Gemcitabine, and 5-fluorouracil (5-FU) appear to be effective single agents for treating cholangiocellular carcinoma (1, 2). Moreover, gemcitabine-based combination regimens such as gemcitabine plus cisplatin or S-1 (an oral combination of 5-FU plus two enzyme inhibitors) are superior to gemcitabine alone in clinical trials (1, 2). In Japan, S-1 is active in patients with gemcitabine-refractory and chemotherapy-naıve intrahepatic cholangiocarcinoma (ICC) (1). However, cholangiocellular carcinomas arising from the epithelial cells of the intrahepatic and extrahepatic bile ducts are chemoresistant and carry a poor prognosis. The treatment options are limited for patients with advanced ICC. Therefore, the development of new treatment modalities, such as chemoimmunotherapy, is of great importance for the treatment of patients with ICC. However, little is known about the phenomena underlying the immunogenic modulation elicited by sublethal doses of the chemotherapeutic agents gemcitabine or 5-FU, or the immunomodulating agent interferon (IFN)-γ in patients with ICC.

Some chemotherapies have been demonstrated to induce immunogenic

modulation in various types tumors by altering the phenotype of surviving tumor cells, rendering them more susceptible to antigen-specific cytotoxic T-lymphocyte (CTL)-mediated lysis (3, 4). Previously, we showed that several human pancreatic cancer cell lines exhibit up-regulated expression of the tumor-associated antigen (TAA) Wilms' tumor 1 (WT1) following treatment with gemcitabine, which is a standard cytotoxic agent for pancreatic cancer and ICC (5). Moreover, gemcitabine sensitized these cell lines to the cytotoxic effects of WT1-specific CTLs *in vitro* and *in vivo* (5, 6). These reports suggest that immunostimulatory modulation of tumor cells by chemoimmunotherapeutic agents may contribute to the treatment of patients with ICC.

Only tumor cells that have undergone immunogenic tumor cell death ectopically expose the Ca^{2+}-binding chaperone calreticulin, which allows TAAs to traffic to the antigen-presenting compartment in dendritic cells (DCs) (7, 8). It has been reported that anthracyclins efficiently induce pre-apoptotic exposure of calreticulin in anthracyclin-treated tumor cells, resulting in immunogenic tumor cell death (7). Therefore, one of the successful goals of chemoimmunotherapy is

induction of immunogenic tumor cell death.

In this study, we cultured primary ICC cells from the ascites of a patient (human leukocyte antigen (HLA)-A*02:01^{+}/-A*24:02^{+}) who received DCs pulsed with a WT1 peptide mixture restricted with HLA-A*02:01 and -A*24:02 (DC/WT1-I). Moreover, we examined whether chemotherapeutic agents such as gemcitabine and 5-FU and the immunomodulating agent IFN-γ can alter the immunogenicity of ICC cells.

Materials and Methods

Patient with ICC. The study protocol was reviewed and approved by the Ethics Committee of the Jikei Institutional Review Board, Jikei University School of Medicine, as well as the Clinical Study Committee of the Jikei University Kashiwa hospital [No. 14-60 (3209) and 21-204 (6082)]. The patient with stage IV ICC was a 58-year-old female with a large tumor (maximum size: 83 mm) and metastasis to the peritoneum and lymph nodes. Ultrasound-guided fine-needle biopsy of the tumors showed a cholangiocellular carcinoma. The patient received gemcitabine followed by oral 5-FU, both combined with DC/WT1-I.

Cells and complete medium. Ascites from the patient with ICC due to carcinomatous peritonitis was obtained with written informed consent, and the primary ICC cells were cultured in Roswell Park Memorial Institute (RPMI) 1640/high glucose Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (2:1:1) medium supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 20% fetal calf serum (FCS).

Treatment of ICC cells with gemcitabine, 5-FU or IFN-γ. ICC cells were treated with gemcitabine (Sigma Aldrich, St. Louis, MO, USA) (0, 1, 10, 100 and 300 ng/ml) for 2 h, or 5-FU (Kyowa Hakko Kirin Co., Tokyo, Japan) (0, 15, 45 and 100 μg/ml) for 90 min and washed three times with Dulbecco's phosphate-buffered saline (D-PBS) (Nissui Pharmaceutical, Tokyo, Japan). They were then cultured for 48 h at 37°C in complete medium. Moreover, the ICC cells were cultured with IFN-γ (0, 1, 3, 10 ng/ml) for 48 h in complete medium. The concentrations of the agents added to the culture were based on

the serum concentrations of clinically treated patients with cancer. The ICC cells maintained stable numbers of viable cells, albeit with impaired growth.

Phenotype analysis. To determine the phenotype of ICC cells treated or not with gemcitabine, 5-FU, or IFN-γ, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against MUC1 (HMPV), epidermal growth factor receptor (EGFR)-2/neuregulin (HER2/neu) (Neu 24.7), programmed death receptor ligand-1 (PDL1), cluster of differentiation (CD)274, HLA-ABC (W6/32), HLA-DR (L243) (BD Pharmingen, San Jose, CA, USA), HLA-A2 or HLA-A24 (MBL, Nagoya, Japan). Intracellular staining was performed using an FITC-conjugated mAb against calreticulin (FMC75) (Abcam, Cambridge, MA, USA). Matched isotype-control IgG was used as a control. The cells were washed, fixed and analyzed using MACSQuant Analyzers (Miltenyi Biotec Inc., CA, USA) and FlowJo analysis software (Tree Star, OR, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cells were lysed directly in BuVer RLT Plus (Qiagen, Hilden, Germany) and homogenized. Reverse transcription (RT) was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan primers and non-fluorescent quencher probes complementary to *WT1* (Assay ID:Hs00240913_m1) and *18S* ribosomal RNA (rRNA, Assay ID:Hs99999901_s1) genes were purchased from Applied Biosystems. qRT-PCR was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems). *WT1* mRNA expression levels were normalized relative to the *18S* rRNA levels. The data were analyzed using the comparative ΔΔCt method. Briefly, the difference between the threshold cycle (Ct) values of the target and reference genes for each sample were calculated and compared to the ΔCt values for each agent-treated and non-treated group.

Statistical analysis. The significance of the differences between the groups was analyzed using one-way analysis of variance. A *p*-value of less than 0.05 was considered statistically significant.

Results

Characterization of the patient with ICC. The patient with stage IV disease (HLA-A*02:01/-A*24:02) with a large ICC (83 mm) and metastasis to the peritoneum and lymph nodes received gemcitabine (total dose was 7600 mg/m^2) followed by oral 5-FU, S-1 (total dose was 3920 mg), both combined with DC/WT1-I (a total of 15 times) (9). The patient refused full doses of chemotherapeutic agents because of grade 2 depression and anorexia based on the Common Terminology Criteria for Adverse Events (CTCAE) v4.0 (10) (Figure 1). Although the patient could not receive full doses of the chemotherapeutic agents during treatments, she showed stabilization of disease (SD) for 160 days and survived 232 days after the first treatment. Moreover, both HLA-A*02:01- and -A*24:02-restricted WT1 peptide-specific delayed-type

hypersensitivity (DTH) was detected after the second vaccination and continued until the eleventh vaccination (Figure 1).

Characterization of primary ICC cells. Primary ICC cells were cultured from the patient's ascites. As shown in Figure 2, the ICC cells displayed a characteristic phenotype, with a high expression of HLA-ABC, -A2, -A24, PDL1 and TAAs, MUC1 and HER2/neu but extremely low levels of *WT1* mRNA. Moreover, the ICC cells also expressed low levels of HLA-DR.

Effects of gemcitabine, 5-FU, and IFN-γ on TAAs of ICC cells. The ICC cells expressed extremely low levels of *WT1* mRNA. Therefore, we first assessed the effects of chemotherapeutic agents such as gemcitabine and 5-FU and the immunomodulatory agent IFN-γ on *WT1* mRNA expression of the ICC cells *in vitro.* A short treatment with low-dose gemcitabine (Figure 3A), but not 5-FU or IFN-γ (data not shown), resulted in up-regulation of *WT1* mRNA in the ICC cells. The degree of the up-regulation of *WT1* mRNA in the ICC cells *via* gemcitabine was dependent on its dose (data not shown). Moreover, MUC1

expression in the ICC cells was also up-regulated *via* gemcitabine, 5-FU, and IFN-γ based on the dose (Figure 3B-D). In contrast, the HER2/neu expression level was not significantly changed by treatment with gemcitabine, 5-FU or IFN-γ (data not shown).

Effects of gemcitabine, 5-FU, and IFN-γ on major histocompatibility complex (MHC) class I and II molecules of the ICC cells. TAAs are expressed on a number of tumor cells, but many tumor cells down-regulate the expression of MHC class I molecules, precluding recognition by antigen-specific CTLs. Therefore, we assessed the phenotypic changes in HLA-ABC, -A2, -A24 and -DR on the ICC cells treated with gemcitabine, 5-FU or IFN-γ. Gemcitabine and IFN-γ, but not 5-FU, induced dose-dependent up-regulation of HLA-ABC, -A2 and -A24 in the ICC cells (Figure 4). Moreover, the ICC cells expressed low levels of HLA-DR; however, the level of expression was enhanced to a large degree by IFN-γ treatment. These results suggest that treating patients with ICC using gemcitabine combined with immunotherapy may make ICC cells more

susceptible to antigen-specific $CD4^+$ and $CD8^+$ T cell-mediated killing through the up-regulation of MHC class I and II molecules.

Effects of gemcitabine, 5-FU, and IFN-γ on PDL1 expression of ICC cells. To assess the immunosuppressive modulation of ICC cells by chemoimmunotherapy, the ICC cells were treated with gemcitabine, 5-FU or IFN-γ. PDL1, an immunoinhibitory molecule, was observed on the ICC cells under basal conditions (Figure 1 and 5). Treatment of the ICC cells with gemcitabine, 5-FU or IFN-γ resulted in a dose-dependent increase in the expression of PDL1 on the ICC cell surface *in vitro* (Figure 5). In particular, PD-L1 expression was extremely increased after treatment with IFN-γ. These results suggest a potential pathway involving IFN-γ by which the ICC cells may modulate the impaired function of T-cells *via* PDL1.

Effects of gemcitabine, 5-FU, and IFN-γ on calreticulin expression of ICC cells. One of the mechanisms whereby some chemotherapeutic agents can augment immunotherapy to destroy tumor cells is known as immunogenic tumor cell

death, which involves expression of calreticulin (7, 8). Therefore, the ICC cells were also assayed by flow cytometry to assess the expression of calreticulin after treatment with gemcitabine, 5-FU or IFN-γ. As shown in Figure 6A, treatment of the ICC cells with gemcitabine dose-dependently resulted in up-regulation of calreticulin in the ICC cell. These results suggest that the ICC cells were sensitive to gemcitabine, resulting in immunological tumor death. Moreover, treatment of 5-FU or IFN-γ did not induce the exposure of calreticulin in the ICC cells (Figure 6).

Discussion

Some chemotherapeutic agents kill rapidly dividing tumor cells and also induce remodeling of the tumor cells towards immunogenic cell death in a way that potently stimulates antitumor immune responses. Therefore, synergy between chemotherapy and immunotherapy might be expected from specific agents at specific doses. Here, we demonstrate that immunogenic modulation of primary ICC cells is induced by low-dose gemcitabine *in vitro* through the up-regulation of MHC class I and II, calreticulin, MUC1 and *WT1* mRNA. However,

gemcitabine, and IFN-γ also induced up-regulation of immunosuppressive PDL1 in primary ICC cells *in vitro*.

Given that WT1, MUC1 and HER2/neu are ideal immunotherapy targets, we first examined the effects of chemoimmunotherapeutic agents on primary tumor cells cultured from the patient with ICC. The ICC cells expressed extremely low levels of *WT1* mRNA; however, treatment with low-dose gemcitabine that maintained stable numbers of viable cells with impaired growth resulted in high levels of *WT1* mRNA. The WT1 protein induces malignant cellular phenotypes, suppresses apoptosis, and promotes cellular proliferation (11). Moreover, patients with WT1-expressing tumors produce WT1-specific CTLs; thus, WT1 protein is highly immunogenic and a promising tumor antigen (11). Other groups and our laboratory have shown the synergistic effects of chemotherapeutic agents such as gemcitabine or 5-FU and immunotherapy when targeting WT1 by peptide- or DC-based vaccines for patients with pancreatic cancer (12-14). We have also reported that gemcitabine induces up-regulation of *WT1* mRNA in some human pancreatic cancer cell lines *in vitro*

and sensitizes them to the cytotoxic effects of WT1-specific CTLs (5). Therefore, our results that primary ICC cells exhibited up-regulation of *WT1* mRNA at high levels after treatment with gemcitabine suggest that the immunogenicity of primary ICC cells is increased for lysis by WT1-specific CTLs. Indeed, this patient with a large ICC, which was metastatic to the peritoneum and lymph nodes, had stable disease for 160 days and survived 232 days after the first treatment,despite receiving insufficient doses of the chemotherapeutic agents gemcitabine and 5-FU. In addition, both HLA-A*02:01- and -A*24:02-restricted WT1 peptide-specific DTH was detected in the patient during treatments with chemotherapeutic agents combined with DC/WT1-I. Like *WT1* mRNA expression, MUC1 expression was also up-regulated by treatment with gemcitabine, 5-FU, or IFN-γ. However, HER2/neu expression was not altered by these treatments, indicating that modulation by TAAs was selective.

$CD8^+$ CTLs recognize MHC class I/antigenic peptide complexes on tumor cells. Therefore, loss or down-regulation of MHC class I molecules, which is observed in approximately 40-90% tumor cells, plays a crucial step in tumor

immune escape, resulting in drastic changes in the immunogenicity of tumors (15). The expression of HLA-ABC, -A2, -A24 and -DR molecules in the ICC cells was up-regulated by gemcitabine and by IFN-γ. As expected, IFN-γ up-regulated the expression of the HLA-ABC, -A2, -A24 and -DR molecules in ICC cells to a high degree. Indeed, IFN-γ production has been detected in $CD8^+$ T-cells in patients with ICC upon stimulation with WT1 peptides *in vitro* (9). Therefore, IFN-γ production from activated T-cells by a DC/WT1-I vaccine in patients with ICC may increase the immunogenicity of ICC cells towards WT1-specific CTL responses.

Chemotherapy has been reported to induce immunogenic tumor cell death, triggering danger signals such as calreticulin protein expression and resulting in antitumor immunity (16, 17). In this study, the chemotherapeutic agent gemcitabine, but not 5-FU, induced calreticulin expression. This finding suggests that gemcitabine induces immunogenic ICC cell death in patients. calreticulin expression in the ICC cells may synergistically augment immunogenic killing by chemotherapy when combined with cancer vaccines

such as DC/WT1-I, resulting in prolonged survival. Moreover, in patients with ICC, the up-regulation of calreticulin in tumor cells may favor the engulfment of apoptotic tumor bodies by DCs, resulting in polyclonal TAA-specific CTL induction (16, 17).

PDL1 is a surface glycoprotein that induces T-cell anergy or apoptosis by binding to a T-cell co-inhibitory receptor (18, 19). Moreover, up-regulation of PDL1 in tumors is associated with evasion of antitumor immune responses (20) through T-cell receptor -mediated activation of T-cells (22) and interactions with CD80 (22). Of extraordinary interest are the PD1/PDL1 pathway-targeting therapies that are now in phase III trials, with excellent clinical results (23). Therefore, we examined the PDL1 expression in the ICC cells treated with gemcitabine, 5-FU or IFN-γ and showed up-regulation of PDL1 in the ICC cells. These results suggest that treatment by PDL1 blockade may be effective in treating tumors expressing high levels of PDL1 induced by chemoimmunotherapy. The appropriately timed use of PDL1/PD1 checkpoint

blockers may result in potent and durable tumor regression and prolong the survival of patients with ICC (23).

In conclusion, immunostimulatory modulation of cholangiocarcinoma cells was induced by gemcitabine *in vitro* through up-regulation of *WT1* mRNA, MUC1, HLA class I, HLA class II and calreticulin molecules. Moreover, treatment with gemcitabine, 5-FU or IFN- γ also induced the expression of immunosuppressive PD-L1. These opposing effects *in vitro* may be involved in the complex results of clinical efficacy.

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Figure legends

Figure 1. *Treatment schedule of a patient with advanced intrahepatic cholangiocarcinoma (ICC).* A patient with advanced ICC was treated with dendritic cells (DCs) pulsed with a Wilms' tumor 1 (WT1)-specific peptide mixture restricted by human leukocyte antigen (HLA)-A*02:01 and -A*24:02 (DC/WT1-I) in combination with low-dose gemcitabine or S-1. 5-FU: Oral 5-fluorouracil.

Figure 2. *Characterization of primary intrahepatic cholangiocarcinoma* (ICC) *cells.* Primary ICC cells were analyzed by flow cytometry for the expression of the indicated antigens [human leukocyte antigen (HLA)-ABC, -A2, -A24, -DR; mucin 1 (MUC1), epidermal growth factor receptor (EGFR)-2/neuregulin (HER2/neu), programmed death receptor ligand-1 (PDL1), and calreticulin)]. The unfilled histogram profile indicates the isotype control, and the solid histogram indicates the fluorescein isothiocyanate (FITC)-labeled specific antibody.

Figure 3. *Effects of chemoimmunotherapeutic agents on tumor-associated antigens of primary tumor cells.* A: Expression of Wilms' tumor 1 *(WT1)* mRNA was analyzed by quantitative reverse transcription polymerase chain reaction. Primary intrahepatic cholangiocarcinoma (ICC) cells were treated with gemcitabine (300 ng/ml), and the expression of *WT1* mRNA was compared to that of cells without gemcitabine treatment (0 ng/ml). The ICC cells were treated with gemcitabine (B), 5-fluorouracil (5-FU) (C) or interferon-gamma (IFN-γ) (D) at the indicated concentrations. The mean fluorescence intensity (MFI) of mucin 1 (MUC1) in the ICC cells was analyzed (left panel). The histogram profile with the dotted line indicates the control (no treatment) and the lined histogram indicates MUC1 expression in the treated ICC cells (right panel). The results are expressed as the mean ± SD. *p<0.05, **p<0.01.

Figure 4. *Effects of chemoimmunotherapeutic agents on major histocompatibility complex (MHC) class I and class II molecules of primary tumor cells.* Primary intrahepatic cholangiocarcinoma (ICC) cells were treated with gemcitabine (A),

5-fluorouracil (5-FU) (B) or interferon-gamma (IFN-γ) (C) at the indicated concentrations. The mean fluorescence intensity (MFI) of human leukocyte antigen (HLA)-ABC, -A2, -A24 and -DR expression was analyzed. The results are expressed as the mean ± SD. *p<0.05, **p<0.01.

Figure 5. *Effects of chemoimmunotherapeutic agents on programmed death receptor ligand-1* (PDL1) *expression by primary tumor cells.* A: Primary intrahepatic cholangiocarcinoma (ICC) cells were treated with gemcitabine (left panel), 5-fluorouracil (middle panel) or interferon-gamma (IFN-γ) (right panel) at the indicated concentrations. The mean fluorescence intensity (MFI) of PDL1 expression was analyzed. The results are expressed as the mean ± SD. *p<0.05, **p<0.01. B: The histogram profile with the dotted line indicates the control (no treatment), and the lined histogram indicates PDL1 expression in the treated ICC cells.

Figure 6. *Effects of chemoimmunotherapeutic agents on calreticulin expression*