor cocktail tablets]. The lysates were incubated with the indicated antibodies for 1 h after preclearance with protein G-Sepharose beads for 30 min. Subsequently, they were incubated with protein G-Sepharose beads for 1 h. The bead-bound proteins were eluted by boiling for 5 min and subjected to immunoblot analysis.

**Statistical analysis.** All 2 × 2 tables were analyzed by the χ² test. Differences between group means were analyzed by the Mann-Whitney U test. P values <0.05 were considered statistically significant.

**Results**

**Induction of galectin-7 by CDDP treatment in bladder cancer cell lines.** The expression of galectin-7 mRNA was analyzed in a panel of bladder cancer cell lines, including RT112, TCCsup, EJ,

Figure 1. CDDP induces galectin-7 in bladder cancer cell lines. A, quantitative and semiquantitative RT-PCR showed the induction of galectin-7 mRNA with (black columns) or without (white columns) CDDP treatment at the concentrations indicated in the figure among five urothelial cancer cell lines. The p53 status of these cell lines was assessed by sequencing analysis. Values of quantitative RT-PCR. Columns, average values of relative expression ratios of galectin-7 (galectin-7/GAPDH × 1,000) from three independent experiments; bars, SD. CDDP concentration was an approximate value of the concentration of IC$_{50}$ for each cell line. IC$_{50}$ concentration was the concentration at which cell growth was inhibited by 50%, calculated from the regression line based on 24-h MTT assay. B, immunoblot analysis showed that CDDP treatment (30 μmol/L) caused the induction of galectin-7 in RT112 in a time-dependent manner. The change of other p53 downstream molecules and the cleavage of caspase-3 were also assessed simultaneously. Arrowhead, procaspase; black arrow, cleaved caspase. C, immunoblot analysis showed that the induction of galectin-7 by CDDP was suppressed in RT112 p53DN-2 compared with RT112 neo. RT112 neo and RT112 p53DN-2 were treated with 30 μmol/L CDDP. Twenty-four hours later, whole-cell lysates were prepared and analyzed by immunoblotting.
Sequencing analysis confirmed the p53 status in these cell lines; RT4 and RT112 had WT p53, whereas J82 and EJ had missense mutation. TCCsup harbored nonsense mutation. Quantitative RT-PCR showed quite low levels of the expression of galectin-7 mRNA in all five cell lines (Fig. 1A). When each cell line was treated with CDDP at its corresponding concentration of IC_{50}, galectin-7 mRNA was induced in RT4 and RT112 18 h after CDDP exposure, whereas it was not induced in other three cell lines. To confirm the induction of galectin-7 at the protein level, RT112 cells were analyzed by Western blotting (Fig. 1B). Treatment with CDDP induced a gradual increase in p53 expression and, subsequently, changes in the expression of p53-related genes, such as p21, Bax, and Bcl-2 occurred. The expression of galectin-7 was induced significantly 24 h after CDDP treatment, when apoptosis was recognized as an increased cleavage of caspase-3. To confirm the necessity of functional p53 to induce galectin-7 expression, the p53 dominant-negative mutant (p53DN) was transfected into RT112 cells, and two stable clones (RT112 p53DN-2 and RT112 p53DN-11) were established. Western blot analysis showed that the induction of galectin-7 by CDDP was suppressed in RT112 p53DN-2 compared with RT112 neo (control). RT112 p53DN-11 also showed similar results (data not shown). These results indicated that galectin-7 expression was under the control of functional p53.

Enhancement of CDDP-induced cytotoxicity in p53-mutant cells by galectin-7 transfection. To examine the roles of galectin-7 in CDDP-induced cytotoxicity, we generated stable transfectants expressing galectin-7 in TCCsup and EJ, both of which had mutated p53 and did not express detectable levels of endogenous galectin-7 when exposed to CDDP. As a reference, we transfected the galectin-7 gene into RT112, which had WT p53 and induced galectin-7 by CDDP. Western blot analysis confirmed the expression of galectin-7 in TCCsup Gal7 and EJ Gal7 (Fig. 2A). Under normal culture conditions (10% FCS), the growth rate of these cells was similar to that of control transfectants (EJ neo, TCCsup neo, and RT112 neo). Cell viability. Points, mean percentage of three independent experiments; bars, SD.

Figure 2. The effect of overexpression of galectin-7 in urothelial cancer cells on CDDP-induced cytotoxicity. A, immunoblot analysis confirmed galectin-7 expression in stable transfectants of pEF1-galectin-7 into TCCsup and EJ bladder cancer cell lines. As controls, vector alone (pEF1-neo)–transfected TCCsup and vector alone (pEF1-neo)–transfected EJ did not express galectin-7. neo, vector (pEF1-neo)–transfected cell; Gal7, galectin-7–transfected cell. B, there was no significant difference in growth rates between galectin transfectants and control transfectants. Each of 4 × 10^4 cells was plated in 96-well plates, and cell proliferation was measured by MTT assay every 24 h. C, Hoechst 33342 nuclear staining showed the acceleration of apoptosis in galectin-7–transfected TCCsup treated with 60 μmol/L CDDP for 36 h compared with TCCsup neo. White arrowheads, apoptotic cells. D, galectin-7 transfection to p53-mutant cells (TCCsup and EJ) enhanced the susceptibility to CDDP but not in p53 WT cells (RT112). Cell viability was evaluated 24 or 48 h after CDDP treatment at the indicated concentrations using MTT assay in galectin-7–transfected EJ, TCCsup, and RT112 cells compared with vector-only transfectants (EJ neo, TCCsup neo, and RT112 neo). Cell viability. Points, mean percentage of three independent experiments; bars, SD.
induced apoptosis as evidenced by nuclear fragmentation in galectin-7 transfectants but not in control transfectants (Fig. 2B).

In addition, a decrease in cell survival rates after CDDP exposure was observed in galectin-7 transfectants of p53-mutant cells (TCCsup Gal7 and EJ Gal7) but not in control transfectants (TCCsup neo and EJ neo). No significant difference was seen between galectin-7 and control transfectants of RT112 after CDDP exposure (Fig. 2D). In this case, we consider that it is because there is no significant difference in the levels of galectin-7 expression after CDDP exposure between galectin-7 and control transfectants of RT112 (data not shown). These results clearly indicated that the forced expression of galectin-7 in p53-mutant cell lines rendered these cells more sensitive to CDDP-induced cytotoxicity.

Modification of cisplatin-induced apoptosis by galectin-7 via reactive oxygen species-JNK-Bax pathway. To investigate the molecular mechanisms underlying the effects of galectin-7 in CDDP-induced cytotoxicity, the activation of JNK, Bax, and caspases was examined after CDDP treatment in a time-dependent manner (Fig. 3A). Western blot analysis showed that transfection of galectin-7 did not change the expression level of JNK nor Bax, but it sustained highly accelerated JNK phosphorylation from 8 h after CDDP exposure. JNK phosphorylation was also seen in control transfectants after CDDP exposure; however, their levels are much lower than galectin-7 transfectants. The activation of caspase-8/caspase-9/caspase-3, as shown by an increase in their cleaved products, occurred from 16 h after CDDP exposure. Concomitant cleavage of PARP as a result of caspase activation was also observed in galectin-7 transfectants. This concentration of CDDP, on the other hand, did not activate caspases nor increase PARP cleavage in control transfectants. Interestingly, compared with control transfectants, in which there was no change in Bax expression throughout CDDP exposure, a significant increase in its expression 36 h after CDDP exposure was observed in galectin-7 transfectants. This observation suggested that up-regulation of

Figure 3. Enhancement of CDDP-induced cytotoxicity in p53-mutant cells by galectin-7 transfection was dependent on the JNK-Bax pathway. A, galectin-7–transfected (TCCsup Gal7) or control-transfected TCCsup (TCCsup neo) was treated with 60 μmol/L CDDP. After the indicated periods, whole-cell lysates were prepared and analyzed by immunoblotting as described in Materials and Methods, using the indicated antibodies. B, cotreatment with specific JNK inhibitor (SP600125) suppressed galectin-7–mediated enhancement of CDDP cytotoxicity. Left, TCCsup cells were treated with either CDDP alone (○, TCCsup Gal7; □, TCCsup neo) or CDDP plus 40 μmol/L SP600125 (△, TCCsup Gal7; ●, TCCsup neo) for 48 h; right, EJ neo or EJ Gal7 was treated by 200 μmol/L CDDP with or without 40 μmol/L SP600125 for 48 h. Cell viability is shown as the percentage of viable cells to each control, based on the results of MTT assays. Points and columns, average of at least three independent experiments; bars, SD. C, the status of molecules associated with apoptosis was analyzed by immunoblotting. Cells were harvested after treatment with 75 μmol/L CDDP in the presence or absence of 40 μmol/L SP600125 or no treatment for 24 h. Activated Bax and caspases as well as cleavage of PARP were seen only in TCCsup Gal7. SP600125 inhibited activation of Bax and caspases resulting in the prevention of apoptosis in TCCsup Gal7. For assessing active Bax, conformationally changed Bax protein was immunoprecipitated using anti-Bax 6A7 antibody and subjected to immunoblot analysis using polyclonal Bax antibody.
Bax gene was induced at this time point in CDDP-exposed galectin-7 transfectants.

To identify the significance of JNK activation in TCCsup Gal7, cell viability was measured in the presence or absence of SP600125, a specific inhibitor of JNK1/2. As shown in Fig. 3B, the inhibition of JNK activation attenuated CDDP susceptibility of TCCsup Gal7 to the same level as TCCsup neo. A similar result was obtained in EJ galectin-7 (Fig. 3B). Western blot analysis also showed that SP600125 suppressed the activation of caspases and cleavage of PARP in TCCsup Gal7 (Fig. 3C).

Bax has been reported to act downstream of JNK and to control its proapoptotic function by conformational change (27, 28) and translocation between cytosol and mitochondria even when the total Bax protein level remains constant (29, 30). The status of Bax was analyzed by immunoprecipitation using anti-Bax monoclonal antibody 6A7, which recognizes the activated form of Bax. At 24 h after CDDP exposure, monomer and dimer forms of active Bax were observed only in galectin-7 transfectants but not in control transfectants (Fig. 3C). A moderate increase in total Bax is shown in Fig. 3A. Furthermore, active Bax in galectin-7 transfectants was partially suppressed when cells were treated with the JNK inhibitor SP600125. These findings suggested that activation of a JNK-Bax-mitochondria pathway played an important role in the enhancement of CDDP-induced cytotoxicity by galectin-7 transfection.

As the generation of reactive oxygen species (ROS) has been reported to play an important role in CDDP-induced cytotoxicity (31) and also to activate JNK (32), the effects of galectin-7 transfection were assessed on the intracellular redox status. As shown in

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**Figure 4.** Accumulation of intracellular ROS was promoted by galectin-7 transfection, leading to apoptosis. A, the effect of CDDP treatment was measured on the fluorescence distribution of DCFH oxidation in galectin-7-transfected (dashed line) or control vector-transfected TCCsup (continuous line). Cells (3 × 10⁶) from each cell line were cultured for 6 h in the absence or presence of CDDP (50 μmol/L), and then the culture medium was replaced with freshly prepared medium containing 10 μmol/L DCFH-DA. After 30 min of incubation at 37°C, fluorescence intensity was measured by flow cytometry (continuous line, TCCsup neo; dashed line, TCCsup Gal7). Only part of TCCsup Gal7 (black arrows) showed accelerated accumulation of intracellular ROS. B, concurrent treatment of antioxidant NAC inhibited galectin-7-mediated enhancement of CDDP cytotoxicity. Fluorescence-activated cell sorting histogram of 7-AAD staining 24 h after continuous drug exposure showed that 10 mmol/L NAC relieved the susceptibility to 75 μmol/L CDDP in TCCsup Gal7 to a similar level to TCCsup neo. C, immunoblot analysis showed that concomitant use of NAC with CDDP suppressed JNK activation and apoptosis in TCCsup Gal7. Cells were harvested after treatment with 75 μmol/L CDDP in the presence or absence of 10 mmol/L NAC or no treatment for 24 h. D, concurrent treatment of NAC relieved the oxidative status generated by CDDP treatment in part of TCCsup Gal7, but SP600125 inversely promoted ROS generation (white arrowheads). Intracellular ROS was measured on the fluorescence distribution of DCFH oxidation 6 h after 50 μmol/L CDDP exposure in the presence or absence of 10 mmol/L NAC or 40 μmol/L SP600125.
Fig. 4A, flow cytometric analysis with DCFH-DA showed that galectin-7 transfection alone had no effects on the intracellular redox status. However, exposure to a relatively low concentration of CDDP (50 μmol/L) caused a significant increase in the accumulation of intracellular peroxides in TCCsup Gal7, whereas the same concentration of CDDP did not change in intracellular ROS in TCCsup neo. To determine the influence of the accumulation of intracellular ROS on the susceptibility to CDDP-induced cytotoxicity, TCCsup Gal7 was treated with an antioxidant NAC. The concomitant use of NAC with CDDP suppressed both JNK activation and apoptosis in galectin-7 transfectants (Fig. 4B and C). As expected, NAC reduced the accumulation of intracellular ROS by CDDP. Interestingly, the concomitant use of JNK inhibitor did not inhibit, but inversely accelerated, the accumulation of intracellular ROS despite its antiapoptotic effect on CDDP-treated TCCsup Gal7. This clearly indicated that ROS generation is upstream of JNK activation in the context of CDDP-induced apoptotic events. Taken together, these results indicated that galectin-7 accelerated CDDP susceptibility through JNK activation by increased generation of ROS.

The relationship of galectin-7 with chemosensitivity of urothelial cancer. To elucidate whether galectin-7 expression is associated with the chemosensitivity of urothelial cancers, its expression levels were analyzed with quantitative RT-PCR in 4 normal controls and 17 invasive bladder cancer samples collected before preoperative MEC chemotherapy. The bladder cancer samples were classified by the reduction ratio of the tumor volume after chemotherapy. A CR was defined as the disappearance of all known disease. A PR was defined as at least a 50% decrease in measurable disease and no evidence of any new lesions or progression of any existing lesions. An inability to show a 50% decrease or a 25% increase in the tumor size, as well as no new lesions, defined no change (NC). A 25% increase in the tumor size or the appearance of any new lesions defined PD. Human skin was used as a positive control of galectin-7 expression. Box plot, the expression values of each category expressed as relative expression ratios of galectin-7 [log2 (galectin-7 mRNA/GAPDH mRNA) × 1,000]. Points, maximum and minimum values for the group. Box, 75% of each group; line within the box plot, median value. The bladder cancer samples were classified by the reduction ratio of the primary tumor volume after chemotherapy. B, the 2 × 2 analysis comparing the high-expression group of galectin-7 with the low-expression group in relation to the response to neoadjuvant chemotherapy. Seventeen bladder cancer samples were divided into two groups by galectin-7 expression level. The median value of all samples was used as the cutoff point. P value was calculated by the χ2 test.

Discussion

Galectin-7 was identified as PIG1, one of the genes highly induced by p53 transfection into the colon cancer cell line DLD-1 (13). Galectin-7 was also reported to be expressed mainly in normal stratified epithelial cells represented by the skin (19), although there has been no report about its expression in urothelial cancer. In the present study, we showed that CDDP exposure induced p53 and subsequently galectin-7 in bladder cancer cell lines with WT p53 but not in lines with mutant p53. Furthermore, transfection of galectin-7 sensitized these cancer cells with mutant p53 to CDDP via the promotion of intracellular ROS generation. Moreover, we showed that lower expression of galectin-7 was related to unfavorable sensitivity to CDDP. These results indicated that galectin-7 played an important role acting downstream of p53 in urothelial cancer cells and is a novel candidate to influence chemosensitivity to CDDP.
Galectin-7 has been reported to have various functions. As a lectin existing on the cell surface, it reduces neuroblastoma cell proliferation or, in contrast, acts as a factor promoting tumorigenesis via matrix metalloproteinase-9 up-regulation in lymphoma (33, 34). Besides its lectin function, however, galectin-7 has been recognized as a molecule that can promote the apoptotic process intracellularly. In human epidermal keratinocytes, UVB irradiation induced high levels of galectin-7 expression, resulting in apoptosis (35). Moreover, galectin-7 overexpression made HeLa cells more sensitive to various cytotoxic agents (20). In the present study, galectin-7 transfection into bladder cancer cells (TCCsup and EJ), which harbored mutated p53, did not seem to cause apoptosis nor change cellular proliferation but promoted their susceptibility to genotoxic stress by CDDP exposure. This result is compatible to previous reports that galectin-7 itself did not cause apoptosis but it enhanced sensitivity to apoptotic stimuli in vitro (20, 21).

With regard to proapoptotic functions of galectin-7, we have suggested previously that galectin-7 acts upstream of JNK activation (20). In this study, galectin-7–transfected TCCsup showed highly accelerated JNK phosphorylation compared with control transfectants. The activation of JNK as well as p38 MAPK is necessary for apoptosis induced by several stresses, such as cytokine, chemotherapy, and serum deprivation (36–39). Over-activation of the JNK pathway has also been reported to augment CDDP-mediated cell death. Therefore, JNK activation is suspected to have a central mechanism to promote CDDP susceptibility by galectin-7. It is known that stress-induced activation of JNK has dual functions and can contribute variably to cell survival as well as apoptosis (40). We confirmed the contribution of JNK activation to apoptosis using SP600125, a specific inhibitor of JNK1/2, by showing the attenuation of CDDP-induced apoptosis in galectin-7 transfecteds by this reagent. Conformational change of Bax by JNK has been reported to cause translocation of Bax from cytosol to mitochondria and to form Bax active homodimers (27, 28, 41). By using the JNK inhibitor, we also showed that conformational change of Bax protein mediates apoptosis through JNK activation.

As for the mechanism underlying the activation of JNK, intracellular ROS generation by CDDP has been considered a possible upstream activator of JNK (30, 31). Our result of the measurement of intracellular ROS showed the elevation of intracellular ROS in galectin-7–transfected cells exposed to a relatively low concentration of CDDP that had no effect on control transfectants. Thus, we considered that, in galectin-7 transfectants, the acceleration of ROS generation by CDDP caused activation of the JNK-Bax pathway resulting in apoptosis via an intrinsic pathway. In this regard, it is of note that we have shown by DNA microarray analysis that galectin-7 affected the expression of redox-related genes (20). Therefore, as future work, it may be interesting to elucidate whether galectin-7 functions through redox-related molecules.

Recently, the modulation of several genes, such as Bcl-2, NF-κB, or PTEN, has been reported as a candidate to enhance chemosensitivity in urothelial cancer by increasing ROS produced by conventional chemotherapeutic agents (42–44). In addition, the pharmacologic or genetic modulation of thioredoxin or glutathione S-transferase-π has been reported to effectively increase the intracellular ROS level (26, 45, 46). Previously, we also reported that the combinational use of CDDP and dicoumarol, an enzymatic inhibitor of antioxidant enzyme NADPH quinone oxidoreductase 1, accelerated CDDP-induced apoptosis via the activation of JNK (12), but it was effective only in bladder cancer cells with WT p53.

Now, we consider that galectin-7 may be a key molecule to transmit the apoptotic signal via JNK activation and its manipulation become a new tool of therapeutic value to potentiate CDDP cytotoxicity in bladder cancer cells with mutant p53.

As well as searching for new therapeutic molecular targets, it has become very important to detect new markers that predict the response to chemotherapy. The significance of p53 tumor suppressor gene, in this context, has been controversial as to whether it is responsible for the effects of CDDP-based systemic chemotherapy. Moreover, recent reports using high-throughput microarray analysis showed that RASL11B (RAS like, family 11, member B) or PHKA2 (phosphorylase kinase 2) can be a new candidate for a predictive marker (47), but there has been no report referring to the relationship between galectin-7 expression and sensitivity to CDDP. As our in vitro data showed a relationship between galectin-7 expression and CDDP cytotoxicity, we examined whether galectin-7 expression levels in clinical samples of bladder cancer would correlate with CDDP-based chemotherapy. Our data showed that bladder cancers expressing higher galectin-7 mRNA tended to respond to neoadjuvant chemotherapy better than those with lower expression.

In conclusion, galectin-7 expression may be related to the chemosensitivity of urothelial cancer and our results will provide potential for its targeted expression as a useful therapeutic modality in the treatment of urothelial cancer.

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References


Sensitizing Effect of Galectin-7 in Urothelial Cancer to Cisplatin through the Accumulation of Intracellular Reactive Oxygen Species

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