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Inhibition of nuclear factor-kappaB suppresses peritoneal dissemination of gastric cancer by blocking cancer cell adhesion

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Currently, patients with peritoneal dissemination of gastric cancer must accept a poor prognosis because there is no standard effective therapy. To inhibit peritoneal dissemination it is important to inhibit interactions between extracellular matrices (ECM) and cell surface integrins, which are important for cancer cell adhesion. Although nuclear factor-kappa B (NF-κB) is involved in various processes in cancer progression, its involvement in the expression of integrins has not been elucidated. We used a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), to study whether NF-κB blocks cancer cell adhesion via integrins in a gastric cancer dissemination model in mice and found that DHMEQ is a potent suppressor of cancer cell dissemination. Dehydroxymethylepoxyquinomicin suppressed the NF-κB activity of human gastric cancer cells NUGC-4 and 44As3Luc and blocked the adhesion of cancer cells to ECM when compared with the control. Dehydroxymethylepoxyquinomicin also inhibited expression of integrin (α2, α3, β1) in *in vitro* studies. In the *in vivo* model, we injected 44As3Luc cells pretreated with DHMEQ into the peritoneal cavity of mice and performed peritoneal lavage after the injection of cancer cells. Viable cancer cells in the peritoneal cavities were evaluated sequentially by *in vivo* imaging. In mice injected with DHMEQ-pretreated cells and lavaged, live cancer cells in the peritoneum were significantly reduced compared with the control, and these mice survived longer. These results indicate that DHMEQ could inhibit cancer cell adhesion to the peritoneum possibly by suppressing integrin expression. Nuclear factor-kappa B inhibition may be a new therapeutic option for suppressing postoperative cancer dissemination. (*Cancer Sci* 2011; 102: 1052–1058)

Peritoneal dissemination is the most frequent process through which gastric cancer recurs,[1] and patients with this condition must currently accept a very poor prognosis.[2,3] Standard chemotherapy is currently not sufficiently effective for improving the survival of patients with peritoneal dissemination of gastric cancer. To inhibit peritoneal dissemination, it may be important to control the adhesion of cancer cells to the peritoneum. During cancer cell dissemination in the abdominal cavity, cancer cells make contact with the basement membrane through gaps between mesothelial cells.[4,5] The basement membrane beneath mesothelial cells comprises extracellular matrices (ECM) consisting of type 1 and 4 collagen, fibronectin or laminin,[6] and mesothelial cells also produce ECM.[7] The interactions between these ECM and cell surface integrins play very important roles in cancer cell adhesion and, therefore, cancer progression.[8]

Integrins are membrane-bound proteins that form heterodimers of α- and β-subunits at the cell surface. The α-subunits vary between 120 and 180 kD, and are non-covalently associated with β-subunits (90–110 kD). To date, 14 α subunits and eight β subunits have been identified, and after mutual dimerization, these subunits contribute to cell adhesion or regulation of signal transduction required for cell survival by making contact with appropriate ECM.[9,10] It has been reported that integrins α2, α3 and β1 play important roles in the peritoneal dissemination of gastric cancer,[11] and that antibodies to these integrins suppress peritoneal dissemination of gastric cancer in a mouse model.[12]

Nuclear factor-kappaB (NF-κB) was first identified and reported in 1986[13] and studied in the context of immune and inflammatory responses.[14] Nuclear factor-kappaB is a generic term for dimers of NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-Rel, RelA (p65/NF-κB3) and RelB.[15] To date, involvement of NF-κB in cancer-related molecules such as cyclin D1,[16] intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1),[17] the Bcl family,[18] inhibitor of apoptosis (IAP), X-linked inhibitor of apoptosis protein (XIAP),[19] p53,[20] vascular endothelial growth factor (VEGF), interleukin (IL)-8,[21] MMP[22] and multidrug resistance protein 1 (MDR1),[23] has been elucidated. However, NF-κB has not been reported to be involved in cancer cell adhesion to the peritoneum via integrins.

A low-molecular-weight NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), was newly developed by Umezawa.[24] Dehydroxymethylepoxyquinomicin specifically inhibits the nuclear translocation of p65 and prevents it binding to DNA[25]; it also has various anti-cancer effects in mouse models without obvious side-effects. Thus far, the following anti-cancer effects of DHMEQ have been reported: G1 arrest by inhibition of cyclin D1 expression;[26] and induction of apoptosis by inhibition of cIAP and XIAP,[27] or Bcl-2 and Bcl-xL.[28] Antitumor effects of DHMEQ have also been reported in *in vivo* models such as those for thyroid cancer,[27] prostate cancer,[29] hepatic cancer,[30] breast cancer,[31] pancreas cancer,[32] multiple myeloma,[28] malignant lymphoma[33] and leukemia.[26]

In the present study, we showed that NF-κB is associated with integrin expression in gastric cancer cell lines and that NF-κB inhibition by DHMEQ suppresses cancer progression by inhibiting the adhesion of gastric cancer cells to the peritoneum in a mouse model of peritoneal dissemination of gastric cancer.

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Materials and Methods

Cell cultures. The human gastric cancer cell line NUGC4 was obtained from the Japanese Cancer Research Resources Bank (JCRB, Osaka, Japan), and 44As3Luc with luciferase activity was constructed by one of the authors (K.Y.).[34] The 44As3Luc cells were derived from 44As3 cells, which is a highly peritoneal metastatic cell line, and were stably transfected with a pEGF-PLuc plasmid with CMV promoter (Clontech, Palo Alto, CA, USA). Human breast cancer cell lines MCF7 with constitutively low NF-κB activity and MDA-MB231 with constitutively high NF-κB activity were obtained from the American Type Culture Collection (Rockville, MD, USA).[31] The NUGC4 cells were cultured at 37°C in RPMI1640 (Sigma, St Louis, MO, USA) along with 10% fetal bovine serum (FBS); the 44As3Luc cells were cultured at the same temperature with RPMI1640 containing 100 μg/mL geneticin (Sigma); and the MCF7 and MDA-MB231 cells were also cultured at 37°C in 95% air and 5% CO_2 in DMEM (Sigma) along with 10% FBS.

Dehydroxymethylepoxyquinomycin (DHMEQ). We have originally designed and developed DHMEQ (molecular weight (MW): 261), a derivative of the natural antibiotic epoxyquinomycin C, to specifically target NF-κB.[24]

DNA-binding activity of NF-κB. To evaluate the DNA-binding activity of NF-κB in the steady state, 70% confluent cultures of NUGC4, 44As3Luc, MCF7 and MDA-MB231 in 10-cm dishes were stored at −80°C. To evaluate the effect of DHMEQ, the medium in the 70% confluent cultures of NUGC4 and 44As3Luc was replaced with 10 μg/mL DHMEQ solution, incubated for an appropriate time and stored at −80°C. The following day, nuclear proteins were extracted and examined using a p65 TransAM kit (ActiveMotif, Carlsbad, CA, USA). The absorbance was determined using a plate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was performed in triplicate.

NF-κB reporter gene assay. A GFP reporter gene construct was transfected using Cignal Reporter Assay kits (SA Biosciences, Frederick, MD, USA). Cultured cells were trypsinized and resuspended in Opti-MEM (Invitrogen, Carlsbad, CA, USA) with non-essential amino acids (Invitrogen) without antibiotics at a concentration of 2×10^5 cells in a 96-well plate. Cells were transfected with the reporter by culturing for 16 h with Surefect (SA Biosciences). After the medium was replaced with Opti-MEM with penicillin/streptomycin, the cells were incubated for an additional 8 h. The medium was then replaced with Opti-MEM containing 10 μg/mL of DHMEQ (or 0.024% of DMSO for the controls). The intensity of fluorescence was measured at appropriate times in triplicate using Varioskan Flash (excitation, 470 nm; emission, 515 nm).

mRNA expression of integrins in DHMEQ-treated cells. Real-time PCR was used to examine mRNA expression. The 44As3Luc cells were cultured in triplicate in 0.024% DMSO solution (controls) or in 10 μg/mL DHMEQ for the appropriate times. Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. For cDNA synthesis, ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) with Oligo(dT) 20 primer (Toyobo) was used in accordance with the manufacturer's instructions. For relative quantification by PCR, each cDNA product was analyzed in a LightCycler (version 1.4) using a QuantiTect SYBR Green PCR kit (Qiagen).

Flow cytometric analysis of integrin expression. p65 silencing was performed using p65 siRNA2 (BD Biosciences, Bedford, MA, USA). Next, 50% confluent cells were incubated for 24 h in medium without antibiotics in 10-cm dishes. Then, 33 nM p65 siRNA was added to each dish and transfected for 48 h. p65 silencing was confirmed by western blot analysis using primary antibodies against ×500 α-tubulin and ×1000 p65 protein (Cell Signaling, Beverly, MA, USA) and ×5000 goat anti-mouse IgG for tubulin or anti-rabbit IgG for the p65 protein. With regard to DHMEQ treatment, the medium in 70% confluent cell cultures in 10-cm dishes was replaced with 10 μg/mL DHMEQ solution (0.024% DMSO for the controls) and cultured for the appropriate times. These cells were trypsinized and analyzed using flow cytometry (FACS Caliber; Becton Dickinson, Franklin Lakes, NJ, USA). The antibodies used for these assays were integrin α2, integrin α3, integrin β1 and isotype controls for these integrins. All antibodies were obtained from R&D Systems (Minneapolis, MN, USA).

Adhesion assay. We evaluated the anti-adhesive effect of DHMEQ by using a plate pre-coated with ECM constituting the peritoneal basement membrane. The medium in 70% confluent cell cultures in 10-cm dishes was replaced with 10 μg/mL DHMEQ solution (or 0.024% DMSO for the controls), and the dishes were incubated for 24 h. These cells were trypsinized, assembled, adjusted to a concentration of 1×10^6 cells/mL with RPMI and distributed on the pre-coated plates (80 μL per plate). Next, the cells were incubated at 37°C for 1 h. Except for the non-treated plate, all plates were washed three times with 100 μL of FBS-free RPMI. After washing, 10 μL of ×50 diluted Cell Counting kit F (CCKF; Dojindo, Osaka, Japan) was added to each well, and the fluorescence intensity of the remaining live cells (adhesive cells) was evaluated using Varioskan Flash at 30 min after CCKF administration (excitation, 490 nm; emission, 515 nm). Pre-coated plates were manufactured by BD Biosciences and the ECM coated on the plates were types 1 and 4 collagen, fibronectin and laminin.

DHMEQ cytotoxicity assay. The cells were seeded into 96-well plates at 5×10^3 cells/well in 10% FBS-containing medium. Twenty-four hours later, the medium in the wells was replaced with different concentrations of DHMEQ solution or 0.048% DMSO solution, and the cells were then incubated again for 24 h. Lactate dehydrogenase (LDH) activity of the supernatant was measured using an LDH cytotoxicity detection kit (Takara Bio, Shiga, Japan).

Animal experiments. Six-week-old male BALB/c-nu/nu mice, each weighing approximately 20 g, were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were grouped as follows: (i), implantation of DMSO-treated cells; (ii) implantation of DHMEQ-treated cells; (iii) implantation of DMSO-treated cells with peritoneal lavage; and (iv) implantation of DHMEQ-treated cells with peritoneal lavage. Each group comprised four mice. Then, 2×10^6 44As3Luc cells, which had been treated with 10 μg/mL DHMEQ (or 0.024% DMSO for the controls) for 24 h, were injected intraperitoneally into the above mentioned mice. One hour after injection, laparotomy and peritoneal lavage were performed using phosphate-buffered saline (PBS). Peritoneal lavage was performed through a 1-cm incision through which 5 mL of PBS was slowly injected. Bio-imaging was performed before and after the peritoneal lavage, and on days 2, 5, 10, 15 and 20 in order to evaluate cancer progression. Luminescence was evaluated at approximately 7 min after intraperitoneal injection of 1500 μg/mouse D-luciferin potassium salt (Synchem OHG, Altenburg, Germany). *In vivo* imaging was performed using Photon Imager Hu (Biospace Lab, Paris, France) with the mice under isoflurane anesthesia (Abbott Japan, Tokyo, Japan). Images were captured using Photo Acquisition 2.6 (Biospace Lab) with 0.5 min exposure and processed using Photo Vision Plus. Signal intensity was quantified as the sum of all detected photon counts (count per minute [CPM]) within the region of interest (ROI). All procedures involving animals and their care were approved by the Ethics Committee of Hokkaido University in accordance with institutional and Japanese governmental guidelines for animal experiments.

Scanning electron microscopy (SEM) of the peritoneal wall. The peritoneal walls of mice injected with cancer cells

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were fixed with 10% formaldehyde for 180 min and then overnight at 4°C with 1.25% glutaraldehyde solution. The fixed samples were dehydrated in a 30–100% graded ethanol series and immersed in tert-butyl alcohol overnight at −20°C. These samples were dried using ES-2000 (Hitachi High-Technologies Co., Tokyo, Japan) for 3 h and ion-sputtered using E-1030 (Hitachi) for 120 s. The peritoneal surface was observed under a scanning electron microscope (S-3500N; Hitachi).

Statistics. The mean and SD were calculated for all variables, except the data from the flow cytometry. Between-group statistical significance was determined using the Student's *t* test. $P < 0.05$ was considered as statistically significant.

Results

DHMEQ effectively suppresses p65-DNA binding activity in gastric cancer cells. In the steady state, the p65-DNA binding activities in NUGC4 and 44As3Luc cells were as high as that in MDA-MB231 cells, a positive control cell with high binding activity. The activity in MCF7 cells is constitutively low as previously reported [31], and hence these cells were used as the negative control (Fig. 1A). The binding activities in both cells reached their lowest levels 2 h after the addition of 10 μg/mL DHMEQ (as a final concentration) and returned to initial conditions within 24 h (Fig. 1B). A GFP reporter assay showed that DHMEQ significantly suppresses transcriptional activity in both cells (Fig. 1C). On the basis of these results, we considered that DHMEQ had a similar effect in NUGC4 and 44As3Luc cells. Therefore, we used 44As3Luc cells in the following experiments. We planned to evaluate cancer progression using bioimaging.

Effect of NF-κB inhibition on integrin expression. In 44As3Luc cells, the mRNA of all integrins – α2, α3 and β1 – were significantly suppressed 2 h after the addition of 10 μg/mL DHMEQ (as a final concentration) compared with the control to which DMSO was added (Fig. 2A). The percentage reduction in the expressions of integrins α2, α3 and β1 was 27%, 31% and 8%, respectively. Flow cytometric analysis revealed that the expressions of all cell surface integrins on 44As3Luc cells were gradually suppressed after the addition of DHMEQ (Fig. 2B). Reductions in integrin expression (α2, α3 and β1) following DHMEQ addition was 68%, 83% and 45% at 24 h, respectively. Similarly, flow cytometric analysis of integrins α2, α3 and β1 revealed that the expressions of cell surface integrins in p65-deleted cells were suppressed to the same degree as in DHMEQ-treated cells (Fig. 2C). Reductions in integrin expression after p65 deletion were 34% (α2), 76% (α3) and 41% (β1). p65 silencing was confirmed by western blotting for nuclear and cytoplasmic p65 proteins (Fig. 2D).

Anti-adhesive effect of DHMEQ-treated cells in an *in vitro* assay. Significantly fewer 44As3Luc cells treated with 10 μg/mL DHMEQ (final concentration) remained alive on plates pre-coated with ECM after they were washed (ECM-adhesive cells) than 44As3Luc cells treated with DMSO (Fig. 3A). Reductions in the numbers of adhesive cells following DHMEQ addition were 18.3% (laminin), 34.8% (fibronectin), 38.2% (type 1 collagen) and 43.5% (type 4 collagen). The LDH value, which represents the cytotoxic effect, was significantly elevated in the supernatant of cells treated with DHMEQ at concentrations >17.5 μg/mL (Fig. 3B).

Effect of peritoneal lavage on implantation of DHMEQ-treated cancer cells on the abdominal wall. The number of cancer cells decreased in mice injected with DHMEQ-pretreated cells and subjected to peritoneal lavage (Fig. 4A). The intensity of bioluminescence after lavage was significantly reduced (reduction rate, 39%) in mice that were injected with DHMEQ-pretreated cells and subjected to peritoneal lavage compared with in mice injected with DMSO-pretreated control cells and subjected to

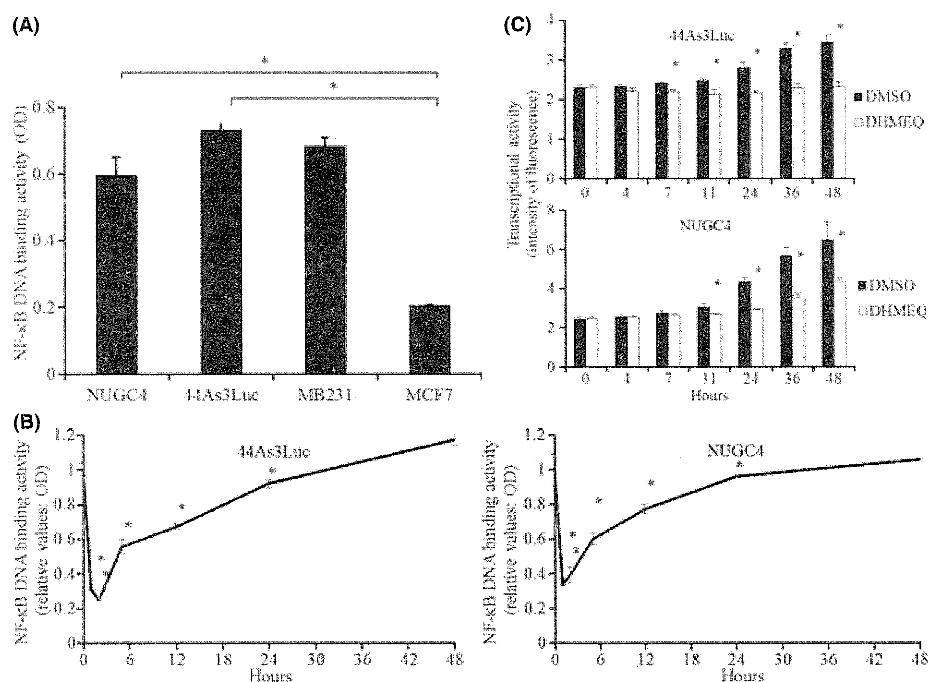

Fig. 1. Dehydroxymethylepoxyquinomicin (DHMEQ) effectively suppressed p65-DNA binding activity in gastric cancer cells. (A) Nuclear p65 protein binding activity to DNA in a steady state. MDA-MB231 cells were used as a positive control, and MCF7 cells were used as a negative one. *$P < 0.05$. (B) Time course of binding activity of nuclear p65 proteins to DNA in DHMEQ-treated cells. The binding activities of both cells were assessed at 2, 6, 12, 24 and 48 h after DHMEQ administration. *Significantly <0 h ($P < 0.05$). (C) Nuclear factor-kappa B (NF-κB) GFP reporter assay. The black bars show cells treated with DMSO, and white bars show those with DHMEQ. *Significantly more than controls ($P < 0.05$). OD, optical density.

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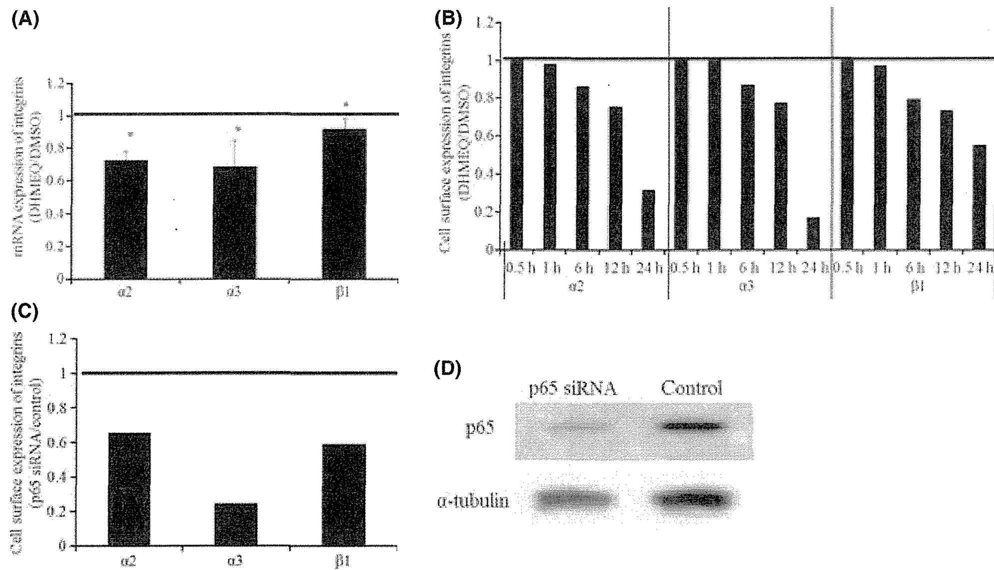

Fig. 2. Effect of nuclear factor-kappa B (NF-κB) inhibition on expression of adhesion molecules. (A) Quantitative evaluation of mRNA of integrins by real-time PCR. The graph shows the average of the ratio of copies of dehydroxymethylepoxyquinomicin (DHMEQ)-treated 44As3Luc cells to DMSO-treated cells at 2 h after DHMEQ administration. When the longitudinal value is below 1 (bold line), the integrin expression of DHMEQ-treated cells is lower than that of DMSO-treated cells. *Significantly less than controls ($P < 0.05$). (B) Expression of cell surface integrins of DHMEQ-treated cells. The graph shows the expression rate of cell surface adhesion molecules of 44As3Luc cells treated with DHMEQ compared with that of DMSO-treated cells for each time point. The bold line is as described above. (C) Expression of cell surface adhesion molecules of cells knocked down by p65 siRNA. The graph shows the rate of cell surface integrins of 44As3Luc cells knocked down by p65 siRNA. The bold line is as described above. (D) p65 deletion. The p65 deletion was confirmed by western blotting.

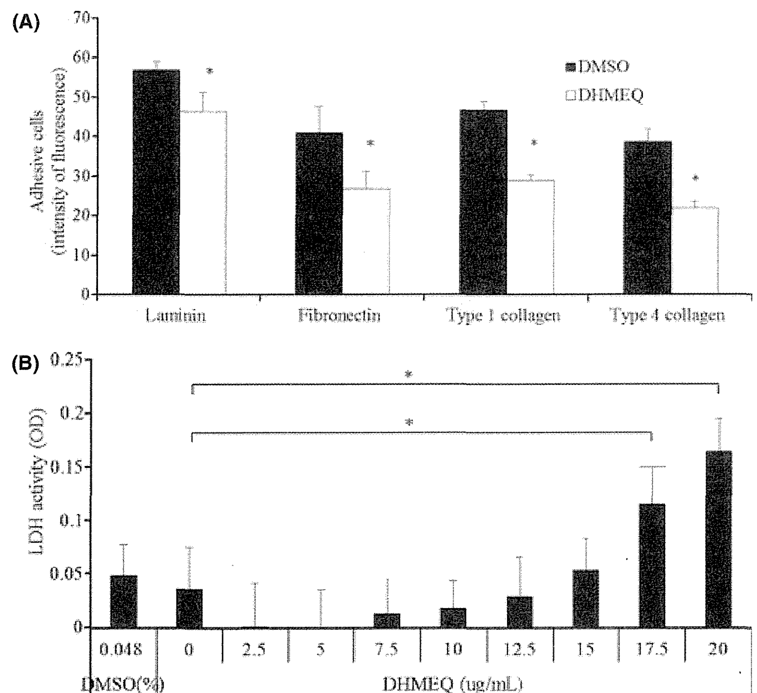

Fig. 3. Anti-adhesive effect of dehydroxymethylepoxyquinomicin (DHMEQ) pretreated cells in the *in vitro* study. (A) Adhesion assay. The bars show the fluorescence intensity of the remaining live cells on the plates. The black bars show cells pretreated with DHMEQ, and white bars show those with DMSO. *Significantly less than controls ($P < 0.05$). (B) Evaluation of cytotoxicity of DHMEQ. The graph shows lactate dehydrogenase (LDH) activity of the supernatant of the 44As3Luc cells treated with DHMEQ or DMSO. *$P < 0.05$. OD, optical density.

peritoneal lavage (Fig. 4B). The SEM revealed that cancer cells adhered less to the basement membrane of the peritoneum in mice injected with DHMEQ-pretreated cells than in those injected with DMSO-pretreated control cells (Fig. 4C).

Follow up of gastric cancer dissemination by *in vivo* imaging. The DHMEQ-pretreated 44As3Luc cells injected in mice grew slowly compared with the DMSO-pretreated cells (Fig. 5A). The increase in the CPM/mm^2 value of the

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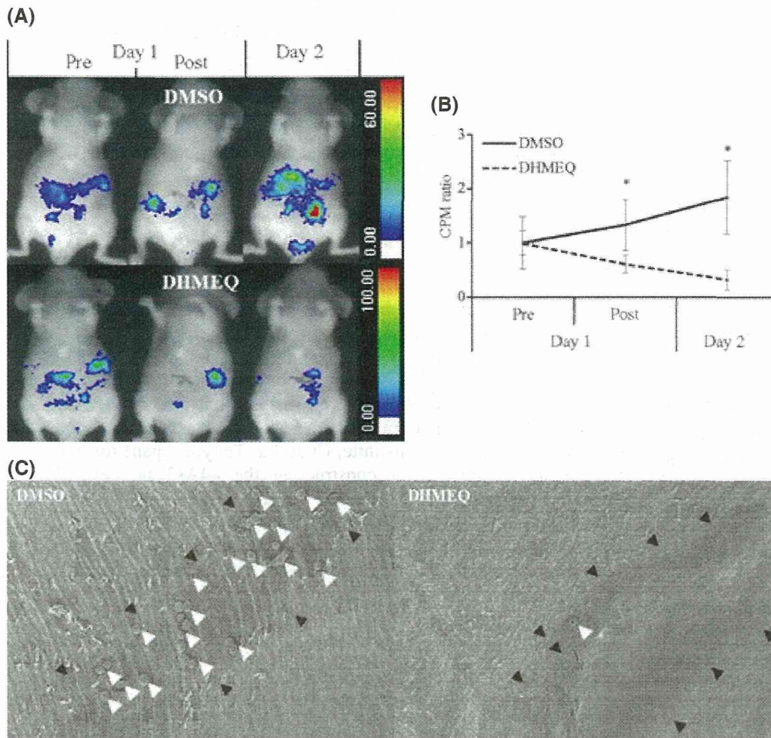

Fig. 4. Peritoneal lavage inhibited cancer cells pretreated with dehydroxymethylepoxyquinomicin (DHMEQ) from implanting into the abdominal wall. (A) *In vivo* imaging at around the time of peritoneal lavage. The luminescent value indicates the number of live cells in the abdominal wall. Pre/Post means before/after the peritoneal lavage. (B) Count per minute (CPM)/mm^2 value of pre/post peritoneal lavage. The graph shows the time course of the CPM/mm^2 value compared with the time of cancer cell injection. Initial values were adjusted to 1. *Significantly less than controls ($P < 0.05$). (C) SEM findings of the peritoneum. Left: abdominal wall injected with 44As3Luc cells pretreated with DHMEQ. Right: those with DMSO. The area indicated by black arrowheads is the area exposed to the peritoneal cavity. White arrowheads show the adhesive cancer cells.

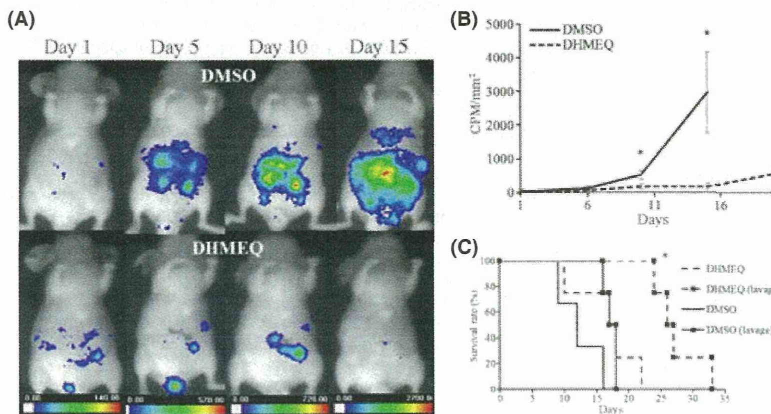

Fig. 5. Follow up after peritoneal lavage. (A) Follow-up imaging of mice subjected to peritoneal lavage. The range bars were adjusted for mice injected with DMSO pretreated cells at every evaluation day. (B) Time course of the count per minute (CPM)/mm^2 value. The black line represents the mice that were injected with DMSO-pretreated cells, and the broken line represents those injected with dehydroxymethylepoxyquinomicin (DHMEQ)-pretreated cells. *Significantly less than controls ($P < 0.05$). (C) Kaplan–Meier analysis of the survival of all groups. The line is as described above. The line with markers represents mice subjected to peritoneal lavage. *Significantly prolonged than all other groups ($P < 0.05$).

DHMEQ-treated cells was significantly delayed. The error bar of the CPM/mm^2 value of the DMSO-pretreated group ranged widely, because malignant ascites possibly obscured luminescent emission at the terminal stage (Fig. 5B). Survival was only significantly prolonged in mice injected with DHMEQ-treated cells and subjected to peritoneal lavage (Fig. 5C).

Discussion

NF-κB is undoubtedly involved in various biological properties of cancer cells.[35] However, its involvement in the expression of integrins, which are associated with cancer cell adhesion to the peritoneum, has not been reported. In the present study, we investigated whether NF-κB is involved in cell adhesion to the peritoneum via regulation of integrin expression, and whether DHMEQ, as a novel NF-κB inhibitor, suppresses the dissemination of gastric cancer in a mouse model.

Several investigators reported that NF-κB activity is associated with peritoneal dissemination of cancer cells.[36–38] Sasaki *et al.*[39] evaluated human gastric cancer tissues by immunohistochemical analysis, where NF-κB activation was significantly correlated with peritoneal metastases and survival. Our results in the present study support the previously reported data that NF-κB activity of gastric cancer cell lines was markedly activated and with highly metastatic behavior, and that DHMEQ sufficiently inhibited NF-κB activity and eventually suppressed the peritoneal dissemination.

Integrins are also associated with malignant potential.[40–42] Integrins play an important role in cancer cell adhesion to the peritoneum by enabling contact with appropriate ECM. Oosterling *et al.*[43] showed that anti-β1 integrin antibody reduces surgery-induced adhesion of colon carcinoma cells to traumatized peritoneal surfaces. Fishman *et al.*[44] showed similar findings using ovarian cancer cell lines in the *in vitro* analysis. With

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regard to gastric cancer, integrins α2, α3 and β1 are key molecules in animal models and humans.[11,12,45,46] The ligands of integrin α2β1 are collagens and laminin, and those of α3β1 are fibronectin, laminin,and collagens.[10] In our *in vitro* study, DHMEQ suppressed cancer cell adhesion to the peritoneum via p65-mediated suppression of integrin expression. Also, Takatsuki *et al.*[12] reported that anti-α3 antibody strongly suppressed the adhesion of gastric cancer cells to mice peritoneum. This integrin α3 was suppressed most by DHMEQ in this study. Therefore, DHMEQ may suppress cancer cell adhesion mainly via integrin α3, while DHMEQ may associate with other adhesion molecules that are not examined in this study.

In our *in vivo* study, viable cells in mice injected with DHMEQ-treated cells and subjected to peritoneal lavage still decreased on day 2 and only this group survived significantly longer. This finding might suggest that DHMEQ exerts another effect via the anti-adhesive effect. Jiang *et al.*[47] reported that NF-κB inhibition by IκBβ reduces anchorage-independent growth in a lung cancer cell line. Scaife *et al.*[48] showed that NF-κB inhibitor causes anoikis in a human colon cancer cell line. It might be possible that DHMEQ is associated with a pro-anoikis effect in gastric cancer dissemination.

In the present study, we first demonstrated that NF-κB could play a pivotal role in the progression of gastric cancer via the regulation of integrin expression and promotion of adhesion of cancer cells to the peritoneal wall. In our *in vivo* study, a specific deletion of NF-κB (p65) by siRNA was not performed, because we considered that transient deletion of p65 protein does not reflect the same result of DHMEQ-administered cells. Additionally, we could not clarify whether the DHMEQ effect on integrins is unique to the integrin pathway or concomitant with other phenomenon such as apoptosis. Further studies are required to clarify the involvement of integrins or other molecules in the anti-adhesive effect of DHMEQ against cancer cells. We believe that NF-κB inhibitors such as DHMEQ may be potential therapeutic options to prevent gastric cancer progression during peri-operative periods.

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Disclosure Statement

The authors have no conflict of interest.

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Phase I clinical trial of a novel peptide vaccine in combination with UFT/LV for metastatic colorectal cancer

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Abstract. To test the safety and immune responses of a novel peptide vaccine derived from RNF43 (ring finger protein 43) and TOMM34 (34-kDa translocase of the outer mitochondrial membrane) administered in combination with chemotherapy in patients with metastatic colorectal cancer, a phase I clinical trial with 21 HLA-A2402-positive metastatic colorectal cancer patients was conducted. Patients received a weekly peptide vaccine (1 mg of each peptide in incomplete Freund's adjuvant) in combination with oral UFT (300 mg/m^2/day) and UZEL (75 mg/day) for 4 weeks, followed by 1 week of rest. The protocol consisted of at least two cycles of this regimen. After the 2nd cycle, vaccinations were given biweekly or monthly, depending on the condition of the patient. Clinical responses were judged 10 weeks after the 2nd cycle by performing computed tomography (CT) scans and assessing the cytotoxic T lymphocyte (CTL) responses against RNF43 and TOMM34 in peripheral lymphocytes. The vaccinations were well tolerated without any serious adverse events. CTL responses were induced against both antigens in 8 patients and against one antigen in 12 patients, while 1 patient had no CTL response. The rate of stable disease was 83%. The group with CTL responses against both antigens had the most long-term survivors, followed by the group showing CTL responses against one antigen (p=0.0079). The patients with no CTL responses had the lowest survival. The safety and immunological responsiveness of the present combination therapy suggests that it is clinically beneficial for metastatic colorectal cancer. Further clinical trials are warranted.

Introduction

Genes that are frequently up-regulated in colorectal cancer (CRC) can be identified by genome-wide analysis with cDNA microarray profiling. This strategy has been used to identify gene products that are essential for the proliferation and/or survival of CRC cells (1). Two novel tumor-associated antigens (TAAs), RNF43 (ring finger protein 43) and TOMM34 (34-kDa translocase of the outer mitochondrial membrane), were found to be up-regulated in more than 80% of CRC tissues as compared to the corresponding noncancerous mucosa (2,4). RNF43 expression cannot be detected in normal human adult organs with Northern blotting. Thus, the function of RNF43 has been associated with the proliferation of tumor cells. Since suppression of TOMM34 by siRNA was found to markedly reduce the growth of colon cancer cells, the gene product is a potential therapeutic target for human CRC (3). HLA-A24-restricted epitope peptides from RNF43 and TOMM34 for cancer vaccination for CRC patients were recently identified (2,4).

We previously reported a phase I trial involving vaccination with cancer peptides in combination with UFT and LV (UZEL) for advanced CRC patients (5). UFT is an oral anticancer drug consisting of tegafur (FT), a prodrug of 5-fluorouracil (5-FU) and uracil, an inhibitor of 5-FU degradation. LV is an oral drug consisting of calcium folinate which modulates 5-FU. We previously demonstrated that the standard dose of UFT and LV did not impede the immunological responses of advanced CRC patients to the peptide vaccination.

To investigate the safety and immunological responses of a peptide vaccination with RNF43 and TOMM34 in combination with UFT and LV, we conducted a phase I clinical study involving patients with metastatic CRC.

Materials and methods

Patients and eligibility criteria. The study protocol was approved by the Institutional Ethics Review Boards of Kinki University (approval no. 18-15) and was registered in the UMIN Clinical Trials Registry as UMIN000003728 (http://www.umin.ac.jp/ctr/index.htm). Complete written informed consent was obtained from the patients at the time of enrollment. The patients (n=23) had histologically confirmed metastatic CRC unsuitable for surgical resection and were HLA-A*2402-positive. A total of 19 patients failed to respond to prior standard chemotherapy, and the remaining 4 patients agreed to receive this immunochemotherapy (Table I). Patients were required to have completed prior chemotherapy at least 4 weeks before trial enrollment and to have

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Key words: peptide vaccine, metastatic colorectal cancer, cytotoxic T lymphocytes, immunochemotherapy

fully recovered from any adverse event with a toxicity of grade 3 or higher according to the Common Terminology Criteria for Adverse Events (CTCAE) scale. The patients were required to have an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, to be older than 20 years of age and to have a life expectancy of at least 3 months. Adequate bone marrow (white blood cell count ≥3,000/mm^3, hemoglobin ≥10 g/dl and platelet count ≥75,000/mm^3), renal function (serum creatinine ≤1.4 mg/dl) and liver function (bilirubin ≤1.5 mg/dl and transaminase within 2.5 times the institution's upper limit of normal) were required. Patients were excluded if they were pregnant or had hepatitis B or C virus antigens or human immunodeficiency virus (HIV).

Peptides. The RNF43-721 (NSQPVWLCL) and TOMM34-299 (KLRQEVKQNL) peptides were synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA) according to a standard solid-phase synthesis method and purified by reverse-phase high performance liquid chromatography (HPLC) (4,6). The purity (>95%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. RNF43-721, TOMM34-299 and the epitope peptide derived from the human immunodeficiency virus-envelope (HIV-Env) protein restricted with HLA-A*2402 (RYLRDQQLL) were used to measure the cytotoxic T lymphocyte (CTL) response.

Clinical protocol. The present open-label phase I study involved a vaccine consisting of two peptides (1 mg of each peptide) derived from RNF43 and TOMM34 mixed with incomplete Freund's adjuvant (IFA) and Montanide ISA 51 (Seppic) administered to patients with locally advanced, recurrent, or metastatic colorectal cancer. The patients received a subcutaneous injection of vaccine into the thigh or back once a week for 5 weeks. Simultaneously, patients received orally administered UFT (300 mg/m^2/day) and UZEL® (75 mg/day) for 4 weeks, followed by 1 week of rest (one cycle). The immunological responses to the inoculated peptides and clinical responses were examined after every five vaccinations. The protocol consisted of two cycles. After the second cycle, vaccinations were given biweekly or monthly (depending on patient condition), while UFT/UZEL administration was continued for 4 weeks followed by a 1-week rest period during the entire treatment period. A complete blood count and results of serum chemistry tests were obtained every 2 weeks. Clinical responses were evaluated at the end of every cycle by examining computed tomography (CT) scans and tumor markers. The vaccinated patients (n=21) were assessed for immunological and clinical responses according to the Response Evaluation Criteria in Solid Tumors (RECIST). Signs of toxicity were assessed according to CTCAE version 3.0. Overall survival rates were analyzed by the Kaplan-Meier method, and survival was measured in days from the first vaccination to succumbing to the disease. p-values were assessed using a log-rank test.

Cells. TISI cells and HLA-A*2402-positive B-lymphoblastoid cell lines were purchased from the IHWG Cell and Gene Bank (IHW no. 9042; Seattle, WA, USA) in November 2008 and stored at -80˚C. Within 2 months of purchase, the cells were resuscitated and maintained in RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO_2 incubator at 37˚C. The peripheral blood was periodically collected from the enrolled patients. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and density gradient centrifugation and were frozen immediately after isolation. PBMCs from each patient were simultaneously thawed and used to measure the CTL response.

Enzyme-linked immunospot assay. For detecting antigen-specific immune responses, enzyme-linked immunospot (ELISPOT) assays were performed with the human γ-interferon (IFN-γ) ELISPOT kit (Mabtech, Nacka Strand, Sweden). Plates with 96 wells and nitrocellulose membranes (Millipore, Molshelm, France) were precoated with primary anti-IFN-γ antibody (1-D1K) at 4˚C overnight.

Measurement of the cytotoxic T lymphocyte response. The IFN-γ ELISPOT assay was performed to measure the specific CTL response against the peptide. PBMCs were obtained from patients and frozen prior to vaccination and at the end of each treatment course. The frozen PBMCs were thawed and *in vitro* sensitization was performed. In brief, PBMCs were stimulated with 10 μg/ml of each peptide and 20 IU/ml of interleukin (IL)-2 at 37˚C, in 5% CO_2 for 2 weeks. Peptides were added on day 0 and 7. Following incubation, the harvested cells were used as responder cells, and RNF43-721 or TOMM34-299 peptide-pulsed TISI cells were used as stimulator cells (10^5 cells per well). The HLA-A*2402-restricted epitope peptide derived from the HIV-Env protein was used as a control peptide. The IFN-γ ELISPOT kit and the AEC substrate set (BD Biosciences Pharmingen, San Diego, CA, USA) were used to measure the CTL response. Spots were captured and analyzed using an automated ELISPOT reader, ImmunoSPOT 4S (CTL Ltd., Cleveland, OH, USA). The ELISPOT assays were performed in triplicate wells. The number of peptide-specific spots was calculated by subtracting the number of spots when stimulated with the HIV-Env peptide from the number of spots when stimulated with the RNF43-721 or TOMM34-299 peptide. The percentage of specific spots was calculated by dividing the number of peptide-specific spots by the number of spots when stimulated with the RNF43-721 or TOMM34-299 peptide. CTL induction was defined as positive when more than 10 specific spots were detected or the percentage of specific spots was greater than 5%. The number of peptide-specific spots was detected as the responder/stimulator ratio-dependency.

Statistical analysis. Overall survival rates were analyzed by the Kaplan-Meier method, and survival was calculated in days from the first vaccination to succumbing to the disease. The statistical analyses were performed with SPSS statistics 17.0 (SPSS, Chicago, IL, USA).

Results

Characteristics of the patients and vaccinations. Between January 2007 and June 2009, 23 HLA-A*2402-positive patients with metastatic colorectal cancer were enrolled in the present trial. All the patients had one or more metastatic

Table I. Patient characteristics.

Patient no.	Age	Gender	Primary cancer	Sites of metastases	PS	Previous treatment
1	56	M	R	Pelvis	0	UFT, CPT-11
2	64	F	S	Lung	0	5-FU, UFT/LV
3	57	F	R	Lymph nodes	1	5-FU/LV, CPT-11, S-1
4	42	M	R	Pelvis	0	None
5	53	F	S	Lung	0	UFT/LV, vaccine
6	54	M	R	Lung	0	None
7	74	F	S	Lymph nodes	0	5-FU, UFT/LV
8	78	M	R	Lung, lymph nodes	1	5-FU, UFT/LV, CPT-11
9	58	M	R	Lung	1	None
10	46	M	T	Liver, lymph nodes	1	FOLFOX, FOLFIRI, vaccine
11	59	M	S	Primary cancer, liver, lymph nodes	1	FOLFIRI, FOLFOX
12	66	M	S	Lung, liver, lymph nodes	0	S-1
13	66	F	RS	Lung	0	UFT/LV
14	49	M	S	Lung, liver	0	None
15	51	F	S	Liver, lymph nodes	1	UFT/LV, CPT-11
16	66	M	R	Lung, liver, lymph nodes	1	UFT/LV
17	61	F	C	Liver, lymph nodes	1	FOLFOX+Bev, FOLFIRI+Bev
18	54	M	S	Primary cancer, liver, lymph nodes	0	FOLFOX+Bev, UFT/LV
19	83	M	S	Lung	0	UFT
20	66	M	R	Lung, pelvis, bone	0	FOLFOX+Bev, FOLFIRI+Bev
21	61	M	R	Lung, pelvis	1	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
22	73	M	R	Lung, pelvis, lymph nodes	0	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
23	65	M	R	Lung, pelvis	0	FOLFOX+Bev, FOLFIRI+Bev, IRIS

PS, Eastern Cooperative Oncology Group performance status; R, rectal cancer; S, sigmoid colon cancer; T, transverse colon cancer; RS, rectosigmoid cancer; C, cecal cancer; Bev, bevacizumab; Cet, cetuximab; IRIS, irinotecan+S-1.

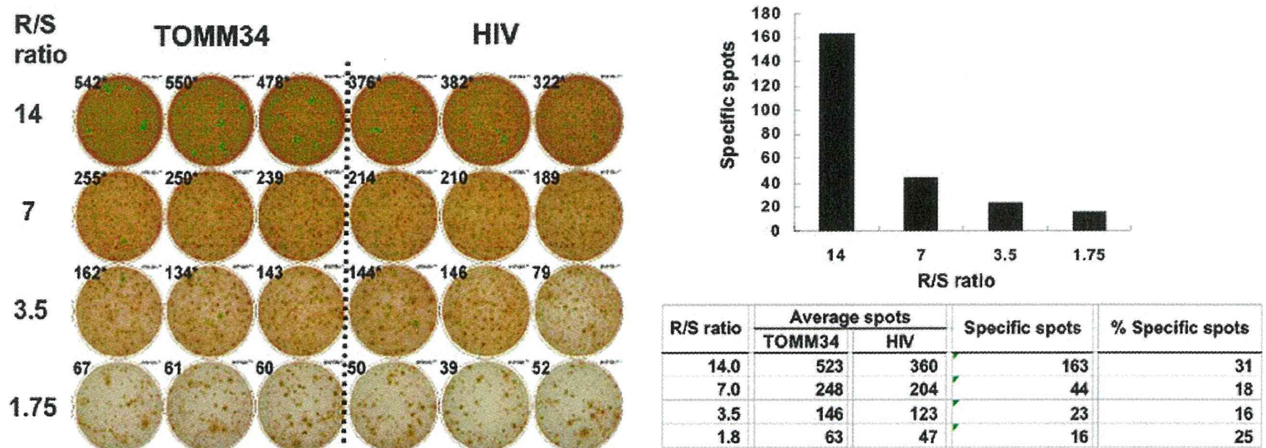
Table II. Adverse events.

Toxicity	Total n (%)	Grade 1	Grade 2	Grade 3
Anemia	5 (23.8)	5	0	0
Transaminase elevation	3 (14.3)	3	0	0
Hyperbilirubinemia	2 (9.5)	2	0	0
Anorexia	5 (23.8)	5	0	0
Nausea	2 (9.5)	2	0	0
Malaise	3 (14.3)	3	0	0
Vaccination site reaction	15 (71.4)	15	0	0
Renal dysfunction[a]	1[a] (4.8)	0	0	1[a]

[a]A double-J catheter was placed by a urologist into one patient who experienced acute grade 3 renal dysfunction, which led to the disappearance of the hydronephrosis and the resumption of therapy.

foci that were unsuitable for surgical resection. A total of 19 patients had not responded to prior standard chemotherapy, and the remaining 4 patients agreed to receive this immunochemotherapy (Table I). A total of 2 patients (nos. 10 and 17) were disqualified as they did not meet the inclusion criteria. The final subject group thus consisted of 21 patients (15 men and 6 women) with a median age of 61 years (range 42-83). A total of 727 vaccinations were administered with a median of 31 vaccinations per patient (range 7-69). The vaccination with chemotherapy protocol was well tolerated by all patients.

Toxicities. The overall toxicities are shown in Table II. The most frequent adverse events were vaccination-site reactions (n=15), anemia (n=5), anorexia (n=5), malaise (n=3) and



R/S ratio	Average spots TOMM34	Average spots HIV	Specific spots	% Specific spots
14.0	523	360	163	31
7.0	248	204	44	18
3.5	146	123	23	16
1.8	63	47	16	25

Figure 1. Enzyme-linked immunospot (ELISPOT) assays detecting TOMM34-specific T-cell activity. Peripheral blood lymphocytes collected from patient no. 5 at the end of the second course were cultured in recombinant interleukin-2 without any antigen stimulation for 14 days and subjected to the ELISPOT assay to detect the antigen-specific T-cell response induced by the vaccination.

Table III. Immunological and clinical responses.

Patient no.	No. of vaccinations	Vaccination site reaction	CTL response	Clinical response	TTP (days)	OS (days)
1	69	Ind, red	RNF, TOMM	SD	252	1226 (alive)
2	7	(-)	RNF, TOMM	-	38	1026
3	17	Ind, red	TOMM	SD	169	448
4	16	(-)	RNF, TOMM	SD	211	741
5	69	Ind, red	RNF, TOMM	SD	365	1086 (alive)
6	31	Ind	RNF, TOMM	SD	428	1054 (alive)
7	37	Ind, red	RNF, TOMM	PD	49	1012
8	8	(-)	RNF	-	36	80
9	69	Ind, red	TOMM	SD	694	904 (alive)
11	11	(-)	RNF	PD	36	183
12	29	Ind	RNF	SD	219	387
13	37	Ind	TOMM	SD	219	521
14	54	Ind	RNF, TOMM	SD	260	512 (alive)
15	22	Ind	RNF	SD	107	197 (alive)
16	16	Ind, red	TOMM	SD	73	132
18	41	Ind	TOMM	PD	70	414 (alive)
19	52	Ind, red	RNF, TOMM	SD	309	414 (alive)
20	46	Ind	RNF	SD	218	330
21	50	Red	TOMM	SD	246	365 (alive)
22	15	(-)	TOMM	SD	69	151
23	31	(-)	(-)	SD	176	288

Two patients (no.10 and 17) were disqualified for failure to meet the inclusion criteria. CTL, cytotoxic T lymphocyte; TTP, time to progression; OS, overall survival. Ind, induration; red, redness; SD, stable disease; PD, progressive disease.

elevation of serum transaminase (n=3). With the exception of one incident of grade 3 acute renal dysfunction (no. 20) due to hydronephrosis, all of the adverse events were grade 1. A double-J catheter was placed by a urologist into the patient who experienced acute renal dysfunction, which led to the disappearance of the hydronephrosis and the resumption of therapy. This patient had a large area of tumor recurrence in the pelvis prior to therapy; therefore, the renal dysfunction due to ureteral obstruction was considered to be caused by the metastasis and not related to the therapy.

Immunological monitoring. Peripheral blood lymphocytes obtained before, during, and after the vaccination periods were cultured in rIL-2 without any antigen stimulation for

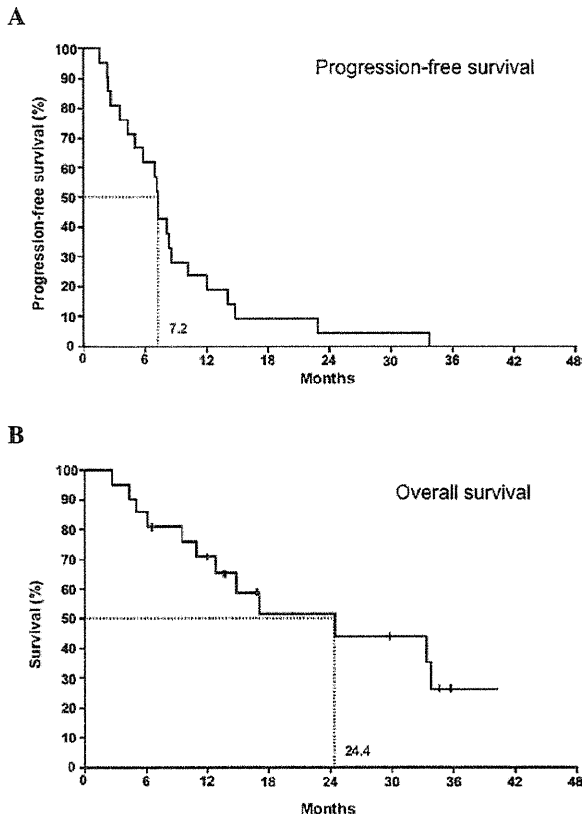


Figure 2. Survival analysis of 21 patients with metastatic colorectal cancer treated with the peptide vaccination in combination with oral chemotherapy. (A) Progression-free survival; (B) overall survival.

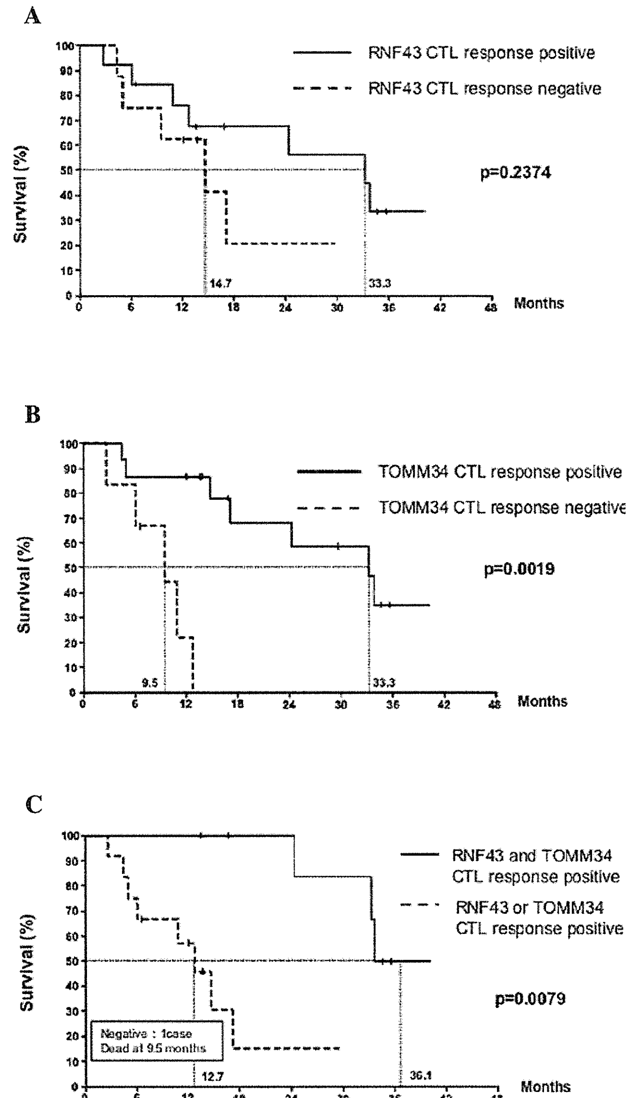


Figure 3. The relationship between cytotoxic T lymphocyte (CTL) responses and overall survival in patients with metastatic colorectal cancer treated with peptide vaccination in combination with oral chemotherapy. (A) CTL response to RNF43 and survival, (B) CTL response to TOMM34 and survival, (C) CTL responses to RNF43 and/or TOMM34 and survival.

14 days and subjected to the ELISPOT assay to detect the antigen-specific T-cell response induced by the vaccination. The CTL response was considered to be positive when more than 10 specific spots were detected or the percentage of specific spots was greater than 5%. In addition, the number of peptide-specific spots was detected as the responder/stimulator ratio-dependency. Representative CTL-positive data from ELISPOT assays against the TOMM34 antigen are shown for patient no. 5 (Fig. 1). Among the 21 patients, 8 patients had positive CTL responses against RNF43 and TOMM34, 12 patients had a positive response against one of the antigens, and the remaining patient had a negative response (Table III). The magnitude of the CTL response varied depending on the timing of the vaccinations. However, there was a clear separation between positive and negative CTL responses.

Clinical response and overall survival. Among the 21 patients, 19 patients were assessed for clinical response at the end of the 10th vaccination (2nd cycle) according to the RECIST criteria (Table III). The clinical responses of the remaining 2 patients were not assessed as they received fewer than 10 vaccinations (6 and 8, respectively). None of the patients showed a complete response or a partial response. A total of 16 patients had stable disease and 3 patients had progressive disease. The median time of progression-free survival was 7.2 months (Fig. 2A), and the mean survival time was 24.4 months (Fig. 2B).

Effect of a cytotoxic T lymphocyte response against RNF43 and TOMM34 on overall survival. The effect of a positive CTL response to RNF43 or TOMM34 on overall survival was analyzed. The Kaplan-Meier estimates for the overall survival of patients with detected CTL responses as compared to patients with no response are shown in Fig. 3. No statistical difference was found between the two groups with or without a response to RNF43 (p=0.2374) (Fig. 3A). However, there was a statistical difference between the two groups based on the TOMM34 response (p=0.0019) (Fig. 3B). Furthermore, we investigated the relationship between CTL response to both antigens and overall survival. The best long-term survival was observed in the group with CTL responses against both antigens, followed by the group showing CTL responses against only RNF43 or TOMM34 (p=0.0079). The patient with no response had the lowest survival (Fig. 3C).

Discussion

In this clinical trial, cancer vaccination with two peptides in combination with oral UFT/LV chemotherapy was well tolerated without any severe side effects in metastatic CRC patients. Common adverse events included vaccination site reaction, anemia, anorexia, malaise and elevation of transaminase. With the exception of the skin reaction, the rates of other adverse events did not exceed those of the UFT/LV chemotherapy (7). Therefore, addition of the peptide vaccination did not increase the adverse events (beyond mild vaccination site reactions) in this combination therapy. The design of this clinical trial was based on the results of two previous phase I trials. These previous trials found that vaccination with multiple peptides derived from novel cancer-testis antigens in advanced cancer was feasible and that antigen-specific T-cell responses were induced with objective clinical responses (8). These trials also showed that the peptide vaccination combined with oral UFT/LV chemotherapy was well tolerated in the metastatic CRC patients and induced peptide-specific IgG responses that correlated well with overall survival (5).

The combined chemo-immunotherapy approach has been criticized on the grounds that chemotherapy is immunosuppressive. This opinion is based on the fact that most cytotoxic drugs kill granulocyte precursors in bone marrow and thus induce leucopenia, which is associated with the occurrence of bacterial and mycotic infection. However, there is no evidence that cytotoxic chemotherapy affects the antigen-specific CTL response. Recently, Correale *et al* (9) reported that the antigen-specific killing ability of human CTL lines *in vitro* is not affected by 5-FU or oxaliplatin when exposure to these drugs does not occur during the stimulation phase. Moreover, they found that chemotherapy i) up-regulated tumor-associated antigen expression including CEA or other target molecules such as TS; ii) down-regulated tumor cell resistance to the death signals induced by tumor antigen-specific CTL; iii) reduced the percentage of PBMCs containing immune-suppressive regulatory T cells ($CD4^+CD25^+$T reg) and the number of cells expressing the FAS receptor (CD95); and iv) induced the complete restoration of the CD4/CD8 T-cell ratio, which is often reduced in advanced cancer patients resulting in a progressively deteriorating immune response (10). Based on these considerations, we believe that the rationale for chemo-immunotherapy in advanced cancer patients will be accepted.

The two cancer-specific peptides, RNF43 and TOMM34, used in the present study are novel cancer-testis antigens specific for CRC. More than 80% of colorectal cancers express these antigens, and these antigens can induce potent CTLs against colon cancer cell lines (4,6). RNF43 and TOMM34 are defined as oncoantigens. They are highly expressed in cancer cells, are involved in the critical functions of cancer cells (i.e., proliferation) and can induce potent CTL responses. In this context, it is of note that common antigens, such as MUC-1 or CEA, in colorectal cancer, are not critical for tumor cell survival; therefore, they can be lost under the selective pressure of a vaccine-induced antigen-specific immune response without significantly damaging tumor development (11-14).

Using the two crucial cancer-testis antigen-derived peptides, CTL responses were observed in 95% of the study patients (20 of 21 patients). Potent CTL responses against both antigens were induced in 8 patients (38%), and a CTL response against one peptide occurred in 12 patients (57%). Therefore, the use of two peptides allowed CTL responses to occur in almost all patients who received the vaccinations.

Overall survival was well correlated with the response to TOMM34. The patients exhibiting a response to RNF43 also experienced longer survival, although the correlation was not statistically significant. Notably, the patients exhibiting CTL responses to both peptides (n=8) had the longest survival, followed by the patients who showed a CTL response to one peptide (n=12). The patient exhibiting no response had the lowest survival (n=1) (Fig. 2). We do not have evidence to prove that the induced CTLs interacted directly with the cancer lesions in the patients with metastatic CRC to control the cancer lesions and thus contribute to the longer survival. However, we can conclude that the CTL response is a useful biomarker for patients receiving peptide vaccination therapy.

In conclusion, this study suggests that vaccination with two colorectal cancer-specific peptides in combination with UFT/LV is well tolerated and can induce potent and specific CTL responses to at least one peptide antigen in 95% of patients. Furthermore, the patients who developed potent CTL responses against both antigens showed the longest survival. This treatment approach warrants further clinical study.

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Recent Advances in Active Specific Cancer Vaccine Treatment for Colorectal Cancer

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Abstract: Cloning techniques to identify genes and peptides of tumor-associated antigens have created new possibilities for the immunotherapy of patients with advanced cancer. Here, we review recent clinical trials of specific cancer vaccines, mainly HLA-restricted peptides, and epitope-encoding vectors for advanced colorectal cancer (CRC). Many researchers initially focused on carcinoembryonic antigen (CEA) as an immunologic target antigen that is overexpressed on virtually all CRCs. A recombinant vaccine containing the CEA gene and dendritic cells (DCs) loaded with CEA peptide was administered to patients with CEA-elevated CRC. Although CEA-specific responses were detected, the clinical responses were limited. Recently, new types of clinical trials—namely, a personalized protocol to take into account the immunological diversity of cytotoxic T cell responses among patients and a novel cancer-testis antigen protocol that uses multiple peptides derived from genes identified by the cDNA array method—have been introduced. The personalized protocol seemed to be better than the classical (non-personalized) protocol in terms of clinical response and survival. Novel cancer-testis antigen protocols that use multiple CRC-derived peptides were recently conducted in patients with advanced CRC. The preliminary study yielded promising results regarding specific T cell responses to peptides and survival benefits. In this review, we summarize these results and discuss future perspectives.

Keywords: Active specific cancer vaccine, cancer-specific peptide vaccine, colorectal cancer (CRC), personalized peptide vaccine.

INTRODUCTION

Despite advances in treatment modalities, colorectal cancer (CRC) is still a leading cause of cancer-related mortality in industrialized countries. Improved treatment is urgently needed. Since the discovery of tumor-associated antigens during the early 1990s [1], rapid progress has been made in identifying antigens and describing immune interactions in cancer patients. Immunotherapeutic approaches have entered the clinical phase [2]. The goal of active specific immunotherapy is to induce an *in vivo* tumor-directed immune response. Thus, active specific immunotherapy must be distinguished from passive immunotherapy and nonspecific immunotherapy including cytokines or immunostimulants.

RATIONALE OF IMMUNOTHERAPY FOR COLORECTAL CANCER

The survival advantage of pronounced lymphocytic infiltration in CRC has been known for many years. The pioneering study by Jass showed the improved survival of CRC patients when prominent lymphocytic infiltrate was present [3]. Improved survival in patients with an increased number of peritumoral and stromal tumor-infiltrating lymphocytes (TILs) was demonstrated by Ropponen *et al.* [4]. However, in studies by Nanni *et al.* [5], Nielsen *et al.* [6], and Roncucci *et al.* [7], the number of TILs at the tumor margin or in the stroma did not influence survival in multivariate analysis. Notably, these authors did not investigate the role of intraepithelial lymphocytes (IELs). When Naito *et al.* [8] examined the role of TIL location in relation to prognosis, they found that stromal and peritumoral lymphocytes had no influence on survival, whereas the presence of IELs and CD8+ T cells in cancer cell nests was a predictor of improved outcome, independent of stage. In the same fashion, Funada *et al.* [9] demonstrated that patients with a high level of macrophage and CD8+ T cell invasion at the invasive margin had a 5-year overall survival rate of 92%, compared with a 72% survival rate in patients with a low level of infiltration Table (1).

These contradictory results may result from the complex interactions between lymphocytes, tumor, and microenvironment. It is clear, however, that the presence of activated CD8+ T lymphocytes in cancer cell nests suggests that the lymphocytes are recognizing a tumor antigen, resulting in a better prognosis.

Approximately 15% of sporadic CRCs and most hereditary nonpolyposis CRCs (HNPCCs) exhibit microsatellite instability (MSI) caused by a defect in the DNA mismatch repair system. CRCs with high MSI are usually proximal, poorly differentiated, and associated with pronounced lymphocyte infiltrate, and they have a better prognosis in comparison with MSI-negative tumors [10]. The increased im-

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Table 1. Tumor-Infiltrating Lymphocytes and Survival in CRC

Investigator	Pts N	TIL Location	RFS	OS	Follow-up
Roncucci [7] (1996)	397	Tumor margin	NR	Rectal cancer: 62% vs 36% Colon cancer: 61% vs 54%	5 Years
Ropponen [4] (1997)	195	Margin, stroma	HR, 0.72 (P<0.05)	HR, 0.55 (P<0.05)	14 Years
Naito [8] (1998)	131	Margin, stroma, IEL	NR	HR,0.91 (P=NS), HR,0.81 (N=NS), HR, 0.54 (P<0.05)	5 Years
Nielsen [6] (1999)	584	Tumor margin	NR	HR, 0.66 (P=0.03)	5 Years
Nanni [5] (2002)	263	Stroma	65% vs 58% (P=0.2)	81% vs 72% (P=0.09)	4 Years
Funada [9] (2003)	97	Margin, CD8+T cells	NR	92% vs 72% (P<0.05)	5 Years

Studies comparing patients with colorectal cancer exhibiting prominent amounts of tumor-infiltrating lymphocytes or not and comparing of survival of CRC patients.
Pts N: patients number; TIL: tumor-infiltrating lymphocytes; IEL: intraepitherial lymphocytes; RFS: relapse-free survival; OS: overall survival; HR: hazard ratio; NR: not reported; NS: not significant

munogenicity may result from a large number of mutated proteins, which can serve as tumor-rejection antigens.

The studies described here suggest that there is a significant host response to CRCs and that the presence of the host response is associated with improved survival. These findings suggest that appropriate immunologic approaches may improve patient prognosis.

VACCINE THERAPY: SPECIFIC IMMUNOTHERAPY FOR CRC

Numerous studies have been done on vaccination in colorectal cancer patients. Among them, representative studies of antigen pulsed dendritic cells (DCs) vaccination (three studies), viral vector based vaccination (one study), personalized peptide vaccination (three studies), and colorectal cancer-specific antigen derived peptide vaccination (two studies) are summarized in Table (2).

Peptide-Pulsed Dendritic Cells

Dendritic cells (DCs) are the pivotal antigen-presenting cells (APCs) for triggering T cell immunity. Autologous DCs have been used in cancer vaccines for CRC patients. DC-based vaccines can induce tumor-specific immune responses and objective clinical responses in CRC patients with marginal adverse effects.

Liu *et al.* [11] documented an increased number of CEA-specific T cells in 7 of 10 (70%) CRC patients who received a DC vaccination. Two (20%) of these patients had stable disease for at least 12 weeks, and 1 of these 2 patients experienced a transient decrease in CEA levels during the treatment period. In a study by Weihrauch *et al.*, 17 patients received CEA-derived peptide (CAP-1) or CAP-1-pulsed DCs in combination with chemotherapy (irinotecan/ high-dose 5-fluorouracil (5-FU)/ leucovorin (LV) [12]. Five of these patients experienced a complete response, 1 patient had a partial response, 5 patients had stable disease and 6 patients had progressive disease. Favorable results may depend on concurrent chemotherapy. It is noteworthy that increases in CAP-1-specific T cells were observed in 47% of patients after vaccination, whereas the EBV/CMV recall antigen-specific CD8+ cells decreased by an average of 14% during chemotherapy. In a study by Kavanagh *et al.* [13], 13 patients with advanced CRC were treated with DCs loaded with multiple peptides derived from CEA, MAGE, and HER2/neu. When the T cell responses were examined by enzyme-linked immunospot (ELISPOT) assay, 3 patients had T cell responses to one CEA-derived peptide, and 2 patients had T cell responses to multiple peptides. However, all patients showed progressive disease.

Collectively, these results indicate that DC-based vaccination could be a promising strategy for CRC. However, multiple problems, including high cost, conflicting results, and the large amount of time required for vaccine development, must be addressed before an affordable DC-based vaccination can be developed as a standard treatment. Moreover, reliable biomarkers must be identified, and vaccines and protocols must be standardized.

Viral Vector-Based Vaccine

A recombinant vaccinia virus encoding antigen sequences, such as the CEA gene and gene products, is capable of infecting professional antigen-presenting cells (APCs) and presenting CEA peptides to T lymphocytes in the context of HLA class I and II molecules, which activate the corresponding CD8+ or CD4+ T cells. In a phase I study, the safety of the vaccine was documented, and a CEA-specific T cell response was detected; however, no significant clinical effect was observed [14]. Approaches such as boost vaccination, T cell costimulation, and granulocyte-macrophage colony-stimulating factor (GM-CSF) administration enhanced the CEA-specific T cell responses in the majority of patients [15]. A trend towards an enhanced CEA-specific immune response to vaccination and an increase in progression-free survival and overall survival was documented. However, the subject group consisted of several small cohorts with different types of cancers, including 35 CRCs and 9 lung cancers;

Table 2. Specific Vaccine Trials for Colorectal Cancer

Investigator	Vaccines	Chemotherapy	Pts N	Clinical Response	Survival
Liu [11] (2004)	DC + CEA	-	10	2 SD, 8 PD	NR
Weihrauch [12] (2005)	DC * + CEA	Irinotecan, high-dose 5-FU, LV	17	5 CR, 1 PR, 5 SD, 6 PD	OS 17mo. with survival rate of 35% (6/17)
Kavanagh [13] (2007)	DC + peptides **	-	11	11 PD	NR
Marshall [15] (2005)	Virus expressing CEA + costimulator (TRICOM)	-	35	0 CR, 0 PR	Trend towards enhanced CEA-response and an increase in PFS
Sato [18] (2004)	Peptide (personalized)	-	10	1 PR, 1 SD, 8 PD	NI
Sato [21] (2007)	Peptide (personalized)	TS-1-based	7	1 SD, 6 PD	NI, 2/7 patients still alive at follow-up (17, 30mo.)
Hattori [22] (2009)	Peptide (personalized)	UFT/LV	13	6 SD (3 MR), 7 PD	PFS 10.7 wk (range 5.0-51.0 wk), OS correlated with peptide-specific IgG
Hazama (unpublished data)	Peptides (multiple) ***	FOLFOX	26	13 PR, 12 SD, 1 PD	PFS (has not been calculated)
Okuno (unpublished data)	Peptides	UFT/LV	19	17 SD, 2 PD	PFS (7.2 mo)

*A few patients with DC; ** CEA, MAGE, HER2/neu; *** RNF43, TOMM34, KOC1, VEGFR1, VEGFR2; Pts N, patients number; NR, not reported; NI, not identifiable; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response; OS, overall survival; PFS, progression free survival

therefore, definitive conclusions regarding this method cannot de drawn.

Peptide Vaccines

Rosenberg *et al.* [16] summarized the clinical responses to peptide-based vaccine therapy in 2004. Objective response rates for peptide vaccines and viral vaccines administered to patients with metastatic cancer at the National Cancer Institute (Bethesda, Maryland, USA) were 2.9% (11 of 381 cases) and 1.9% (3 of 160 cases), respectively. In a subsequent study, those trials and other trials of cell-based therapies were analyzed collectively, giving a combined objective response rate of 3.8% (29 of 765 patients, 36 protocols). These results indicate that the classical types of cancer vaccines, including peptide vaccines, do not have a promising future as a new treatment modality for cancer.

Personalized Peptide Vaccines

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific cytotoxic T lymphocyte (CTL) precursors are pre-existent. The initiation of immune boosting through vaccination was better than that of immune priming to induce prompt and strong immunity. Based on this concept, Itoh *et al.* [17] conducted a new regimen that included pre-vaccination measurement of peptide-specific CTL precursors in the circulation, followed by vaccination of only CTL-reactive peptide (CTL precursor-oriented vaccine). In a pilot study, 10 patients with advanced CRC were treated with up to four peptides that had been positive in the pre-vaccination measurement [18]. Post-vaccination peripheral blood mononuclear cells (PBMCs) from 5 patients demonstrated an increased peptide-specific immune response to the peptides. An increased CTL response to cancer cells was detected in post-vaccination PBMCs of 5 patients. Interestingly, antipeptide immunoglobulin G (IgG) became detectable in post-vaccination sera of 7 patients. One patient had a partial response, and another patient had stable disease for 6 months. These results are promising, but the clinical response was not satisfactory. In another protocol, the combination of this type of vaccination with chemotherapy in refractory prostate cancer patients was beneficial. This chemoimmunotherapy may break through the impasse in the clinical efficacy of cancer vaccines [19,20].

In a subsequent study, personalized peptide vaccination in combination with the oral administration of a 5-fluorouracil derivative (TS-1) in advanced CRC/ gastric cancer patients was investigated [21]. Eleven patients who did not respond to prior TS-1-based chemotherapy were enrolled. The combination therapy was generally well tolerated. The vast majority of patients experienced an increase in peptide-specific IgG after the sixth vaccination, irrespective of the dose of TS-1. In the patients who received 80 mg/m^2/day of TS-1, the CTL-mediated cytotoxicity against cancer cells was maintained at the prevaccination level. These results indicate that the standard dose (80 mg/m^2/day) of TS-1 in combination with personalized peptide vaccination does not impede immunological responses in cancer patients and could maintain or augment the immunological responses.

The combination of oral UFT® and UZEL® (LV) is a standard chemotherapy for CRC. UFT is an oral anticancer drug consisting of both Tegafur (FT), a prodrug of 5-FU, and uracil, an inhibitor of degradation of 5-FU. UZEL is an oral

drug consisting of calcium folinate, which modulates 5-FU. Therefore, we investigated the safety and immunological responses of personalized peptide vaccination in combination with UFT and LV in 14 patients with metastatic CRC [22]. Peptides were determined based on the presence of peptide-specific CTL precursors and IgG in each patient. A maximum of four peptides was administered weekly with UFT and LV for 4 weeks, followed by the standard 1-week rest period. This therapy was well tolerated, although 1 patient developed a grade 3 skin reaction at the vaccine site. After the tenth vaccination, 9 of 10 patients tested had an increase in peptide-specific interferon-γ production, and 8 of 10 patients tested had an increase in peptide-specific IgG. Six patients had stable disease, and 7 patients had progressive disease, as determined by the RECIST (Response Evaluation in Solid Tumors) criteria. Three of the 6 patients with stable disease showed a minor response; all 3 of these patients showed both strong CTL and IgG responses to at least one of the vaccinated peptides.

Interestingly, IgG responses correlated with overall survival (P= 0.0215) Fig. (1).

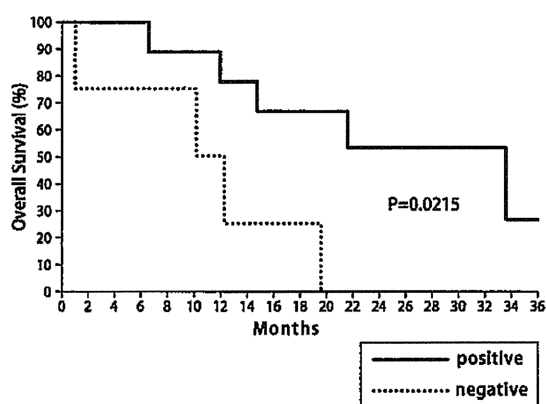

Fig. (1). Correlation between survival and peptide-specific IgG responses.
Overall survival was well correlated with increased levels of peptide-specific IgG (P=0.0215). Solid line: positive peptide-specific IgG response, dotted line: negative peptide-specific IgG response. (Ref. [22])

A similar correlation has been reported for CRC patients receiving a recombinant CEA vaccine [23]. However, the biological roles of IgGs specific to CTL epitopes are unknown. One possibility is that 9-mer peptide-recognizing CD4+ T cells were involved in this phenomenon. Peptides that bind to MHC class II molecules are generally considered to be 12 – 25 amino acids in length; however, the core sites anchored to MHC class II molecules are sufficient even at a length of about nine amino acids [24]. Indeed, our collaborator reported that the 9-mer peptide could induce peptide-specific and HLA-DR-restricted CD4+ T cells [25]. Another possibility is that CD4+ helper T cells might recognize the inoculated peptides presented on the HLA-A24 or -A2 molecules of antigen-presenting cells, resulting in both the activation of helper T cells and the subsequent promotion of IgG production [26]. CD4+ helper T cells are necessary to maintain CD8 T cell immunity [27]. If increased levels of peptide-specific IgGs reflect the activation levels of CD4+ helper T cells, the measurement of peptide-specific IgGs would be worthwhile as an immunological biomarker to predict the clinical benefits of peptide vaccination therapy for cancer patients.

In conclusion, personalized peptide vaccination combined with UFT/LV in patients with metastatic CRC is well tolerated and can induce cellular and humoral immune responses. Increased peptide-specific IgGs may be immunological biomarkers predictive of longer survival. Further trials of these vaccines are merited.

Peptides Derived from Novel Colorectal Cancer-Associated Antigens

cDNA microarray technology coupled with laser microdissection has been used to identify HLA-A24-restricted epitope peptides as potential targets for cancer vaccination in CRC patients [28, 29]. HLA-A24-positive is a dominant population in Japan (approximately 60%), subsequently HLA-A2-positive (approximately 20%). Therefore, to identify the binding epitope to HLA-A24 is essential issue for the successful anti-cancer vaccination in Japan. These antigenic peptides were derived from two different cancer-testis antigens, RNF43 (*ring finger protein 43*) [28] and TOMM34 (34 kDa-translocase of the outer mitochondrial membrane) [29]. Gene expression profiling revealed that RNF43 and TOMM34 were highly expressed in more than 80% of CRC samples, while these transcripts were hardly detectable in normal organs, with the exception of the testis and/or placenta. These peptides could stimulate CTLs that recognized and killed CRC cells. Therefore, RNF43- and TOMM34-derived peptides are promising candidates for the treatment of metastatic CRC. To evaluate the safety and immune response of vaccination with these peptides in combination with oral chemotherapy of UFT and LV for metastatic colorectal cancer, 20 HLA-A2402-positive patients were enrolled in a phase I clinical trial (Okuno *et al.* unpublished data). Eighteen patients were treated with peptides subcutaneously every week and two courses of UFT/LV chemotherapy for 10 weeks. Ten weeks later, the clinical responses were judged by CT scans, and cytotoxic T lymphocyte (CTL) responses against RNF43 and TOMM34 in peripheral lymphocytes were assessed by enzyme-linked immunospot assays. The vaccinations were well tolerated without any serious adverse events. Of the 18 patients, CTL responses were induced against both RNF43 and TOMM34 in 6 patients and against RNF43 or TOMM34 in 9 patients, while 3 patients had no CTL response. The rate of stable disease was 83%, as determined by RECIST criteria. Long-term survivors were observed in the group showing CTL responses against both RNF43 and TOMM34, followed by the group showing CTL responses against only RNF43 or TOMM34. The patients with no CTL responses had the worst survival Fig. (2).

Hazama *et al.* have been investigating a phase I trial of three peptides highly expressed in CRC (RNF43, TOMM34, KOC1), and the epitope peptide of vascular endothelial growth factor receptor 1(VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) in combination with

FOLFOX (combination of oxaliplatin, 5-FU, and LV) chemotherapy for metastatic CRC patients. Among 26 patients, 13 patients had a partial response, 12 patients had stable disease, and 1 patient had progressive disease. The median progression-free survival has not been calculated (Shoichi Hazama, personal communication).

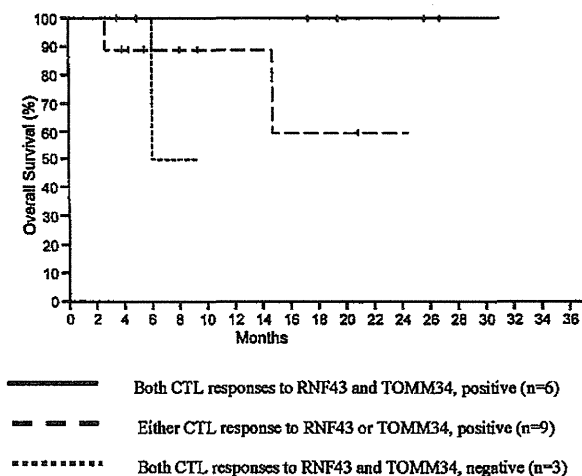

Fig. (2). Overall survival of patients with specific T cell responses to RNF43 and TOMM34.
Patients with responses to both RNF43 and TOMM34 had the best survival. Patients having no responses had worst survival. Patients with one response had intermediate survival.

RATIONALE OF COMBINATION THERAPY

Cancer is an extremely complex and heterogeneous disease that robustly resists host-defense systems and therapeutic efforts. The loss of MHC class I expression is a major mechanism of tumor cell escape from immune surveillance, whereas the appearance of multidrug resistance is the major mechanism of tumor cell resistance to chemotherapy. One approach to overcome the resilience of cancer is the design of a new combination therapy in which each modality imposes independent selective pressure to the acquired mutations of cancer [17].

Chemo-Immunotherapy in CRC

The combined chemo-immunotherapy approach has been criticized on the grounds that chemotherapy is immunosuppressive. This opinion is based on the fact that most cytotoxic drugs can kill granulocyte precursors in bone marrow and thus induce leucopenia, which is associated with the occurrence of bacterial and mycotic infection. However, there is no evidence that cytotoxic chemotherapy affects the antigen-specific CTL response. Recently, Correale *et al.* [30] reported that the antigen-specific killing ability of human CTL lines *in vitro* is not affected by FU, oxaliplatin, or gemcitabine (GEM) if exposure to these drugs does not occur during the stimulation phase. Moreover, they found that chemotherapy (1) up-regulated tumor-associated antigen expression, including CEA or other target molecules such as thymidylate synthase (TS); (2) down-regulated tumor cell resistance to the death signals induced by tumor antigen-specific cytotoxic T lymphocytes; (3) reduced the percentage of PBMCs containing immune-suppressive regulatory T cells (CD4+CD25+T reg) and the number of cells expressing the FAS receptor (CD95); and (4) induced the complete restoration of the CD4/CD8 T cell ratio, which is often reduced in advanced cancer patients showing a progressively deteriorating immune response [31].

HLA Loss or Down-Regulation in Cancer Progression

For successful CTL-based immunotherapy, it is essential to eliminate the loss of major histocompatibility complex (MHC) class I on cancer cells. A large population (30 – 60%) of cancer cells do not express MHC class I molecules, which are crucial for CTL-mediated elimination of cancer cells [32]. This problem, however, could be overcome by the combined use of another type of peptide vaccine, such as peptide of VEGFR1, or VEGFR2 [33], and either chemotherapy [31] or cytokine therapy capable of activating innate immunity including natural killer cells and macrophages. From this viewpoint, the development of an effective vaccine against tumor angiogenesis is suitable, because endothelial cells are genetically stable, do not down-regulate HLA class I molecules, and are critically involved in the progression of a variety of tumors. Furthermore, the CTLs could directly cause damage to the endothelial cells without penetrating any other tissue, and the lysis of even low numbers of endothelial cells within tumor vasculature may result in the destruction of vessel integrity, leading to the inhibition of many tumor cells. The results of a phase I study of multiple peptide vaccination including VEGFR1 and VEGFR2 in combination with FOLFOX chemotherapy for patients with metastatic CRC by Hazama *et al.* are anticipated.

FUTURE PERSPECTIVES

Numerous studies of vaccination in CRC patients have been performed. Antigen-specific responses were induced to some extent, depending on the individual immunizing methods in the trials; however, the clinical responses were marginal. In a meta-analysis by Nagorsen *et al.* [34], the objective response rate was only 0.9% for 527 CRC patients treated with active specific immunotherapy in 32 different studies. There are several possible approaches to improve the poor clinical outcome of vaccine immunotherapy in CRC.

Adjuvant Setting

Despite the nearly complete lack of a clinical response in patients with advanced colorectal cancer, a few studies have shown that adjuvant active specific immunotherapy may be beneficial in subgroups of patients after CRC resection [35, 36]. As we do not expect vaccination in patients with a high tumor burden to be highly clinically effective, we may be able to obtain a better impact on clinical outcome from the adjuvant setting. Recently, we started a randomized trial of CRC-specific peptides (RNF43, TOMM34) in combination with UFT/ LV chemotherapy as adjuvant immunochemotherapy in stage III colorectal cancer patients.

Helper-Peptide Vaccines

Cancer vaccine therapy first focused on the activation of CD8+ cytotoxic T cells (CTLs), which eradicate tumors *in*