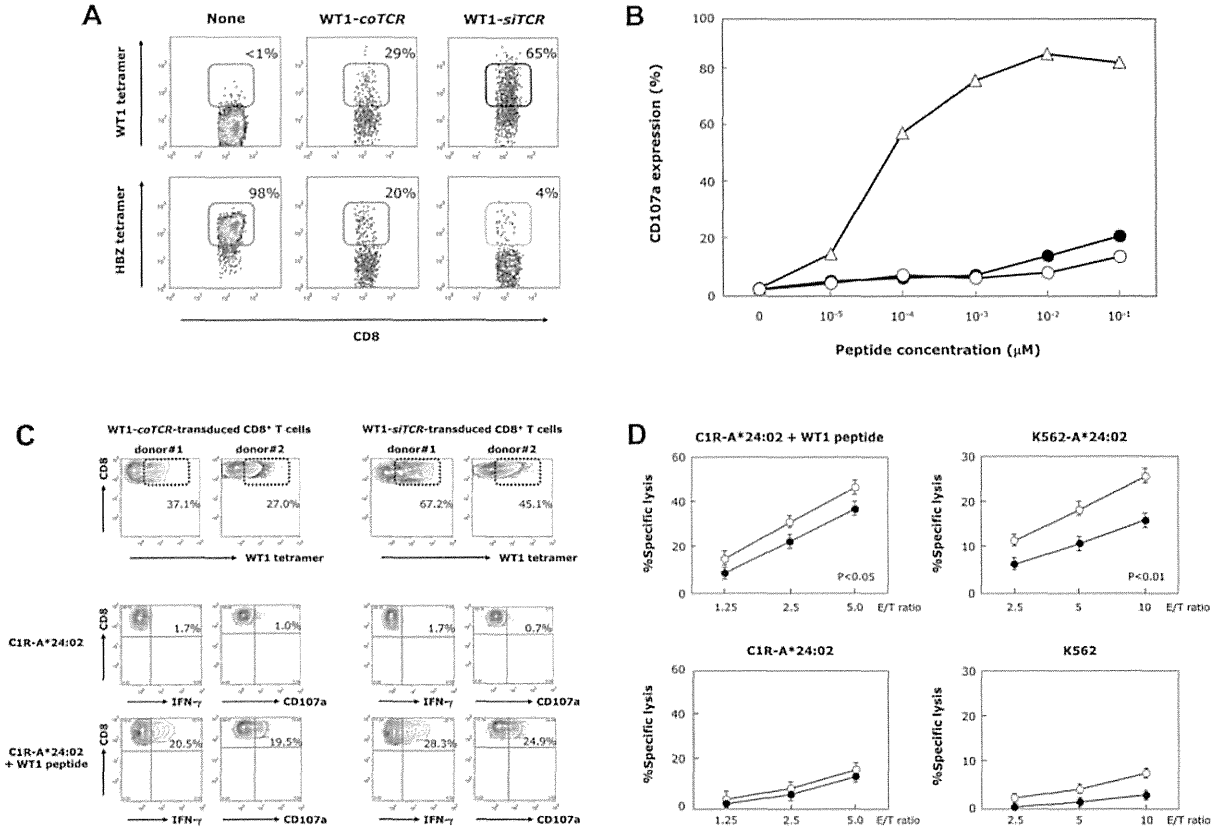

Figure 1. Enhanced expression of introduced WT1-specific TCR and augmented functionality in WT1-*siTCR*–transduced CD8$^+$ T cells. (A) An HLA-A*02:01-restricted $HBZ_{26–34}$-specific CTL clone (HBZ-1) was transduced with the WT1-*siTCR* or WT1-*coTCR* vector. Expression of the introduced WT1-specific and intrinsic HBZ-specific TCRs in *TCR* gene-modified HBZ-1 cells was examined using either HLA-A*24:02/WT1 tetramer or HLA-A*02:01/HBZ tetramer. A non–gene-modified HBZ-1 clone was used as a negative control. (B) HBZ-1 cells (△), WT1-*coTCR*–transduced HBZ-1 cells (●), and WT1-*siTCR*–transduced HBZ-1 cells (○) were cocultured with HLA-A*02:01-positive T2 cells loaded with various concentrations of HBZ peptide for 3 hours. Thereafter, surface CD107a expression was analyzed as detailed in "Detection of CD107a and intracellular IFN-γ expression in WT1-*TCR* gene–transduced CD8$^+$ T cells." (C) IFN-γ production and degranulation of WT1-*siTCR*–transduced and WT1-*coTCR*–transduced CD8$^+$ T cells in response to stimulation with WT1 peptide. Populations of WT1 tetramer-positive cells in WT1-*coTCR*– and WT1-*siTCR*–transduced CD8$^+$ T cells before stimulation are shown in the upper column. The CD8$^+$/WT1-tetramer$^+$ cells shown with a broken line in each sample were analyzed for intracellular IFN-γ production and surface CD107a expression. One set of data obtained from experiments performed using CD8$^+$ T cells from 2 different donors are representatively shown. (D) Cytotoxic activities of WT1-*siTCR*–transduced CD8$^+$ T cells (○) and WT1-*coTCR*–transduced CD8$^+$ T cells (●) against C1R-A*24:02 cells loaded with or without WT1 peptide and K562 cells transduced with or without *HLA-A*24:02* gene were examined by standard 5-hour ^{51}Cr-release assays at various effector/target (E/T) ratios.

therapy. Therefore, we compared the expandability of WT1-*siTCR*–transduced and WT1-*coTCR*–transduced CD8$^+$ T cells after repeated stimulation with WT1 peptide. Representative data are shown in Figure 2A. WT1-*siTCR*–transduced CD8$^+$ T cells showed good expansion after stimulation with WT1 peptide and maintained their antigen specificity. In contrast, WT1-*coTCR*–transduced CD8$^+$ T cells showed rapid growth, but their WT1 specificity declined rapidly. A summary of this experiment using WT1-*siTCR*–transduced CD8$^+$ T cells generated from 5 donors and WT1-*coTCR*–transduced CD8$^+$ T cells generated from 3 donors is shown in Figure 2B.

We further confirmed the WT1 specificity and HLA-A*24:02 restriction of cytotoxicity mediated by WT1-*siTCR*–transduced CD8$^+$ T cells that had been cultured and expanded for more than 2 months with repeated WT1 peptide stimulation. Representative data for 5 experiments are shown in Figure 3A. WT1-*siTCR*–transduced CD8$^+$ T cells after $>$ 2 months of culture appeared to be totally positive for TCR-Vβ5.1 expression, and $>$ 70% of the cells were positive for HLA-A*24:02/WT1-tetramer staining. These WT1-*siTCR*–transduced CTLs exerted strong cytotoxicity against WT1 peptide-loaded but not peptide-unloaded C1R-A*24:02 cells. Similarly, they exerted strong cytotoxicity against HLA-A*24:02-positive but not HLA-A*24:02-negative leukemia cell lines, as shown in Figure 3B. Their cytotoxicity against HLA-A*24:02-positive leukemia cell lines was significantly abrogated by anti-HLA class I MoAb. These data indicate that stimulation of WT1-*siTCR*–transduced CD8$^+$ T cells with WT1 peptide might be effective for their expansion while maintaining their antigen specificity.

Lysis of autologous leukemia cells and lack of damage to autologous hematopoietic progenitor cells by WT1-*siTCR*–transduced CTLs

For clinical application of adoptive T-cell therapy using WT1-*TCR* gene-engineered CTLs, it is essential to obtain evidence that autologous leukemia cells are indeed lysed and that autologous hematopoietic progenitor cells are not damaged by WT1-*siTCR*–transduced CTLs. We therefore performed cytotoxicity assays using WT1-*siTCR*–transduced CTLs as effector cells, and autologous leukemia cells from leukemia patients and hematopoietic

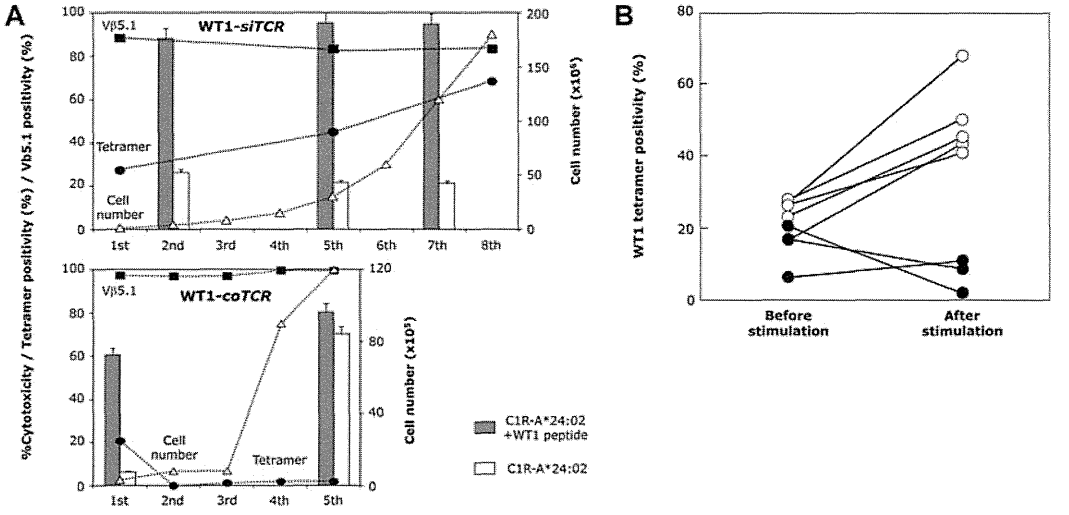

Figure 2. Enhanced expandability of WT1-*siTCR*–transduced CD8$^+$ T cells by repetitive stimulation with WT1 peptide in vitro. (A) WT1-*siTCR*–transduced CD8$^+$ T cells (top) and WT1-*coTCR*–transduced CD8$^+$ T cells (bottom) were repetitively stimulated with HLA-A*24:02-positive LCLs loaded with WT1 peptide in vitro. Total cell number (△), percentage of Vβ5.1-positive cells (■), and percentage of HLA-A*24:02/WT1 tetramer-positive cells (●) were monitored after stimulation with WT1 peptide. Cytotoxic activities of WT1-*siTCR*–transduced CD8$^+$ T cells and WT1-*coTCR*–transduced CD8$^+$ T cells against WT1 peptide-loaded (gray bars) or -unloaded C1R-A*24:02 cells (white bars) are also shown. (B) Percentages of HLA-A*24:02/WT1 tetramer-positive cells among WT1-*siTCR*–transduced CD8$^+$ T cells from 5 donors (○) and those in WT1-*coTCR*–transduced CD8$^+$ T cells from 3 donors (●) before and after stimulation with WT1 peptide.

progenitors from cord blood as target cells. As shown in Figure 4A, WT1-*siTCR*–transduced CTLs generated from peripheral blood CD8$^+$ T cells of patients with leukemia exerted cytotoxicity against autologous leukemia cells. On the other hand, those generated from cord blood CD8$^+$ T cells exerted no cytotoxicity against autologous hematopoietic progenitor cells. As shown in Figure 4B, all WT1-*siTCR*–transduced CTL lines made from cord blood CD8$^+$ T cells used in this experiment appeared to efficiently lyse WT1 peptide-loaded HLA-A*24:02-positive LCLs and HLA-A*24:02-positive leukemia cell lines without the addition of exogenous WT1 peptide.

In vivo antileukemia efficacy of adoptively transferred WT1-*siTCR*–transduced CTLs in a xenograft mouse model

We further examined the in vivo antileukemia efficacy of adoptive transfer with WT1-*siTCR*–transduced CTLs using 3 cohorts of a therapeutic xenograft mouse model. In the first group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with non–gene-modified CD8$^+$ T cells (control CTLs), and additionally treated by intravenous infusion of control CTLs weekly for a total of 5 times. Control CTLs were prepared by stimulation of peripheral blood CD8$^+$ T cells with anti-CD3 MoAb

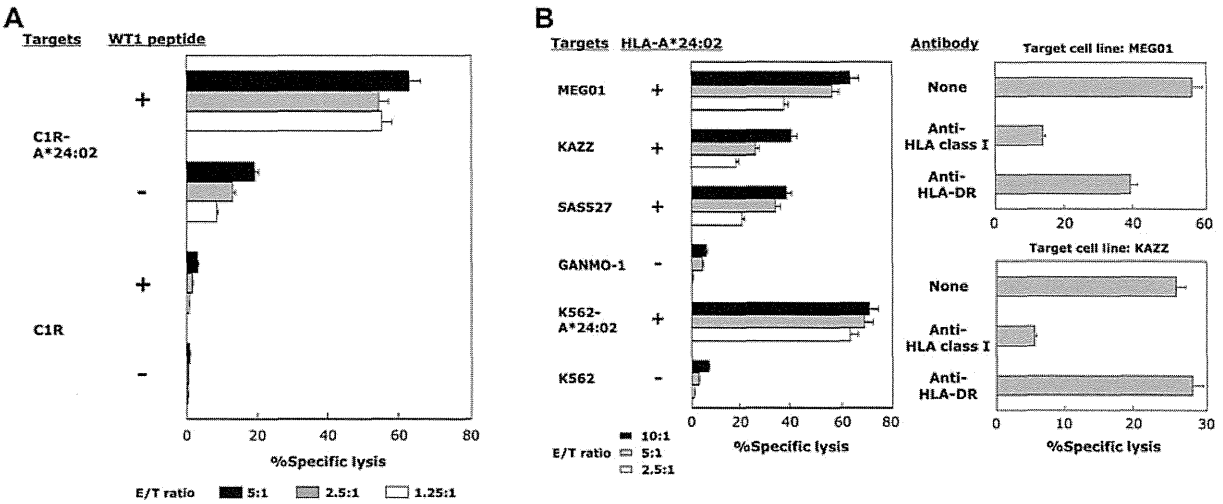

Figure 3. HLA-A*24:02-restricted and WT1 peptide-specific cytotoxicity mediated by WT1-*siTCR*–transduced CTLs after repeated stimulation with WT1 peptide. (A) Cytotoxicity of WT1-*siTCR*-transduced CD8$^+$ T cells that had been cultured continuously for > 2 months against WT1 peptide-loaded or -unloaded C1R-A*24:02 cells and C1R cells was examined by 5-hour ^{51}Cr-release assays. (B) Cytotoxicity of WT1-*siTCR*–transduced CD8$^+$ T cells that had been cultured continuously for > 2 months against HLA-A*24:02-positive and HLA-A*24:02-negative leukemia cell lines was examined by 5-hour ^{51}Cr-release assays. HLA class I-restriction of cytotoxicity mediated by WT1-*siTCR*–transduced CTLs against MEG01 and KAZZ cell lines was examined by 5-hour ^{51}Cr-release assays at an E/T ratio of 5:1 in the presence or absence of anti-HLA class I MoAb or anti-HLA-DR MoAb.

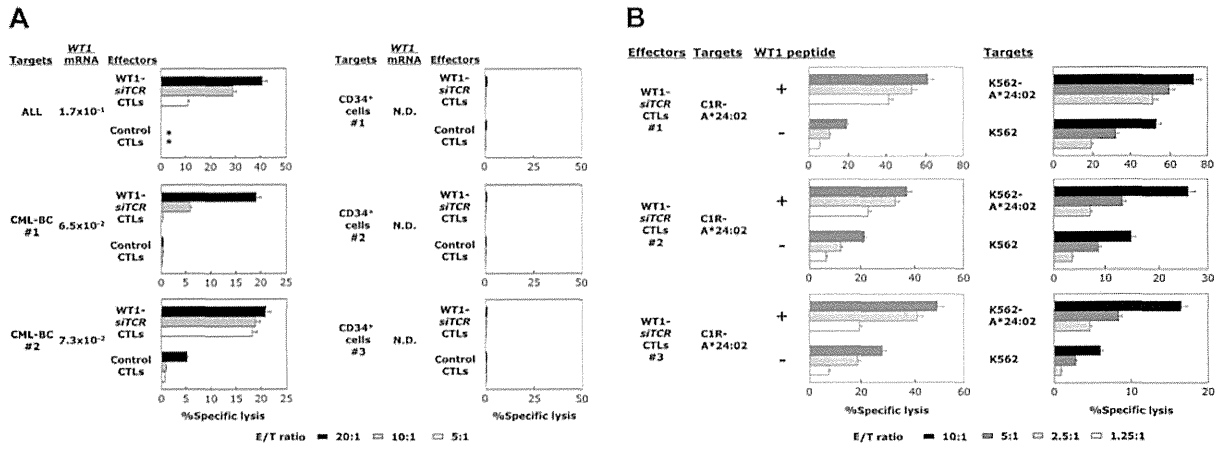

Figure 4. Cytotoxicity mediated by WT1-*siTCR*–transduced CTLs against autologous leukemia cells and hematopoietic progenitor cells. (A) Cytotoxic activities of WT1-*siTCR*–transduced CTLs against autologous leukemia cells and autologous normal hematopoietic progenitor cells were examined by 5-hour ^{51}Cr-release assays. WT1-*siTCR*–transduced CTLs were generated from peripheral blood CD8$^+$ T cells from a patient with acute lymphoblastic leukemia (ALL) in complete remission, 2 patients with blastic crisis of chronic myelogenous leukemia (CML-BC) in chronic phase after chemotherapy, and cord blood CD8$^+$ T cells from 3 donors. Their cytotoxicity against autologous freshly isolated leukemia cells or autologous hCB-CD34$^+$ cells was examined by standard ^{51}Cr-release assays at various E/T ratios. The relative expression levels of *WT1* mRNA in target cells are shown. N.D. indicates not detectable. (B) Cytotoxicity mediated by WT1-*siTCR*–transduced CTLs generated from cord blood CD8$^+$ T cells against C1R-A*24:02 cells with or without loaded WT1 peptide, K562-A*24:02 cells, and K562 cells was examined by 5-hour ^{51}Cr-release assays at various E/T ratios. Each number of effector cells (#1, #2, and #3) corresponds to that of the hCB-CD34$^+$ cell sample shown in Figure 4A, respectively.

and cultured in IL-2–containing medium. In the second group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs, without additional cell transfer. In the third group, NOG mice were inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs and received additional cell transfer with WT1-*siTCR*–transduced CTLs weekly 5 times. The growth curves of the inoculated leukemia cells are shown in Figure 5. K562-A*24:02 cells grew rapidly in all mice treated with control CTLs and died within 40 days. Compared with mice treated with control CTLs, the survival of mice inoculated with K562-A*24:02 cells that had been preincubated with WT1-*siTCR*–transduced CTLs was significantly prolonged. Furthermore, additional transfer of WT1-*siTCR*–transduced CTLs further significantly prolonged the survival period of K562-A*24:02-inoculated mice. Notably, no tumor formation was detected in mice during adoptive transfer of WT1-*siTCR*–transduced CTLs. These results clearly show the efficacy of adoptive T-cell therapy using WT1-*siTCR*–transduced CTLs for treatment of human leukemia.

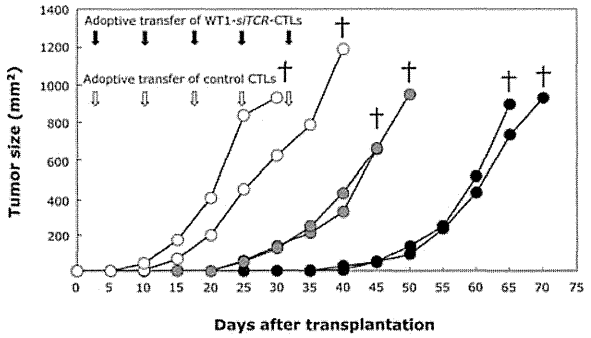

Figure 5. Antileukemia effect of adoptively transferred WT1-*siTCR*–transduced CTLs in a xenograft mouse model. WT1-*siTCR*–transduced CTLs and non–gene-modified human CD8$^+$ T cells (control CTLs) were prepared from peripheral blood CD8$^+$ T cells. NOG mice were inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs or control CTLs with or without additional cell therapy. (○) represents the growth of leukemia cells in 2 control mice inoculated with K562-A*24:02 cells preincubated with control CTLs and into which control CTLs were transferred weekly; gray circles, leukemia cell growth in 2 mice inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs without additional transfer of CTLs; and (●), the growth of leukemia cells in 2 mice inoculated with K562-A*24:02 cells preincubated with WT1-*siTCR*–transduced CTLs, and into which WT1-*siTCR*–transduced CTLs were additionally transferred weekly. The time points (in days) after transplantation of K562-A*24:02 cells when the mice died are indicated.

No deteriorative effect of WT1-*siTCR*–transduced CTLs on engraftment and differentiation of autologous hematopoietic progenitor cells in humanized mice

Finally, we addressed the issue of whether WT1-*siTCR*–transduced CTLs exert an inhibitory effect on the proliferation and differentiation of normal hematopoietic progenitor cells, as it has been reported that WT1 expression is detectable in normal hematopoietic progenitor cells.[21,22,36] The hCB-CD34$^+$ cells that had been preincubated with WT1-*siTCR*–transduced CTLs or control CTLs were transplanted into NOG mice. Three months later, these mice were killed and analyzed for engraftment and differentiation of human hematopoietic cells. HLA-A*24:02 appeared to be efficiently expressed in human blood cells that had proliferated in humanized mice (data not shown). Representative data for 3 experiments are shown in Figure 6A. It is clearly evident that human CD34$^+$ cells preincubated with WT1-*siTCR*–transduced CTLs were successfully engrafted and differentiated into human peripheral blood cell components, including hCD45$^+$/CD33$^+$ myeloid cells, hCD45$^+$/CD19$^+$ B cells, and hCD45$^+$/CD3$^+$ T cells in the spleen, as was the case for NOG mice transplanted with human CD34$^+$ cells that had been preincubated with control CTLs. In bone marrow, not only hCD45$^+$/CD33$^+$ myeloid cells, but also hCD45$^+$/CD34$^+$ hematopoietic progenitor cells, hCD45$^+$/hGPA$^+$ erythroid immature cells, and hCD45$^+$/CD41a$^+$ megakaryocytic immature cells were efficiently engrafted and differentiated from human CD34$^+$ cells preincubated with WT1-*siTCR*–transduced CTLs as well as control CTLs. Although interindividual differences in engraftment efficacy were detected among NOG mice transplanted with human CD34$^+$ cells, it was concluded that

Figure 6. Lack of an inhibitory effect of WT1-*siTCR*–transduced CTLs on human hematopoiesis in a humanized mouse model. HLA-A*24:02-positive hCB-CD34$^+$ cells preincubated with autologous WT1-*siTCR*–transduced CTLs or non–gene-modified autologous CTLs (as a negative control) were transplanted into NOG mice. Three months later, the mice were killed and examined for engraftment and differentiation of human hematopoietic progenitor cells in the spleen and bone marrow. (A) Representative data for 3 experiments. (B) Summary of long-term hematopoiesis of engrafted human hematopoietic progenitor cells in the bone marrow and spleen of mice transplanted with hCB-CD34$^+$ cells that had been preincubated with control CTLs or WT1-*siTCR*–transduced CTLs.

WT1-*siTCR*–transduced CTLs never damage human CD34$^+$ hematopoietic progenitor cells (Figure 6B).

Discussion

In our previous study, we developed a novel retroviral vector system that can express antigen-specific TCR more efficiently based on the concept of siRNA-targeting of the constant regions of the endogenous *TCR*-α and *TCR*-β genes and siRNA-resistant codon-optimization of exogenous *TCR* genes.[25] To apply the basic concept of WT1-*siTCR* clinically for treatment of human leukemia, we investigated in detail the efficacy and safety of this strategy. Consequently, we demonstrated the marked advantages of WT1-*siTCR* gene transfer for adoptive immunotherapy in terms of both enhancement of the antileukemia effect and safety. First, we clearly demonstrated that up-regulated expression of introduced WT1-specific TCR and sufficient inhibition of endogenous TCR could be achieved using an experimental system in which the WT1-*siTCR* gene was transduced into a HBZ-specific T-cell clone. Enhanced expression of the introduced WT1-TCR on WT1-*siTCR*–transduced CTLs resulted in augmentation of WT1-specific cytotoxicity as compared with that mediated by WT1-*coTCR*–transduced CTLs. In addition, through repetitive stimulation with cognate peptide in vitro, WT1-*siTCR*–transduced CTLs showed marked expandability while maintaining their antigen specificity. Furthermore, the WT1-*siTCR*–transduced CTLs were able to successfully lyse autologous leukemia cells but not normal hematopoietic progenitor cells. Importantly, experiments using a xenograft mouse model revealed that adoptively transferred WT1-*siTCR*–transduced CTLs effectively inhibited leukemia cell growth in vivo. In contrast, the engraftment and differentiation abilities of normal hematopoietic progenitors showed no deterioration in the presence of WT1-*siTCR*–transduced CTLs, thus negating the possibility that WT1-specific CTLs might mediate severe bone marrow failure.

One of the major advantages of *TCR* gene-engineered T-cell immunotherapy revealed by our present series of experiments is the establishment of augmented antigen-specific cytotoxicity and safety through silencing of endogenous *TCR* gene expression. Recent clinical studies using *TCR* gene-transduced T cells have indicated

that almost all of these cells disappeared in patients within 2 months after infusion.[8,37] Moreover, there appeared to be a significant correlation between clinical response and the persistence of infused T cells in the peripheral blood of patients.[9] Therefore, it would be important to maintain a sufficient number of *TCR* gene-engineered T cells with adequate antigen specificity in patients for a long period to achieve a good clinical response. In our present study, the antigen specificity of WT1-*coTCR*–transduced CTLs declined rapidly during culture, even though they were stimulated repeatedly with WT1 peptide. This might have been because of the formation of mispaired TCRs that had acquired nonspecific reactivity. In contrast, WT1-*siTCR*–transduced CTLs appeared to be markedly expanded while maintaining WT1 specificity and showing enhanced WT1-specific cytotoxicity for more than 2 months as a result of repetitive stimulation with WT1 peptide. Recently, it has been reported that adoptively transferred gp100-specific murine T cells were expandable up to 1000-fold after cognate peptide vaccination, resulting in an effective antitumor response in vivo.[38] These data strongly support the practical value of WT1-*siTCR* for maintaining the WT1 specificity of *TCR* gene-modified CTLs for a long period in vivo and also suggest that WT1 peptide vaccination after adoptive transfer of WT1-*siTCR*–transduced CTLs would facilitate the expansion of WT1-specific CTLs in human patients.

One of the major concerns related to adoptive transfer of *TCR* gene-engineered T cells is the possibility of evoking severe autoimmunity mediated by mispaired TCR. Recently, an elegant study of *TCR* gene therapy using a mouse model has revealed that mispairing of introduced and endogenous TCR chains in *TCR* gene-modified T cells leads to the formation of self-reactive TCRs that are responsible for lethal graft-versus-host disease.[19] Furthermore, it has been reported that adjustments in the design of gene therapy vectors for preventing the formation of mispaired TCRs could reduce the risk of *TCR* gene therapy-induced lethal autoimmunity.[19] This evidence obtained from basic research strongly supports the clinical advantage of our WT1-*siTCR* vector.

It is also notable that autologous HLA-restricted and foreign antigen-derived peptide-specific TCRs can exert allogeneic HLA responsiveness. Recently, the frequent incidence of allogeneic HLA reactivity mediated by redirected T cells against predefined virus antigens has been reported.[39] Using an LCL panel, we similarly observed that our HLA-A*24:02-restricted and WT1 peptide-specific *TCR* gene-transduced CTLs responded to the HLA-B*57:01 molecule in the absence of cognate WT1 peptide.[40] Therefore, *TCR* gene-engineered T cells should be tested for their allogeneic HLA reactivity against recipient cells before administration.

Another notable finding in the present study was that WT1-*siTCR*-transduced CTLs never exerted a cytotoxic effect on normal hematopoietic progenitor cells. Although it has been proposed that WT1 is an ideal tumor-associated antigen,[41] previous reports have indicated that WT1 expression is certainly detectable in normal hematologic progenitor cells,[21,22,36] suggesting a risk of bone marrow failure mediated by WT1-specific CTLs. Furthermore, the occurrence of severe leukocytopenia after WT1 peptide vaccination in 2 patients with myelodysplastic syndrome has been reported.[42] Therefore, we examined in detail the inhibitory effect of WT1-*siTCR*–transduced CTLs on normal human hematopoiesis in a humanized mouse model. We clearly demonstrated that WT1-*siTCR*–transduced CTLs never damaged normal human hematopoietic progenitor cells. There are 3 possible explanations for the mechanism underlying the resistance of normal hematopoietic progenitors to WT1-specific CTL-mediated cytotoxicity. The first is that the amount of WT1 expressed in normal hematopoietic progenitors is not enough to be recognized by CTLs. This possibility seems likely because quantitative analysis has revealed that the expression level of *WT1* mRNA in normal hematopoietic progenitor cells is relatively low compared with that in leukemia cells.[43,44] The second is that normal hematopoietic progenitors have the potential to resist CTL-mediated cytotoxicity. We previously reported that, although the levels of WT1 expression in myeloma and lymphoma cells were almost the same, only myeloma cells were lysed efficiently by WT1-specific CTLs. The extent of membrane damage induced by purified perforin appeared to be significantly higher in myeloma cells than in lymphoma cells.[32] Therefore, the susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity, and normal hematopoietic cells are relatively resistant to CTL-mediated granule exocytosis. The third is that the introduced WT1-specific TCR in this study might have had an optimal range of avidity for recognition of leukemia cells but that spare normal hematopoietic progenitor cells physiologically expressed WT1, as avidity enhancement of the introduced TCR was able to evoke "on-target" adverse events against normal tissues.[9]

In conclusion, the present study has revealed that our WT1-*siTCR* retrovirus vector system shows considerable promise in terms of efficacy and safety for adoptive immunotherapy for leukemia using *TCR* gene-engineered T cells. On the basis of our data, we intend to begin clinical trials of adoptive WT1-*siTCR*–transduced T-cell therapy with WT1 peptide vaccination for chemotherapy-resistant leukemia.

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Authorship

Contribution: T.O. designed and performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper, and provided financial support; S.O., J.A., K.N., T.S., J.M., and H.S. discussed and interpreted the experimental results and provided materials; K.K. made and supplied the tetramer; and M.Y. discussed and interpreted the experimental results, wrote and edited the paper, and provided financial support.

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ORIGINAL ARTICLE

Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response

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Abstract Wilms' tumor gene (WT1), which is expressed in human pancreatic cancer (PC), is a unique tumor antigen recognized by T-cell-mediated antitumor immune response. Gemcitabine (GEM), a standard therapeutic drug for PC, was examined for the regulation of WT1 expression and the sensitizing effect on PC cells with WT1-specific antitumor immune response. Expression of WT1 was examined by quantitative PCR, immunoblot analysis, and confocal microscopy. Antigenic peptide of WT1 presented on HLA class I molecules was detected by mass spectrometry. WT1-specific T-cell receptor gene–transduced human T cells were used as effecter T cells for the analysis of cytotoxic activity. GEM treatment of human MIAPaCa2 PC cells enhanced WT1 mRNA levels, and this increase is associated with nuclear factor kappa B activation. Tumor tissue from GEM-treated MIAPaCa2-bearing SCID mice also showed an increase in WT1 mRNA. Some human PC cell lines other than MIAPaCa2 showed up-regulation of WT1 mRNA levels following GEM treatment. GEM treatment shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic peptide. In fact, presentation of HLA-A*2402-restricted antigenic peptide of WT1 (CMTWNQMNL) increased in GEM-treated MIAPaCa2 cells relative to untreated cells. WT1-specific cytotoxic T cells killed MIAPaCa2 cells treated with an optimal dose of GEM more efficiently than untreated MIAPaCa2 cells. GEM enhanced WT1 expression in human PC cells and sensitized PC cells with WT1-specific T-cell-mediated antitumor immune response.

Keywords Pancreatic cancer · WT1 · Gemcitabine · NF kappa B · T-cell response

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Introduction

Pancreatic cancer (PC) is a devastating disease with a 5% overall 5-year survival rate [1, 2]. This high mortality rate is due to a combination of factors that include a high incidence of metastatic disease at initial diagnosis, an aggressive clinical course, and the failure of systemic therapies used for treatment. Despite the fact that advanced loco-regional disease is found in 40% of patients [3], only 5–25% of patients with pancreatic cancer are treated surgically [4]. Even in cases where pancreatic cancer is discovered at a resectable stage, only 10–20% of patients are expected to survive for more than 5 years after curative resection [5].

Gemcitabine (GEM) is currently the most commonly used therapeutic drug prescribed in cases of advanced PC [6, 7]. Numerous phase III trials testing gemcitabine in combination with other cytotoxic drugs have failed to reveal any additional benefit compared with gemcitabine alone [8]. Erlotinib, a small molecule inhibitor of the epidermal growth factor receptor tyrosine kinase, is a notable exception in that it is the only drug reported to confer a significant improvement in survival over gemcitabine alone [9]. Recently, Folfirinox was reported to be a more efficient, but more toxic, regimen for pancreatic cancer and might be promising for the patients with good performance status [10]. Ultimately, improved treatment of advanced PC will likely require additional selected and targeted agents that provide the benefit of prolonged survival with minimum risk.

The Wilms' tumor gene WT1 encodes a zinc finger transcription factor. Although the WT1 gene was originally defined as a tumor suppressor gene [11–13], additional reports demonstrate that it is highly expressed in leukemia and various types of malignant tumors [14] and can confer oncogenic functions [15]. WT1-specific cytotoxic T lymphocytes (CTLs) and WT1 antibodies have both been shown to be induced spontaneously in tumor-bearing leukemia patients [16]. These results indicate that WT1 protein is highly immunogenic and establish it as a promising tumor antigen for recognition by specific CTLs [17]. The safety and clinical efficacy of major histocompatibility complex (MHC) class I-restricted WT1 epitope peptides against various malignancies have been confirmed in clinical immunotherapy trials [14, 15].

Reports indicate that WT1 is frequently overexpressed in human pancreatic cancer cells [18]. Recent clinical reports on treatments combining GEM drug therapy with peptide vaccine immunotherapy have demonstrated safe and promising results in cases of advanced PC [19, 20]. In our recent phase I clinical trial that tested a combination of WT1 peptide vaccine and GEM in treatment of advanced PC, several cases showed marked tumor regression (manuscript in preparation). These results suggest that the actions of WT1-targeted antitumor immunity and GEM can function synergistically against PC cells. In the present study, we demonstrate that GEM treatment up-regulates WT1 expression in PC cell lines, and that antitumor immune activity against PC cells via a WT1-specific T-cell response is augmented by GEM treatment.

Materials and methods

Cell lines, antibodies, and mice

Human pancreatic cancer cell lines (MIAPaCa2, PANC-1, AsPC-1, BxPC-3, Capan-1 and Capan-2) were obtained from the American Type Culture Collection (Manassas, VA, USA) [21]. A rabbit polyclonal antibody against WT1 (C-19) and a goat polyclonal antibody against Lamin B (C-20) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Eight- to ten-week-old SCID mice were supplied by Nihon SCL Co., Ltd. (Hamamatsu, Japan) and were maintained in our specific pathogen-free facilities. Mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue or cell samples were lysed directly in Buffer 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, Foster City, CA). WT1 expression levels were normalized relative to those of 18S rRNA.

Inhibition of nuclear factor kappa B (NF-kB)

Inhibition of NF-kB activity in human PC cells was achieved using an NF-kB p65 (Ser276) inhibitory peptide kit (IMGENEX, San Diego, CA, USA). Briefly, MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates and incubated for 24 h. Growth medium was then changed to medium containing GEM (0 or 30 ng/ml) with NF-kB blocking peptide (50 μM) or control peptide (50 μM). After 24-h incubation, cellular expression of NK-kB was determined using qRT-PCR.

Immunoblot analysis

The nuclear fraction of MIAPaCa2 cells used for the detection of WT1 protein was isolated using an Active Motif extraction kit (Carlsbad, CA, USA). Protein samples (30 μg/well) separated by electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk for 1 h, membranes were exposed to antibodies specific to WT1 (1:100) and beta-actin (1:10,000; Sigma–Aldrich, St. Louis, MO, USA) and then to horseradish peroxidase-conjugated secondary antibodies. The ECL-PLUS Detection System (GE Healthcare, Buckinghamshire, UK) was used for chemiluminescent detection of secondary antibodies.

Confocal microscopy

MIAPaCa2 cells cultured on glass coverslips were incubated with or without GEM (30 ng/ml) for 24 h. Cells were then washed and fixed in 4% paraformaldehyde. Immunofluorescent visualization of cells expressing WT-1 was achieved by incubating slides in rabbit anti-WT1 antibody (1/200), followed by Amaxa488-conjugated donkey anti-rabbit IgG antibody (Molecular probes, Eugene, OR, USA). Cell nuclei were stained with TO-PRO-3 iodide (Molecular Probes), and a laser scanning confocal microscope (LSM510, CarlZeiss, Thornwood, NY, USA) was used to obtain fluorescence images.

Positive ion ESI LC–MS/MS analysis of MHC class I binding peptides from MIAPaCa2 cells

MIAPaCa2-bearing mice were injected intraperitoneally with PBS or GEM (3.75 mg/mouse). After 48 h, tumors were resected and digested using collagenase to obtain single cells. MHC class I binding peptides were isolated from 10^8 cells using the method described by Storkus et al. [22]. Isolated peptides were dissolved in 50% methanol and analyzed via electrospray ionization (ESI) liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a triple quadrupole mass spectrometer (Q TRAP) (Applied Biosystems, Foster City, CA, USA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography (Agilent Technologies, Wilmington, DE, USA) was employed. The WT1 antigenic peptide (aa 235–243 CMTWNQMNL; MW = 1,139.5 Da) in 50% methanol was easily produced m/z 1171.5 as a methanol adduct ion $(M + MeOH)^+$. The multiple reaction monitoring (MRM) transition monitored for the detection of this peptide was m/z 1,171.5/1,154.5. This peptide was eluted at a flow rate 0.2 mL/min from an Intersil C8-3 column [50 × 2.1 mm, 3 μm particle size] (GL Science Inc., Tokyo Japan) using a linear gradient of 9.5% min^{-1} of 5–100% acetonitrile containing 1% formic acid. To estimate cellular peptide concentrations, a standard curve was prepared by increasing concentrations (0–1,000 pmol) with chemically synthesized WT-1 antigenic peptide. The response was considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression analysis.

Cytotoxicity assay

WT1-specific cytotoxic effector cells were generated as described below. Full-length WT1-specific T-cell receptor (TCR) a/b genes (Va20/J33/Ca for TCR-a and Vb5.1/J2.1/Cb2 for TCR-b, respectively) isolated from the HLA-A*2402-restricted $WT1_{235-243}$-specific $CD8^+$ CTL clone TAK-1 [23] were cloned into a pMEI-5 retroviral vector (Takara Bio, Shiga, Japan). WT1-specific TCR genes were then transduced into normal $CD8^+$ lymphocytes as described previously [24]. Cytotoxicity assays were performed using a standard 4-h culture ^{51}chromium (Cr) release assay described elsewhere [25].

Statistical analysis

The significance of differences between groups was analyzed using Student's *t* test for two independent groups and with Tukey's test for multiple-group comparisons. Values that did not fit a Gaussian distribution were analyzed with the Bonferroni method for multiple-group comparisons.

Results

Up-regulation of WT1 mRNA in human PC cells by in vitro treatment with GEM

Proliferation of MIAPaCa2 cells was inhibited for 48 h with stable numbers of viable cells following treatment with 30 and 100 ng/ml of GEM (Fig. 1a). Growth of MIAPaCa2 cells was also impaired by treatment with 10 ng/ml of GEM for 72 h. Levels of WT1 mRNA were enhanced significantly by treatment of MIAPaCa2 cells with 10, 30, and 100 ng/ml of GEM for 24, 48 and, 72 h, respectively (Fig. 1b). Enhancement of WT1 mRNA was also observed after 2-h treatment with GEM (100 ng/ml) in following 72 h (Fig. 1c). This GEM-mediated enhancement was suppressed by the addition of NF-kB blocking peptide in the culture (Fig. 1d).

GEM-mediated up-regulation of WT1 mRNA expression was examined in various human pancreatic cancer cell lines. GEM-treated Capan-2 cells showed a significant enhancement of WT1 mRNA expression (Fig. 2a). Low steady-state levels of WT1 mRNA expression in AsPC-1 and BxPC-3 cells were also enhanced by GEM treatment (Fig. 2b). In contrast, expression of WT1 mRNA in Capan-1 and PANC-1 cells was not up-regulated by GEM treatment (Fig. 2b, c).

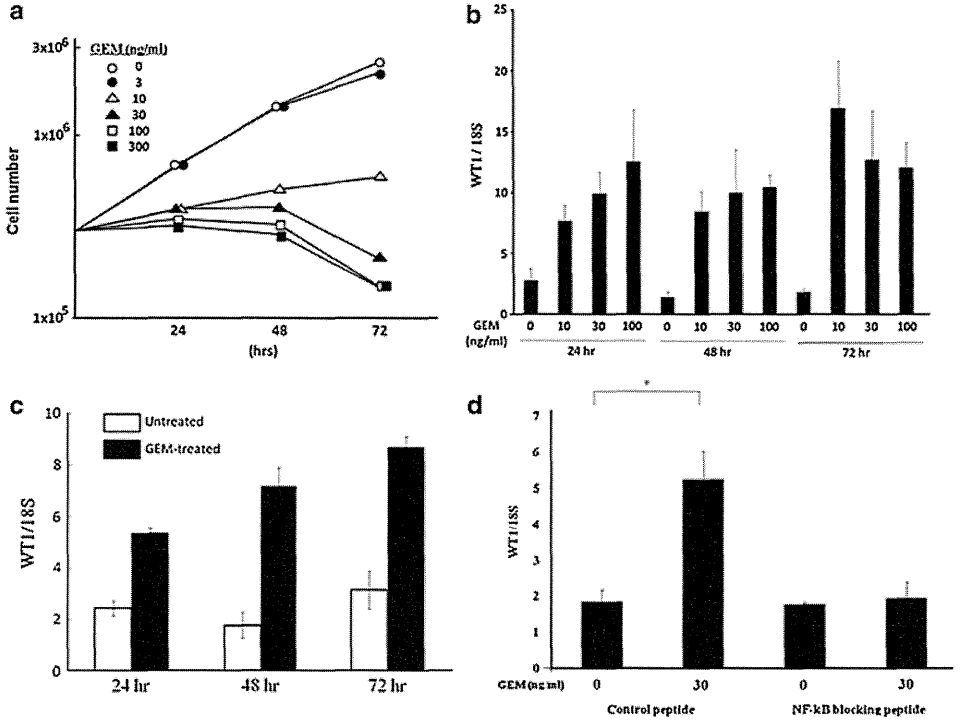

Fig. 1 **a** Proliferation of MIAPaCa2 cells in medium containing various concentrations of GEM. MIAPaCa2 cells (3×10^5/well) were seeded in 6-well culture plates in regular culture medium, which was then exchanged for GEM-containing medium after 24 h. At 24-h intervals, cells were detached using trypsin, and cell numbers were counted using a hemocytometer ($n = 3$). **b** Up-regulation of WT1 mRNA in MIAPaCa2 cells by GEM treatment. Twenty-four hours after plating, culture medium was exchanged to media containing GEM at indicated concentrations (0, 10, 30 and 100 ng/ml). MIAPaCa2 cells were harvested at 24-h intervals, and WT1 mRNA in cell homogenates was analyzed using qRT-PCR. WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **c** Up-regulation of WT1 mRNA in MIAPaCa2 cells after short treatment with GEM. Twenty-four hours after plating, MIAPaCa2 cells were untreated or treated with 100 ng/ml of GEM for 2 h. MIAPaCa2 cells did not proliferate but kept alive for following 72 h by this treatment with GEM. After GEM treatment, cells were washed well, cultured in regular culture medium, and harvested at 24-h intervals. WT1 mRNA in cell homogenates was analyzed using qRT-PCR, and WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **d** NF-kB suppresses GEM-induced up-regulation of WT1 mRNA. MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates. After 24 h, medium was exchanged for media containing GEM (0 or 30 ng/ml) and/or NF-kB blocking peptide (50 μM) or control peptide (50 μM). WT1 mRNA levels were quantified after 24-h incubation using qRT-PCR. $^*P < 0.01$

Changes in WT1 mRNA expression levels were also examined in MIAPaCa2 cells following in vitro treatment with various other chemotherapeutic agents. Oxaliplatin, Doxorubicin, and five-fluorouracil showed significant enhancement of WT1 mRNA expression, but cisplatin and irinotecan did not (Suppl. 1). Because GEM is the standard drug used to treat human PC, its effect on human PC cells was studied thereafter.

In vivo up-regulation of WT1 mRNA in tumor tissue by treatment of MIAPaCa2-bearing SCID mice with GEM

In order to clarify whether in vivo treatment of tumor cells with GEM induces an enhancement of WT1 mRNA expression, SCID mice implanted subcutaneously with MIAPaCa2 cells were treated with a clinical dosage of GEM. We observed a significant increase in the levels of WT1 mRNA 48 h after injection of GEM (Fig. 3).

GEM treatment shifts localization of WT1 from the nucleus to the cytoplasm

We used immunoblot analysis to examine the levels of WT1 protein in MIAPaCa2 cells cultured in the absence or presence of GEM. Relative to untreated cells, WT1 protein levels in GEM-treated MIAPaCa2 cells were augmented; however, after 36 h of cell culture, levels of WT1 protein diminished in both untreated and GEM-treated cells (Fig. 4a). This decline in WT1 protein levels was rescued by treatment with the proteasome inhibitor MG-132, indicating that WT1 protein is susceptible to proteasomal degradation (Fig. 4b).

Confocal microscopy images demonstrate that WT1 protein is primarily located in nuclei of untreated cells (Fig. 5a). However, in MIAPaCA2 cells treated with GEM, localization of WT1 protein shifted to the cytoplasm and the intensity of WT1 immunofluorescence in the nucleus decreased

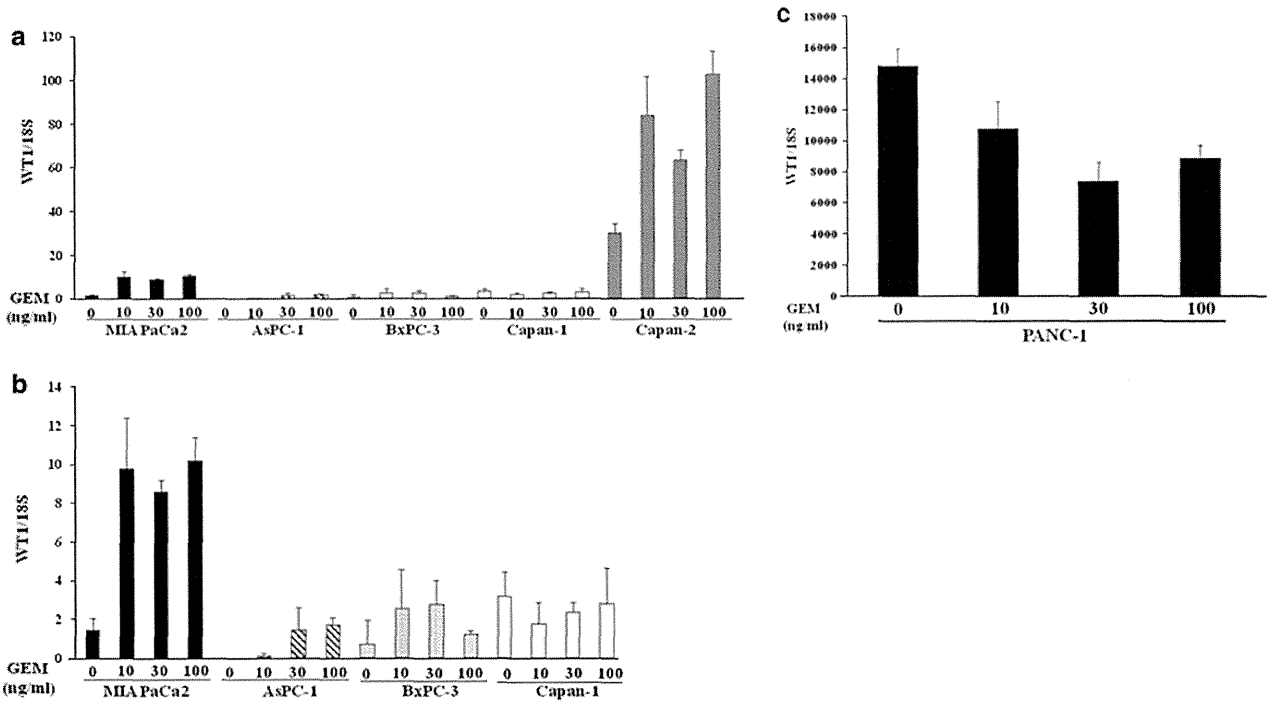

Fig. 2 **a** Up-regulation of WT1 mRNA levels in various human PC cell lines following GEM treatment. Human PC cells (1×10^6 MIAPaCa2, AsPC-1, BxPC-3, Capan-1 or Capan-2) were seeded in 10-cm culture plates. After 24-h incubation, medium was changed to media containing GEM (10, 30 or 100 ng/ml). After 48 h, we used qRT-PCR to quantify the relative ratio of WT1 to 18S mRNA levels in each cell line ($n = 3$). **b** GEM-induced up-regulation of WT1 mRNA in human PC cells with low basal levels of WT1 mRNA (MIAPaCa2, AsPC-1, BxPC-3 and Capan-1). To illustrate these results, we replotted data from (**a**) to represent a considerably narrower range of mRNA level ratios (0–14) on the *y*-axis. (**c**) Expression of WT1 mRNA in human PC cells with high basal levels of WT1 mRNA (PANC-1). To illustrate the results, we plotted data to represent a considerably wider range of mRNA level ratios (0–18,000) on the *y*-axis

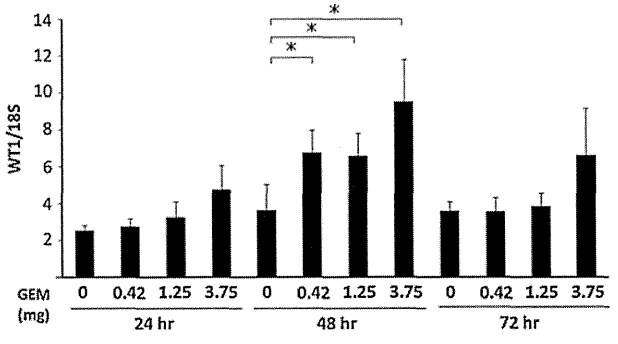

Fig. 3 Tumors in PC-bearing SCID mice treated with GEM show increased WT1 mRNA levels. Ten days after subcutaneous inoculation of SCID mice with 5×10^6 MIAPaCa2 cells (formation of approximately 1-cm diameter tumors), mice were injected intraperitoneally with GEM (0, 0.42, 1.25 and 3.75 mg/mouse). Tumors were resected every 24 h thereafter, and relative levels of WT1 mRNA were quantified using qRT-PCR ($n = 3$). Duplicate trials of the same protocol showed similar results. $^*P < 0.01$

(Fig. 5a). Decline in WT1 protein levels following GEM treatment was also observed in immunoblot analyses of the nuclear fraction of treated MIAPaCa2 cells (Fig. 5b).

Enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide following GEM treatment

Figure 6a shows typical standard curve obtained with increasing quantities of WT1 antigenic peptide. The data indicate a linear relation over a wide range (0–1,000 pmol) of analyte amount with correlation coefficients greater than 0.99. The data in the Fig. 6b demonstrate the sensitivity as well as the noise background of the LC–MS/MS. The noise background is less than 1 cps. The signal from injection of 10 pmol of this peptide spiked to MIAPaCa2 cells is approximately 16 cps, giving an S/N ratio of approximately 16. The low noise background and signal of 10 pmol of this peptide indicated the extrapolated limit of detection is less than 0.8 pmol on column under S/N = 2.

The level of the WT1 antigenic peptide was estimated among MHC class I binding peptides from MIAPaCa2 cells treated with either PBS or GEM to 6.49 pmol/10^8cell or 8.78 pmol/10^8cell, respectively. GEM treatment increased the presentation of HLA-A*2402-restricted WT1 antigenic peptide on MIAPaCa2 cells.

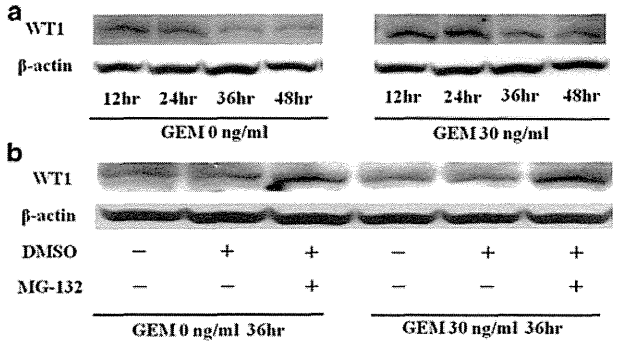

Fig. 4 **a** WT1 protein is degraded by proteasomal enzymes. Twenty-four hours after 3×10^5 MIAPaCa2 cells/well were seeded in 6-well culture plates, medium was exchanged from untreated to media containing GEM (0 or 30 ng/ml). Expression of WT1 protein in the cells was analyzed every 12 h thereafter from immunoblots described in Sect. "Materials and methods". **b** Protease inhibitors block WT1 degradation. Twenty-four hours after incubating MIAPaCa2 cells with GEM (0 or 30 ng/ml), MG-132 in DMSO or DMSO alone was added to each well at a concentration of 5 μM and 0.05%, respectively. Treated and control cells (in 0.05% DMSO alone) were incubated for 12 h before harvesting cells for immunoblot analysis of WT1 and beta-actin proteins

GEM-treated PC cells are killed efficiently by effector cells transduced with genes encoding a WT1-specific T-cell receptor

The susceptibilities of untreated and GEM-treated MIAPaCa2 cells to WT1-specific cytotoxic effector T cells were compared. The cytotoxic effect of WT1-specific effector cells on MIAPaCa2 cells was enhanced significantly when PC cells were treated with either 10 or 30 ng/ml of GEM for 48 h (Fig. 7). Notably, effector cell cytotoxicity was not enhanced by treatment of PC cells with 100 ng/ml of GEM, although this high dose of GEM was more toxic to PC cells than 10 or 30 ng/ml. Up-regulation of MHC class I in MIAPaCa2 cells by GEM treatment that possibly provides the similar results was not observed (data not shown).

Discussion

In the present study, we demonstrate that expression of WT1 mRNA in human PC cells is enhanced by treatment

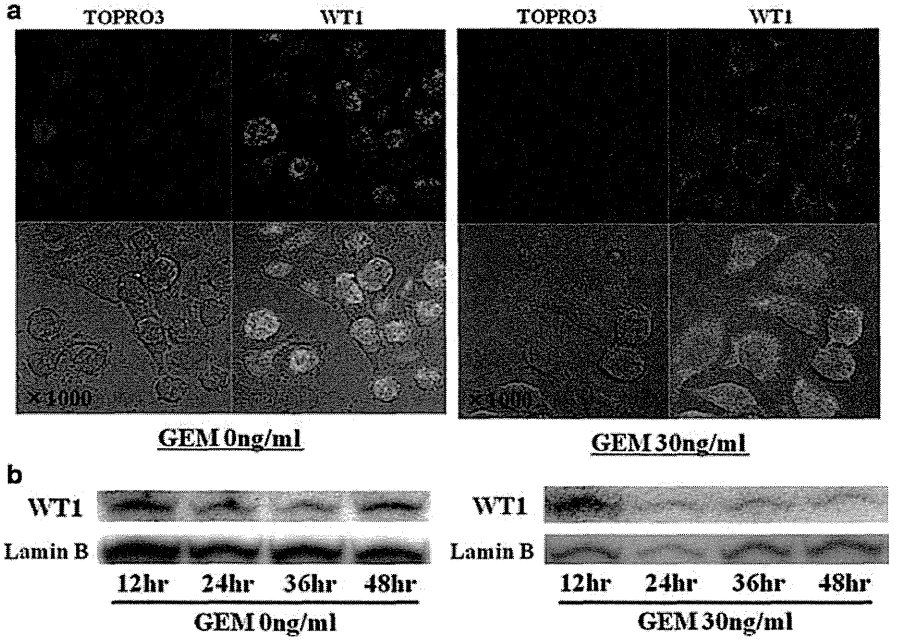

Fig. 5 **a** GEM treatment shifts WT1 protein localization from nucleus to cytoplasm. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, untreated medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). After 24-h incubation, cells were fixed with paraformaldehyde, followed by nuclear staining with TO-PRO-3 iodide (*blue color*) and detection of WT1 with rabbit anti-WT1 polyclonal antibody and anti-rabbit IgG conjugated with fluorescein isothiocyanate (*green color*). Stained cells were observed using confocal microscopy (original magnification ×1,000). **b** GEM treatment diminishes nuclear localization of WT1 protein. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). At 12-hour intervals thereafter, nuclei were isolated and WT1 protein levels of nuclear extracts were analyzed on immunoblots as described in Sect. "Materials and methods"

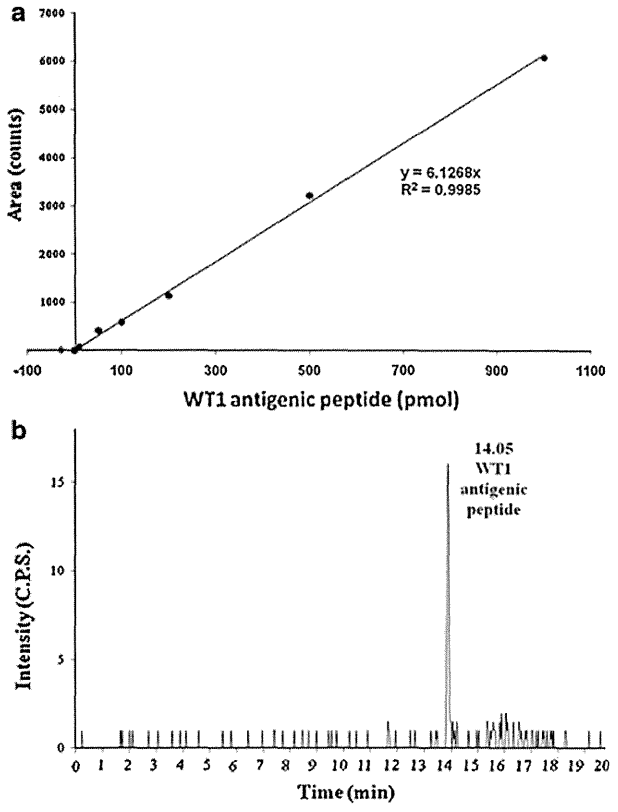

Fig. 6 **a** Standard curve for HLA-A*2402 restricted WT1 antigenic peptide. **b** Trace of MRM signal during LC–MS/MS analysis of spiked HLA-A*2402-restricted WT-1 antigenic standard peptide (10 pmol) in MIAPaCa2 cells

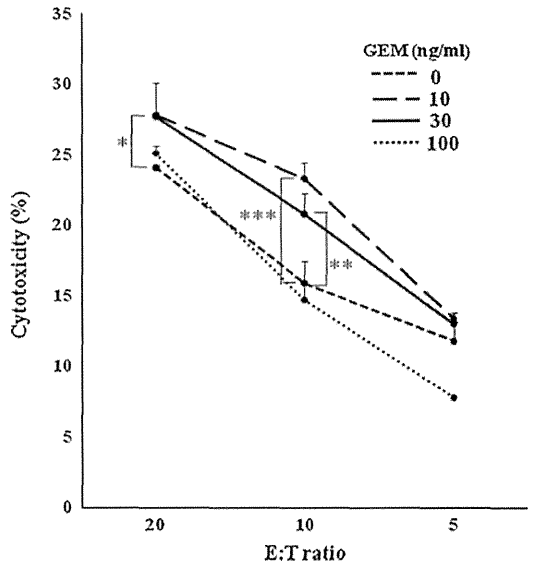

Fig. 7 WT1-specific CTLs kill GEM-treated MIAPaCa2 cells efficiently. MIAPaCa2 cells pretreated with 0, 10, 30, or 100 ng/ml GEM for 48 h were labeled with ^{51}Cr. ^{51}Cr release assays were used to measure the cytotoxic activity of WT1-specific effector cells against untreated or GEM-pretreated MIAPaCa2 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

with GEM. MIAPaCa2 cells demonstrating GEM-mediated enhancement of WT1 mRNA levels did not proliferate but maintained stable numbers of viable cells with impaired growth by continuous treatment with low-dose GEM as well as short treatment with high-dose GEM. WT1 is a transcription factor with oncogenic potential, in that it can induce malignant cellular phenotypes, suppress apoptosis, and promote cell proliferation [15]. We hypothesize that up-regulation of WT1 levels in PC cells aids cell survival by conferring chemoresistance against GEM's toxic effects.

Based on the fact that GEM-mediated augmentation of WT1 mRNA expression was attenuated by addition of an NF-kB blocking peptide in the culture, activation of NF-kB also appears to play a significant role in WT1 enhancement. NF-kB is known to be active in many malignant tumors and has been implicated in cellular resistance to cytotoxic agents and escape from apoptosis [26]. Previous reports demonstrate that GEM activates NF-kB [27] and that the ensuing regulatory cascade activates the WT1 gene downstream [28]. Human PC cell lines with high NF-kB activity are resistant to GEM [27], and that silencing or suppression of NF-kB increases the sensitivity of PC cells to GEM and induces apoptosis [29–31].

It is of note and interest that some chemotherapeutic agents other than GEM showed capability on up-regulation of WT1mRNA expression. Especially, treatment with oxaliplatin (L-OHP) induced marked enhancement of WT1mRNA expression. Folfirinox including L-OHP was recently reported to be a more efficient regimen for metastatic pancreatic cancer (10). However, combined treatment with Folfirinox and WT1 targeting immunotherapy might be unsuccessful because of severe leukopenia by Folfirinox. GEM has relatively low hematologic toxicity and thus seems to be preferable for combination therapy with WT1 targeting immunotherapy.

We also observed up-regulation of WT1 mRNA by GEM treatment in vivo. Within 48 h of treating MIAPaCa2-bearing SCID mice with a clinical dose of GEM, steady-state levels of WT1 mRNA in the tumor increased. Despite its rapid disappearance after intraperitoneal injection, the enhancement of WT1 mRNA expression in tumor tissue was significant. Enhancement of WT1 mRNA expression was also observed after in vitro short treatment with GEM. These results suggest strongly that GEM treatment of human PC in a clinical setting might induce up-regulation of WT1 in PC cells.

In the present study, we found that the localization of WT1 protein shifted from nucleus to cytoplasm following GEM treatment. WT1 protein has been shown to undergo nucleocytoplasmic shuttling [32], and the function of WT1 has been suggested to correlate with its cellular location: Siberstein et al. [33] described that WT1 was localized to

the cytoplasm and not to nuclei in some human breast cancers and suggested that such localization may be regulated by alternative splicing of WT1 mRNA. On the other hand, immunohistochemical studies of Nakatsuka et al. [34] demonstrate a majority of WT1-positive tumors with diffuse or granular staining in the cytoplasm. Ye et al. [35] report that phosphorylation of WT1 protein resulted in cytoplasmic retention of WT1, thereby inhibiting DNA binding and altering transcriptional activity. Through the activation of NF-kB, GEM treatment may mediate a similar phosphorylation and translocation of WT1 protein from nucleus to cytoplasm.

In order for MHC class I-restricted antigen to be presented and recognized by antigen-specific CTLs, tumor antigen must be degraded by proteasomal enzymes located in the cytoplasm [36]. Retention of an intra-nuclear tumor antigen such as WT1 in the cytoplasm should favor tumor antigen processing, and in fact, we observed enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide using ESI LC–MS/MS analyses. GEM-treated MIAPaCa2 cells showed greater susceptibility than untreated cells to the cytotoxic effects of WT1-specific CTLs generated by transduction of a gene encoding a WT1-specific T-cell receptor. Importantly, treatment with 10–30 ng/ml of GEM enhanced the susceptibility of MIAPaCa2 cells to CTL, but treatment with 100 ng/ml did not. This phenomenon indicates that the enhanced susceptibility of GEM-treated MIAPaCa2 cells to CTLs is not due to GEM toxicity, but to augmented expression of the WT1 target antigen.

GEM is a nucleoside analog with clinical relevance to the treatment of several solid tumors, including PC; nonetheless, its antitumor effect is limited. We observed significant clinical response in a phase I clinical study of combined treatment against advanced PC using a WT1 peptide vaccine and GEM (manuscript in preparation). The presumed actions of GEM up-regulating WT1 expression in vivo and WT1-specific CTLs killing GEM-treated tumor cells efficiently may prove valuable for the treatment of human PC. It has been reported that GEM may suppress the activity of myeloid-derived suppressor cells that inhibit antitumor immunity [37]. In addition, GEM has been shown to increase the number of dendritic cells in blood without affecting T-cell activity in patients with PC [38]. We propose that combining GEM's proven role as an immunopotentiator with its ability to up-regulate target WT1 expression of PC cells will enhance the susceptibility of PC cells to WT1-specific CTLs. Furthermore, PC cells already acquired GEM resistance by the activation of NF-kB might be injured by WT1-specific CTLs. Assessment of the clinical response to combined therapy with WT1 peptide vaccine and GEM is presently underway.

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Conflict of interest There are no financial disclosures of any of the authors to declare.

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医療

難治性腫瘍の治療成績向上を目指した外科手術を基軸とする集学的治療戦略

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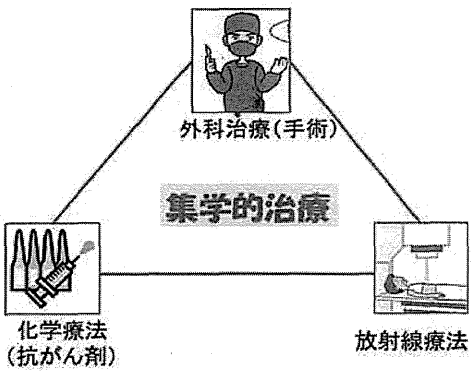
何を目指しているのですか？

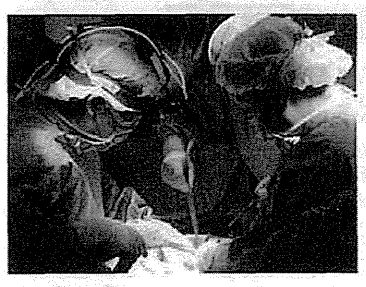
大学院医学研究科消化器外科学分野 II では上部消化管・胆道・膵臓の癌を代表とする悪性疾患やその他の腫瘍性・非腫瘍性疾患を対象として手術治療や臨床・基礎研究を行っています。

当科では超進行癌に対する積極的な拡大根治手術、低悪性度病変に対する適切な縮小手術、早期癌に対する低侵襲内視鏡手術を治療戦略として手術を行っています。その中でも食道癌、胆嚢癌、膵癌はとりわけ悪性度が高く再発しやすいため、**外科**治療をもってしても治療が困難な癌、いわゆる難治癌と呼ばれています。これら難治性で高度に進行した癌の治療においては手術治療だけでは不足であり、術前・術後の手術以外の治療（補助療法）、すなわち抗がん剤による化学療法や**放射線治療**を組み合わせた「集学的治療法」の開発が必要になります。



難治性高度進行癌の治療においては手術治療だけでは不足であり、化学療法や放射線治療と組み合わせた「集学的治療法」の開発が必要になります。

この「集学的治療法」の開発のために私たちが進めているプロジェクトの一つに膵癌術前化学療法の臨床試験があります。術前に化学療法を加えることで術後の癌再発率を下げ、治療成績を向上させるのが目的です。このプロジェクトの特徴は道内にある三つの医育大学の垣根を取り払い、全道各地の 38 施設の内科・外科が共同してチームを組み、文字通り“オール北海道”で大規模な臨床試験を行うというものです。また、診断時に手術適応のない高度進行膵癌・胆道癌の患者さんに対しても新たなプロジェクトがあります。抗癌剤治療や放射線療法が半年以上効果的で病勢の悪化が認められない場合には、通常行わない肝動脈などの血管合併切除再建を伴う積極的な手術治療、すなわち拡大切除術を行うことで良好な成績が得られる可能性があることを世界で初めて報告し、まもなく全国的な多施設共同試験を開始します。これら



高度進行癌に対する化学療法後の手術の様子

の他、手術治療、化学療法、放射線療法など標準的治療法が効かなくなった高度進行あるいは再発癌の患者さんを対象として、副作用の少ない体に優しい補助療法を開発するための癌免疫療法の臨床研究、また最新の分子生物学的な手法を応用した遺伝子治療や分子標的治療の基礎研究にも着手しています。次項では当科の研究実績の中から癌免疫治療の臨床研究を紹介します。

何が課題となっていますか？

癌細胞の表面には癌細胞のみに発現し正常組織には発現しない腫瘍抗原という目印が存在します。当科では多くの癌腫に発現する腫瘍抗原を標的として、「HER2 蛋白 CHP 複合体パルス樹状細胞を用いた癌**ワクチン**第Ⅰ相臨床試験」、「NY-ESO-1 癌抗原蛋白／CpG／リポソーム複合体を用いた癌ワクチン第Ⅰ相臨床試験」など、免疫治療の臨床研究を 2005 年より展開してきました。もともと生体に備わった免疫力を増強させることで抗腫瘍活性を高めることを目的とした、患者さんにとって負担の少ない新しい補助治療です。高度進行・再発癌の患者さんを対象としたこれまでの臨床試験で明らかになったことは、癌免疫治療により高頻度に特異的な免疫反応が誘導されること、また、免疫治療のみで比較的長期間、病状の進行を停滞させることができる症例が存在するという事実です。


実験室で行う"基礎研究"の様子

このように癌ワクチンによって生体内で腫瘍に特異的な免疫反応が誘導される事がわかってきましたが、現在までのところワクチン単独では確実な臨床効果が得られるまでには至っていません。この理由として、腫瘍の免疫逃避機構や免疫抑制性細胞の存在などが知られています。今後はこのような癌ワクチンへの反応良好例を判別するバイオマーカーの開発によりワクチン療法が適応となる症例を選択することや、より強力な免疫反応を誘導できる補助療法の開発と検証が課題と考えています。

次に何を目指しますか？

実験室内での特殊な条件下で得られたデータのみでは臨床現場で使用したり、患者さんに有効な治療にまで発展させることは困難です。実験室で行う“基礎研究”と、それを応用して患者さんを対象として行う“臨床研究”は医学研究の 2 本柱であり、“トランスレーショナルリサーチ”とも呼ばれています。北海道大学病院は平成 24 年に厚労省より臨床研究中核病院に指定され、研究を推進するシステムが整備されています。私たちの診療科でも難治性癌や再発癌を克服するための様々なトランスレーショナルリサーチを展開しており、手術を主軸にし難治癌の集学的治療のエビデンスを創出していきたいと考えています。

3

進行・再発癌に対する新規癌ワクチン CHP-MAGE-A4 の臨床応用と特異的免疫反応の解析

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— Summary —

I. はじめに

固形がんに対する標準治療法は手術、化学療法、放射線療法であるが進行再発癌に対しては十分な治療成績を示しているとは言えない。このような難治性癌に対する補助療法として免疫治療が有効である可能性があり、当科では 2005 年より難治性再発性の進行癌患者を対象に「HER2 蛋白 CHP 複合体パルス樹状細胞を用いたがんワクチン第 I 相臨床試験」、「NY-ESO-1 等癌抗原蛋白／CpG／リポソーム複合体を用いたがんワクチン第 I 相臨床試験」などの臨床研

究を展開してきた。この中で2009年より進行・再発癌患者を対象にCHP-MCP癌ワクチンの第1+2相臨床研究を行っており、ワクチン接種により誘導される特異的免疫反応について解析している（UMIN000001999）。CHP-MAGE-A4ワクチンは、多様な癌腫に発現が認められている癌精巣抗原MAGE-A4蛋白（表1）をコレステリル基置換プルラン（cholesteryl pullulan:CHP）と結合（図1）、ミセル化した蛋白質複合体で、MAGE-A4蛋白をMHC class IとclassIIの両経路に効率的に抗原提示させる作用がある（図2、3）[1]。また、これまでの臨床研究の結果から高率に抗原特異的な免疫反応を誘導することが報告されている（表2）[2,3]。

Cancer types	MAGE-A4 expression (%)
Uterine cancer	63
Ovarian cancer	57
Esophageal cancer	52
Head and neck cancer	51
Melanoma	40
Bladder cancer	38-73
Non small cell lung cancer	28-34
Colorectal cancer	22
Hepatic cell carcinoma	20

表1. 各癌腫におけるMAGE-A4抗原の発現頻度

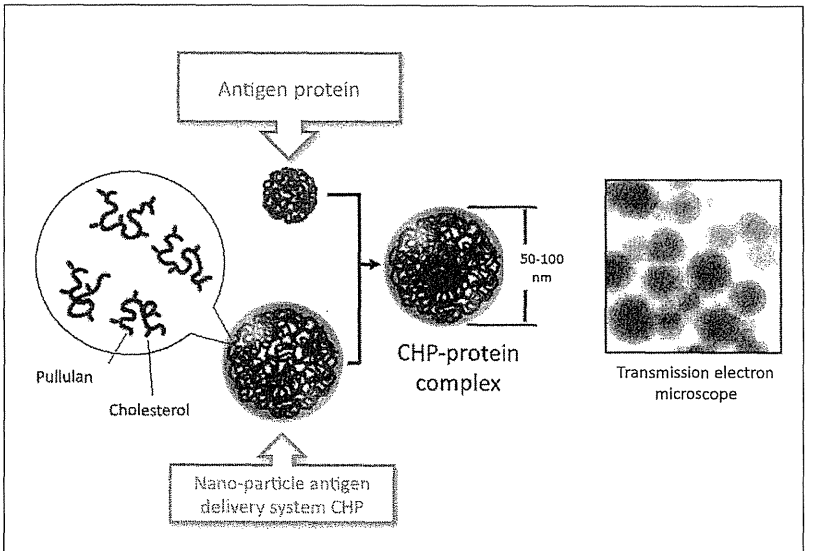

図1.

CHP（cholesteryl pullulan:CHP、コレステリル基置換プルラン）は疎水化多糖類であり、内部に抗原タンパク質を包埋し、ナノ複合体を形成する。

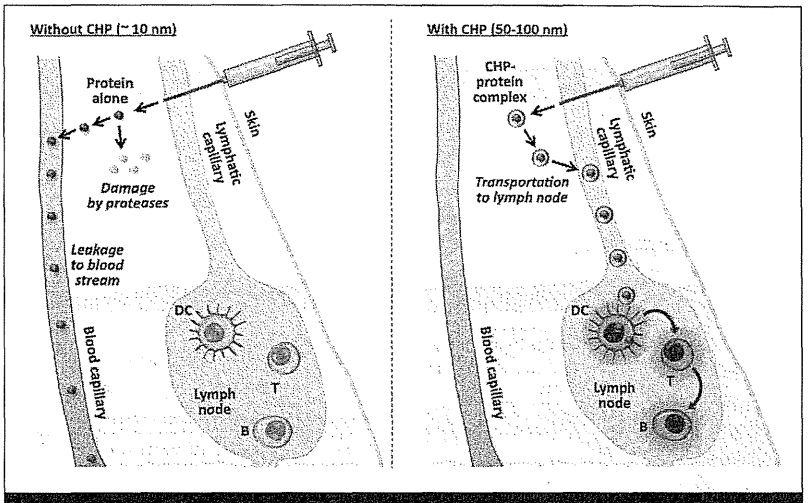

図2.

CHP ワクチンを注射すると、皮膚に存在する樹状細胞などの抗原提示細胞にがん抗原情報が効率よく提示される。

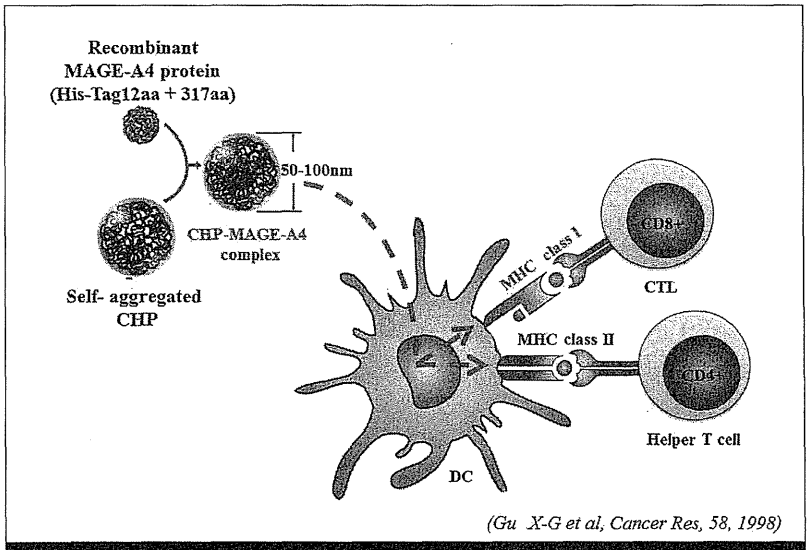

図3. Cholesteryl pullulan(CHP)

CHP-MAGE-A4 ワクチンは癌精巣抗原 MAGE-A4 蛋白を CHP と結合、ミセル化した蛋白質複合体で、MAGE-A4 蛋白を MHC class I と class II の両経路に効率的に抗原提示させる作用がある。従来の抗原タンパクのみの癌ワクチンは、外来抗原であるため class II pathway を介した CD4+Tcell の活性化を担っていた。これに対し、CHP-抗原タンパクの複合体は樹状細胞に取り込まれると、CD4+Tcell はもちろんだが、class I pathway を介し CD8+Tcell も活性化することが可能であり、新規抗原蛋白デリバリーシステムとして期待されている。

II. 対象と方法

対象は 2009 年 4 月から 2011 年 11 月までに第 1+2 相臨床研究に登録した進行・再発癌患者 22 例。免疫染色による MAGE-A4 陽性症例を対象とし、2 週間隔で合計 6 回、ワクチンを皮下投与した。主評価項目として安全性、最大耐性量、用量制限毒性の検証、副次的評価項目