Biosciences) and FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

A24-WT1-specific CTL lines. The research protocols for experiments using human specimens were approved by the medical ethics committees of MBL, Institute for Genetic Medicine, Hokkaido University and the Hokkaido University Graduate School of Medicine. Written informed consent was obtained from each subject. WT1-specific CTL lines were established in our laboratory with A24-positive healthy donors PBMCs purchased from Cellular Technology, Ltd. (Cleveland, Ohio) using a mixed lymphocyte peptide culture (MLPC) method as described (11). Cells were grown in complete RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 12.5 mM HEPES, 5% human AB serum, penicillin/streptomycin, 2 mM L-glutamine (referred to as T-cell medium), and 50 U/mL of human recombinant IL-2 (Shionogi Pharmaceutical Institute Co., Osaka, Japan). The CTL lines were periodically stimulated in the presence of irradiated and peptide-pulsed HLA-A*24:02-positive Epstein-Barr virus-transformed B cells (lymphoblastoid cell lines; LCLs).

Positive selection of antigen-specific T cells. CTL lines were stained with A24-modified WT1 tetramer-PE at 4°C for 15 min. After washing with MACS buffer (phosphate-buffered saline supplemented with 0.5% human serum albumin and 2 mM EDTA), the cells were incubated with anti-PE microbeads (Miltenyi Biotech, Auburn, CA, USA) at 4°C for 15 min. AutoMACS (Miltenyi Biotech) was used to prepare separated cells.

Repertoire analysis of TCR β chains. An IOTest Beta Mark® TCR Vβ Repertoire kit was used for the analysis of TCR β chains with antibodies against the following TCR Vβ regions: Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23.

PCR cloning and sequencing of WT1 peptide-specific TCR α/β genes. The TCR α chain composes a TCR alpha chain variable region (TRAV), a joining region (TRAJ), and a constant region (TRAC). The TCR β chain composes a TCR beta chain variable region (TRBV), a diversity region (TRBD), a joining region (TRBJ), and a constant region (TRBC). Total RNA from sorted CTLs was prepared with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and an aliquot (1 μg) was subjected to reverse transcription using an oligo (dT) primer and SuperScript III (Invitrogen, CA, USA). First strand cDNA was amplified by PCR using a FastStart High Fidelity PCR System (Roche Diagnostics, Rotkreuz, Switzerland) with coding region-specific primers for TRBV5-1 and TRBC1/TRBC2 (WT1 peptide specific TCR β chain), various TRAV primers, and TRAC (TCR α chain) according to the manufacturer's instructions. These PCR products were separated on a 1% agarose gel. A band of the appropriate size (bp) was excised and extracted from the gel. The recovered DNA fragment was cloned into the vectors pCDNA3.1 and pEF6/Myc-His (Invitrogen, CA, USA), and its DNA sequence was determined using BigDye Terminator reagent and a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). The confirmed cDNA sequences of each TCR gene were analyzed using the WEB tool IMGT (8). HLA-A*02:01-restricted Mart-1-specific TCR α/β cDNAs (5) were purchased from IDT (Coralville, IA, USA).

Transduction of TCR genes in lymphoma cells. To establish stable transfectants, TCR α chain/pCDNA3.1 and TCR β chain/pEF6/Myc-His plasmids (10 μg each) were transduced into lymphoma cells by electroporation, and selection was done in a medium containing 0.5 mg/mL of G418 (Roche) and 5 μg/mL of Blastisidin (Invitrogen). Expression levels of the transduced genes were assessed by flow cytometry with tetramer staining.

In vitro cytotoxicity assay. A24-positive and A24-negative LCLs in 100 μL of complete medium were labeled with 3.7 MBq ^{51}Cr for 1 h at 37°C. For peptide reconstitution assays, 1 μM of a synthetic peptide was added 1 h before introducing effector cells. After 4 h of incubation with effector cells, supernatants were determined with a gamma counter. The percentage specific lysis was determined by: [(experimental release − spontaneous release) / (maximum release − spontaneous release)] × 100.

Statistical analysis. Results are given as means and standard deviations. Statistical comparisons were made using two-tailed Student's t-tests; *P*-values of 0.05 were considered significant.

RESULTS

Induction of A24-WT1-specific CTL lines by MLPC

We first attempted to establish WT1-specific CTLs to identify TCR genes that bound to the WT1 peptide-A24 complex. WT1-specific CTLs were induced

using PBMCs from A24-positive healthy donors with A24-modified WT1 peptide (Fig. 1A). After repeated stimulation in MLPC, we established seven WT1-specific CTL lines (data not shown). As shown in Fig. 1B, one CTL line (37F8) was recognized by both A24-modified and natural WT1 tetramers. Although both tetramers reacted with 37F8 cells, their reactivity with the A24-WT1 natural tetramer was lower than that with the A24-WT1 modified tetramer. The frequency and absolute numbers of tetramer-positive T cells among 37F8 cells were much higher than those among the other CTL lines (data not shown). We confirmed that A24-WT1-specific 37F8 cells could be successfully purified using an autoMACS separation system with PE-A24-WT1 modified tetramer, and anti-PE microbeads (data not shown). These results suggested that the established 37F8 cells expressed TCRs that could bind to both A24-modified and natural WT1 peptide-HLA complexes.

Identifying the WT1 specific TCR Vβ repertoire

Next, we sought to identify the TCR Vβ repertoire of the 37F8 cells using a TCR Vβ Repertoire Kit, which could account for about 70% of the variations in TCR Vβ. We confirmed that the TCR β chains of 37F8 cells were recognized by anti-TCR Vβ5.1 (Fig. 2).

TCR cloning of the WT1-specific 37F8 cells

We investigated whole TCR sequences expressed by the 37F8 cells sorted by the AutoMACS system with A24-modified WT1 tetramer. TCR α and β genes were amplified by PCR with coding region-specific primer pairs for TRAC and various TCR α chains, or TRBV5-1 and TRBC1/2. PCR conditions were decided upon using Jurkat cells cDNA (TRAV8-4/TRAJ3/TRAC, TRBV12-3/TRBJ1-2/TRBC1) and human PBMCs cDNA that contained many different TCR genes (data not shown). As a result, we found that the TCR Vα chains of the 37F8 cells comprised TRAV12-2, TRAV12-3, and

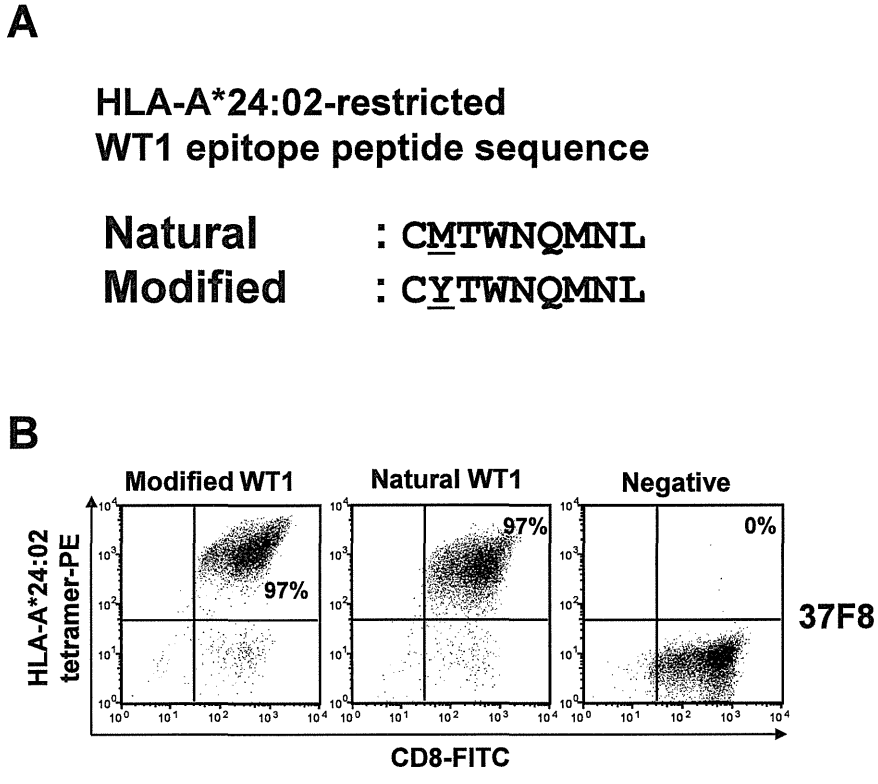

Fig. 1 Establishing WT1-specific CTL lines from PBMCs by MLPC. Upper panel (**A**) shows the CTL epitopes of A24-modified and natural WT1 tetramers used in this study. Lower panel (**B**) shows representative results for WT1-specific CTLs (37F8) induced by MLPC with A24-modified or natural WT1 peptides. The 37F8 cells were stained with A24-modified or natural WT1 tetramers. HIV tetramer was used as a negative control. The percentages of tetramer$^+$ cells among CD8$^+$ T cells are indicated.

TRAV41 (Fig. 3A). Because of high homology, the sequence for the TRAV12-2 PCR product was the same as for the TRBV12-3 PCR product, and the sequence for the TRBV5-1/TRBC1 PCR product was the same as for TRBV5-1/TRBC2. On the basis of the sequence analyses of these TCR α and β PCR products, we concluded that the 37F8 cells had two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and one β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B).

Establishing a T lymphoma cell line that expressed WT1-specific TCRs

To evaluate TCR reactivity with A24-modified and natural WT1 tetramers, we transduced the TCR α/β genes from the WT1-specific 37F8 cells into a Sup-T1 T lymphoma cell line by electroporation with expression vectors. Successful transduction of TCR genes (A12-3 and B5-1) into Sup-T1 T lymphoma cells was confirmed by staining with PE-WT1 tetramers (Fig. 4A). However, the A41 and B5-1 gene-transduced cells did not react with either the A24-modified or natural WT1 tetramers. HLA-A*02:01 Mart-1 TCR was used as a positive control for electroporation and tetramer staining. A stable T lymphoma cell line, SK37, was established by drug selection after transducing the 37F8 TCR A12-3 and B5-1 genes. The reactivity of TCRs expressed on SK37 cells was evaluated by flow cytometry with A24-modified or natural WT1 tetramers. This confirmed that the established SK37 cells reacted with both A24-modified and natural WT1 tetramers whereas an A24-HIV-negative control tetramer did not react with SK37 cells (Fig. 4B).

Functional properties of SK37 TCRs from the 37F8 cells

To investigate SK37 TCR function, cytotoxicity of the 37F8 cells was determined by a ^{51}Cr-release assay. The 37F8 cells showed robust, specific cytotoxicity against A24-positive LCLs that were pulsed with A24-modified or natural WT1 peptides (Fig. 5A and 5B). However, the 37F8 cells did not react with peptide-non-pulsed A24-positive LCLs and A24-negative LCLs (Fig. 5B). These results strongly suggested that the transduced WT1-specific TCR α/β genes could recognize the A24-WT1 epitope peptide.

DISCUSSION

To develop better cancer treatments, we established an accurate immunomonitoring system that could be used to demonstrate the mechanisms of antitumor effects of cancer immunotherapy. An HLA-tetramer reagent is one of the important tools used for immunomonitoring to detect antigen-specific CTLs. It was reported that a tetramer assay using peripheral blood

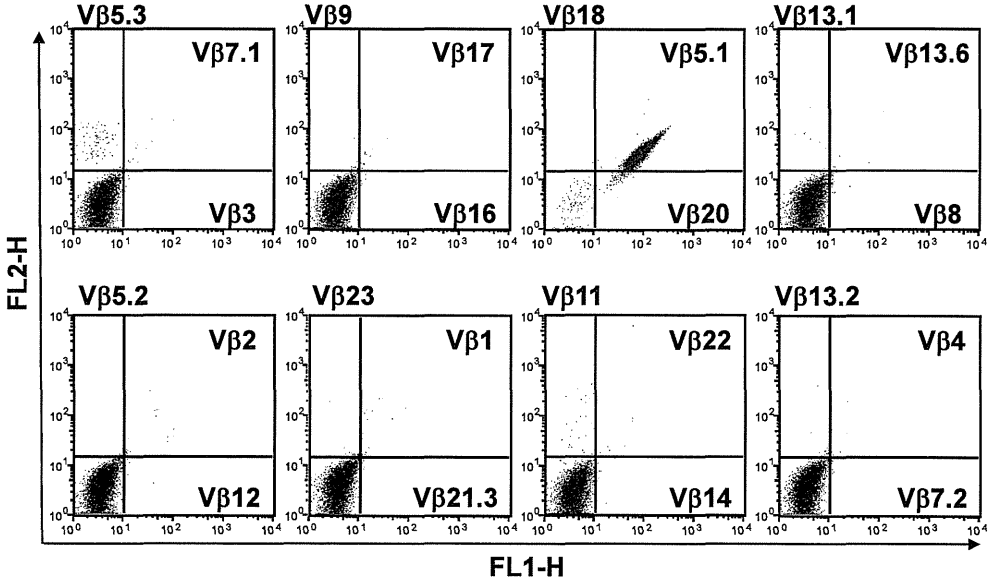

Fig. 2 Analysis of the TCR Vβ chain for the WT1-specific 37F8 cells. 37F8 cells induced by A24-modified WT1 peptide were stained with a variety of anti-TCR antibodies using a TCR Vβ Repertoire Kit. We confirmed that most CTLs reacted with anti-TCR Vβ 5.1. Representative results are shown in this figure.

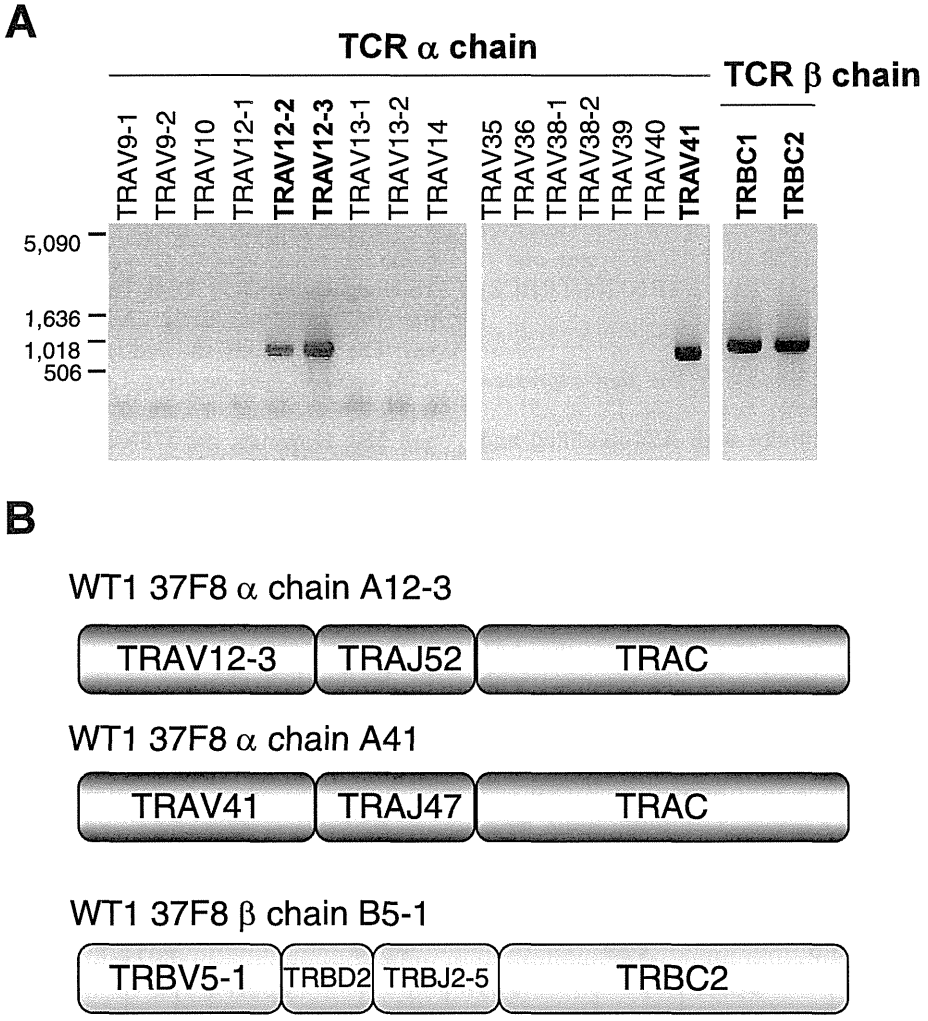

Fig. 3 Identifying the TCR Vα and Vβ repertoires for the 37F8 cells purified with A24-modified WT1 tetramer. TCR α and β genes were amplified with a thermal cycler with PCR coding region-specific primer pairs for TRAC and various TCR α chains, or TRBV5-1, and TCRBC1/2. (**A**) TCR α and β PCR products were separated on agarose gels and visualized by UV illumination. TRAV12-2, TRAV12-3, and TRAV41 genes were detected using PCR with a TCR α primer-set. (**B**) Illustration of TCR α and β chains of the WT1-specific 37F8 cells. The 37F8 cells had two types of α chains (A12-3, A41) and one β chain (B5-1).

from patients could become the best screening procedure, because it could be performed more easily and quickly than conventional procedures (16). However, the frequency of tetramer-positive $CD8^+$ T cells is generally not so high, and it is well-known that they have highly diverse reactivity using current methods. In addition, there is a difficulty with stable quality assurance of tetramer reactivity, because it depends on the specific reactivity between CTLs and HLA-peptide complexes. Therefore, for definitive analysis of antigen-specific immune responses using a tetramer assay, it is necessary to establish a stable cell line that expresses antigen-specific and monoclonal TCR α/β. In this study, we established a lymphoma cell line, SK37, that expressed WT1-specific TCRs, and this cell line could bind to both A24-modified and natural WT1 tetramers (Fig. 4B). These results suggest that SK37 cells could be useful as a positive control to evaluate the quality assurance of A24-modified and natural WT1 tetramers during immunomonitoring.

We successfully identified novel TCR genes composing two types of α chains (A12-3: TRAV12-3/TRAJ52/TRAC, A41: TRAV41/TRAJ47/TRAC) and

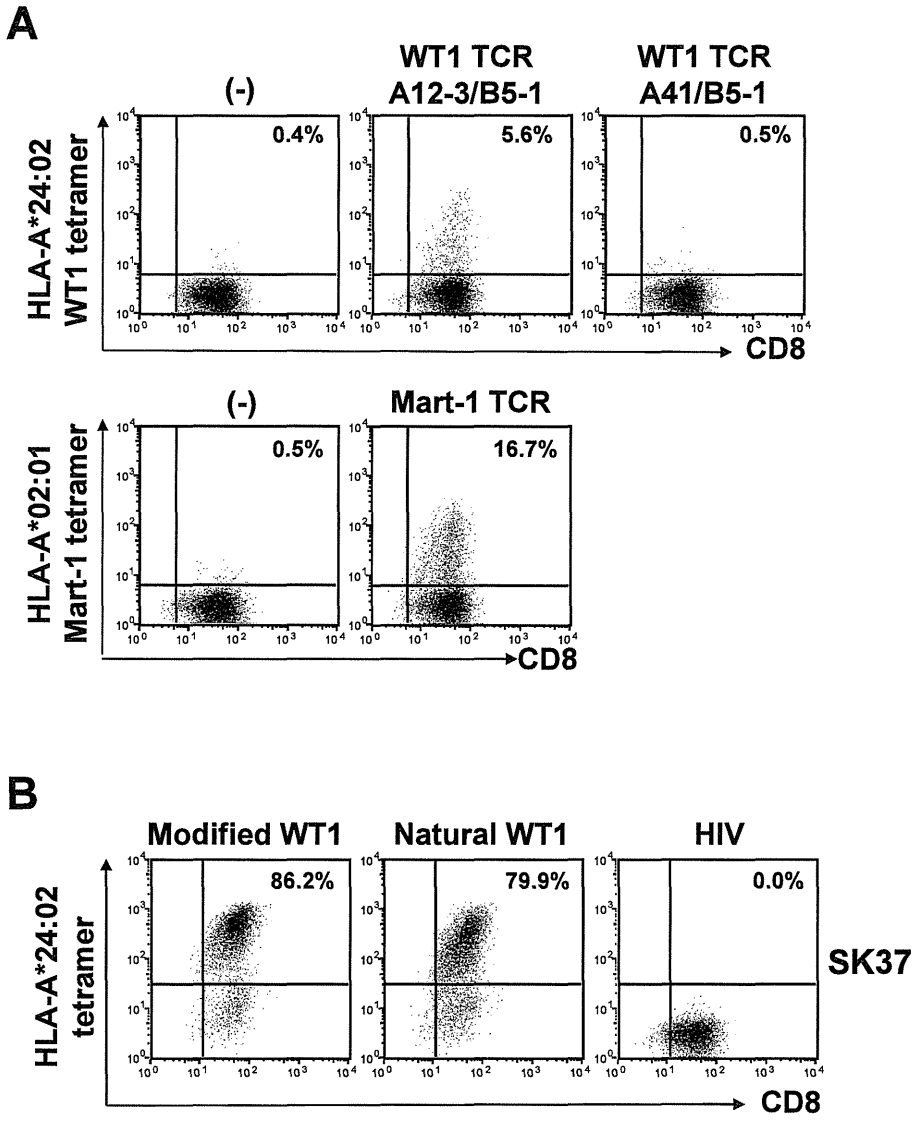

Fig. 4 HLA-tetramer assay for T lymphoma transduced TCR α and β genes from the 37F8 cells. (**A**) WT1 TCR (A12-3/B5-1 or A41/B5-1) genes were transduced into Sup-T1 cells. TCR-transduced T lymphoma cells were stained with A24-modified WT1 tetramer. The A24-WT1 tetramer bound to T lymphoma cells with WT1 TCRs composing A12-3/B5-1 but not with WT1 TCRs of A41/B5-1. A T lymphoma cell line with HLA-A*02:01 Mart-1 TCRs was used as a positive control for electroporation and the subsequent assay with Mart-1 tetramer. (**B**) The SK37 cells were established from T lymphoma cells with WT1 TCRs composing A12-3/B5-1. The TCR reactivity of SK37 cells was evaluated by flow cytometry using A24-modified WT1 (plot at left) or natural WT1 (center plot) tetramer in addition to HIV tetramer as a negative control (plot at right).

a single β chain (B5-1: TRBV5-1/TRBD2/TRBJ2-5/TRBC2) (Fig. 3B) for the WT1-specific 37F8 cells that were established using PBMCs from healthy donors by MLPC (Fig. 1B). It was also important to confirm the various functions of the identified TCR genes that recognized HLA/WT1-peptide complexes. We assessed the cytotoxicity of the established CTLs with A24-restricted WT1 TCRs using a ^{51}Cr release assay. In these experiments, the novel WT1 TCR-transduced CTL line showed specific cytotoxicity against natural WT1 peptide-pulsed A24-positive LCLs (Fig. 5A), although their cytotoxicity against modified WT1 peptide-pulsed LCLs was about four times stronger than natural WT1 peptide-pulsed LCLs. We speculated that the differences in cytotoxicity derived from the different affinities be-

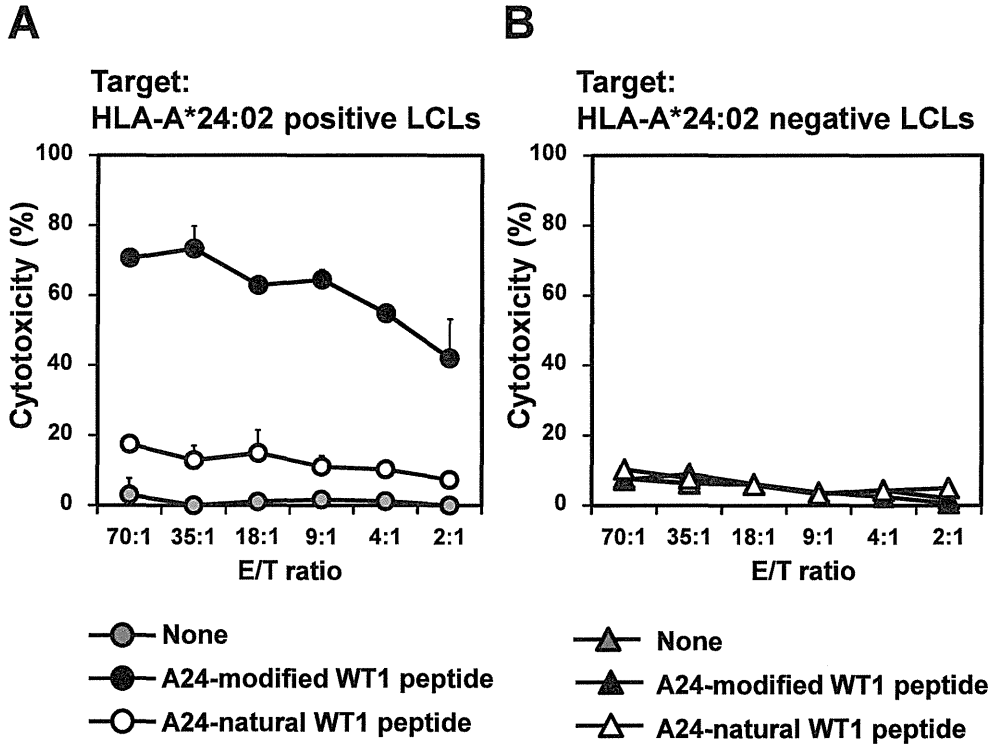

Fig. 5 Cytotoxicity of the WT1-specific 37F8 cells against A24-positive or A24-negative LCLs. A24 positive or A24-negative LCLs, used as target cells, were pre-incubated in the absence (None) or presence of A24-modified or natural WT1 peptides for 1 h, after which 37F8 cells were added as effector cells. CTLs cytotoxicity at the indicated E:T cell ratios was evaluated by 4 h ^{51}Cr-release assay. The cytotoxicity against A24-positive (**A**) and A24-negative (**B**) LCLs are indicated in this figure. 37F8 cells efficiently killed A24-positive target cells pulsed with A24-modified WT1 peptides and also killed fewer target cells pulsed with A24-natural WT1 peptides.

tween HLA molecules and natural or modified WT1 epitope peptides. However, the present findings clearly indicated that the novel WT1-specific TCR-transduced CTLs could recognize naturally processed WT1 peptides and kill endogenously WT1-expressing cancer cells in a WT1-specific A24-restricted manner. Thus, this may become a promising tool for developing TCR gene immunotherapy.

We recently demonstrated that introducing Th1-dominant immunity is essential for inducing fully activated CTLs and immunological memory in tumor-bearing hosts (9, 18, 19, 30). It has also been demonstrated that a mixture of various synthetic long peptides derived from naturally occurring sequences of HPV-16 oncoproteins was superior to short tumor peptides in terms of inducing complete or partial response in vulvar intraepithelial neoplasia (12). Thus, a long peptide vaccine that contains both helper and killer epitopes appears to be a rational strategy to activate Th1-dependent antitumor immunity (18).

As an innovative cancer vaccine, we also developed an artificially synthesized long peptide, H/K-HELP (helper/killer-hybrid epitope long peptide), which was conjugated to a MAGE-A4 class I-binding epitope and our defined helper epitope (3), and used this for a patient with pulmonary metastatic colon cancer. We found that cancer-specific Th1/Tc1 cells were induced in this cancer patient after vaccination with MAGE-A4-H/K-HELP. Therefore, we are now preparing HLA-class II tetramers for accurate immunomonitoring of MAGE-A4- and Survivin-H/K-HELP cancer vaccine therapy.

In summary, we established WT1-specific CTL lines using PBMCs from healthy donors by MLPC. We cloned TCR genes from these CTLs, which were transduced into T lymphoma cell lines by electroporation. As a result, we successfully established a novel A24-WT1 tetramer-positive lymphoma cell line, designated SK37, which was useful for the quality assurance of A24-modified and natural WT1 tetramers for immunomonitoring. Thus, the present

WT1 tetramer reagent that was validated using the SK37 cells could become a diagnostic product for cancer patients' antigen-specific immunotherapy.

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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, stabl 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, antipeptid 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 no satisfactory. In another protocol, the combination of this typ of vaccination with chemotherapy in refractory prostate cancer patients was beneficial. This chemoimmunotherapy may break through the impasse in the clinical efficacy of cance vaccines [19,20].

In a subsequent study, personalized peptide vaccinatio 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 di 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 i peptide-specific IgG after the sixth vaccination, irrespectiv of the dose of TS-1. In the patients who received 8 mg/m^2/day of TS-1, the CTL-mediated cytotoxicity agains 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 cance patients and could maintain or augment the immunologica responses.

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

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).

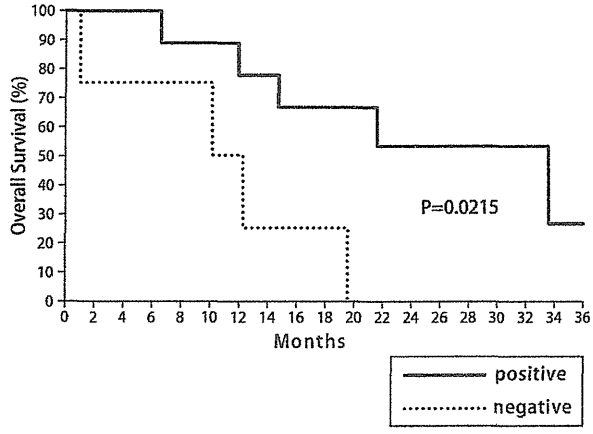

Fig. (1). Correlation between survival and peptide-specific IgG responses.

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 main tain CD8 T cell immunity [27]. If increased levels of pep tide-specific IgGs reflect the activation levels of CD4+ helper T cells, the measurement of peptide-specific IgG would be worthwhile as an immunological biomarker to pre dict the clinical benefits of peptide vaccination therapy fo cancer patients.

In conclusion, personalized peptide vaccination com bined with UFT/LV in patients with metastatic CRC is wel tolerated and can induce cellular and humoral immune re sponses. Increased peptide-specific IgGs may be immu nological biomarkers predictive of longer survival. Furthe trials of these vaccines are merited.

Peptides Derived from Novel Colorectal Cancer-Associate Antigens

cDNA microarray technology coupled with laser micro dissection has been used to identify HLA-A24-restricte epitope peptides as potential targets for cancer vaccination i CRC patients [28, 29]. HLA-A24-positive is a dominan population in Japan (approximately 60%), subsequentl HLA-A2-positive (approximately 20%). Therefore, to iden tify the binding epitope to HLA-A24 is essential issue for th successful anti-cancer vaccination in Japan. These antigeni peptides were derived from two different cancer-testis anti gens, RNF43 (*ring finger protein 43*) [28] and TOMM34 (3 kDa-translocase of the outer mitochondrial membrane) [29] Gene expression profiling revealed that RNF43 an TOMM34 were highly expressed in more than 80% of CR samples, while these transcripts were hardly detectable i normal organs, with the exception of the testis and/or pla centa. These peptides could stimulate CTLs that recognize and killed CRC cells. Therefore, RNF43- and TOMM34 derived peptides are promising candidates for the treatmen of metastatic CRC. To evaluate the safety and immune re sponse of vaccination with these peptides in combinatio with oral chemotherapy of UFT and LV for metastatic colo rectal cancer, 20 HLA-A2402-positive patients were enrolle in a phase I clinical trial (Okuno *et al.* unpublished data) Eighteen patients were treated with peptides subcutaneousl every week and two courses of UFT/LV chemotherapy fo 10 weeks. Ten weeks later, the clinical responses wer judged by CT scans, and cytotoxic T lymphocyte (CTL) re sponses against RNF43 and TOMM34 in peripheral lympho cytes were assessed by enzyme-linked immunospot assays The vaccinations were well tolerated without any seriou adverse events. Of the 18 patients, CTL responses were in duced against both RNF43 and TOMM34 in 6 patients an against RNF43 or TOMM34 in 9 patients, while 3 patient had no CTL response. The rate of stable disease was 83%, a determined by RECIST criteria. Long-term survivors wer observed in the group showing CTL responses against bot RNF43 and TOMM34, followed by the group showing CT responses against only RNF43 or TOMM34. The patient with no CTL responses had the worst survival Fig. (2).

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

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).

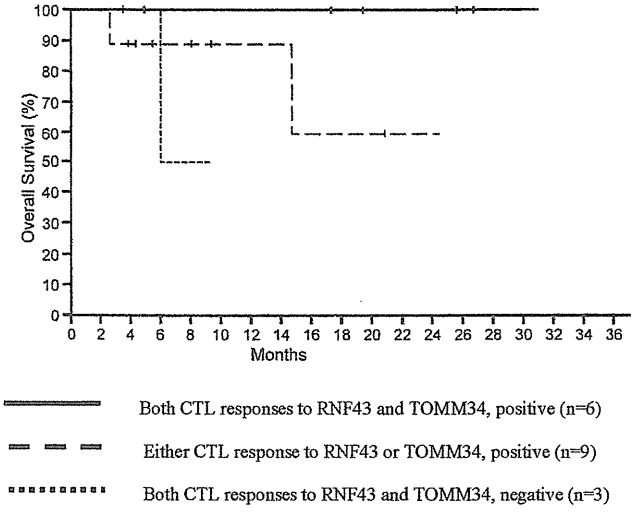

Fig. (2). Overall survival of patients with specific T cell responses to RNF43 and TOMM34.
Patients with responses to both RNF43 and TOMM34 had the best survival. Patients having no responses had worst survival. Patients with one response had intermediate survival.

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*

vivo. Although many investigators approached this problem by using MHC class I-binding peptides, this approach has been hampered by strong immunosuppression and unknown immune-escape mechanisms in tumor-bearing hosts. CD4+ T cells are crucial for the induction of effective antitumor immunity. In particular, the introduction of T helper type 1 (Th1)-dominant immunity in tumor-bearing hosts is critically important to overcome immunosuppression and induce fully activated tumor-specific CTLs. Nishimura *et al.* [37] reported that adoptively transferred tumor-specific Th1 cells exhibited strong antitumor activity *in vivo*. Moreover, they established cancer-specific helper T cell lines from healthy donors by using cancer antigen NY-ESO-1 derived from overlapping 15-mer synthetic peptides bound to HLA-DR molecules [38]. This method is anticipated to be a new cancer vaccine therapy that elicits a cancer-specific helper T cell response in cancer patients. In collaboration with the Nishimura group, we recently started a helper-peptide vaccine study with Survivin or MAGE-A4 antigen-derived helper peptides for the treatment for advanced CRC patients.

CONFLICT OF INTEREST

None declared.

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None declared.

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EUROPEAN JOURNAL OF CLINICAL & MEDICAL ONCOLOGY REVIEW ARTICLE

Intracellular Glutathione in Monocytes are Useful Biomarker of Immune Status of Tumor Bearing Patients

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ABSTRACT

Solid tumor patients, with low immune response, tend to have poor survival outcome after surgery. A new concept has emerged that preoperative determination of patient's immune status will be helpful in determining appropriate therapeutic options to achieve the best possible postoperative outcome for solid tumor bearing patients. Here we propose a method of predicting patient's immune status using peripheral blood specimen. This method is based on the finding that a relationship exists among preoperative monocytes (Mo) status (reductive, R-Mo/oxidative, O-Mo) determined by glutathione levels, presence/absence of tumor infiltrating lymphocytes (TIL) in tumor parenchyma, and the patient's survival after operation. In fact, R-Mo with a higher icGSH can stimulate $CD4^+$ T-cells to produce IFN-γ more effectively than O-Mo and thereby induce antitumor Th1 responses. Based on this concept, we will in this review introduce a method of predicting local antitumor reactions in preoperative solid tumor patients using peripheral blood specimen. In patients who were predicted as having poor immune response, we will overview the probability of inducing antitumor immune response, before patients undergo surgery, by converting O-Mo to R-Mo using biological response modifiers (BRM) such as lentinan. These clinically practical methods can be helpful in determining appropriate therapeutic options to achieve the best possible postoperative outcome for solid tumor bearing patients.

Keywords: glutathione, reductive monocyte, oxidative monocyte, tumor infiltrating T-cell, biological response modifier

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INTRODUCTION

In this brief review we will introduce a new concept of regulating host response prior to surgery, leading to the proposal of a clinically practical method to improve the survival rate of patients with solid tumors using individualized therapy. We will explore the possibility of predicting local antitumor reactions without surgery using biomarkers easily measurable in peripheral blood specimens. Such predictions can be helpful in determining appropriate therapeutic strategies before surgery to achieve the best possible postoperative outcome for solid tumor bearing patients.

Many previous reports have demonstrated the critical role of tumor microenvironments, especially the presence of TIL in tumor stroma, for a good prognosis of solid tumor bearing patients.[1–3] The presence of TIL is usually determined in resected tumors using pathological paraffin sections. Patients with poor antitumor immunity are usually unable to overcome the risk of micrometastasis associated with surgery and, therefore, tend to have poor survival outcome. Predicting antitumor immune response at local tumor growing sites using only peripheral blood specimens enables us to classify the potential clinical outcome of concerned patients based on the presence or absence of antitumor immune response in their local tumor growing sites. This presurgical classification can enable doctors to administer individualized presurgery treatment to patients with limited antitumor immune response. Presurgical determination of antitumor immune responses allows for patient-oriented therapeutic options and may result in better survival outcome of patients. Several treatments such as BRM,[4,5] low dose chemotherapy, or radiation therapy[6,7] are known to be effective at inducing antitumor immune response.

In a prior report, we demonstrated that determining icGSH redox status in peripheral Mo correlated with the existence of TIL in tumor parenchyma.[8] In this review, we will focus on the relationship between antitumor activity and intracellular redox status of peripheral Mo and discuss the following issues: (1) the relationship between icGSH levels of peripheral Mo and antitumor activity, (2) the possibility of using BRM to modulate intracellular redox status of peripheral Mo in order to induce adequate antitumor immune response in solid tumor bearing patients.

A FUNCTIONAL HETEROGENEITY OF MO AND ANTITUMOR IMMUNE-REACTION IN SOLID TUMORS

To induce the efficient sensitization of immune cells with solid tumors, helper T-cells, and CTL are required to transmigrate the endothelium and tumor stromal tissues and penetrate the tumor parenchymal area.[9] In our previous study, we reconciled the relationship among the pathology of the tumor peri-stroma area, the existence of TIL in tumor parenchymas, and the GSH status of peripheral Mo.[8,10]

Many researchers consider Mϕ as suppressors of antitumor immune response, however, we will shed light on the functional heterogeneity of Mϕ. Further, many reports have mentioned Mϕ/Mo plasticity and their ability to change their status based on environmental factors such as cytokines, microbial stimuli, and hypoxia. Hamuro et al previously reported that Mϕs were divided into at least two activated states based on their icGSH content. Mϕs with elevated icGSH are arbitrarily termed as R-Mϕ and those with reduced icGSH as O-Mϕ.[11] They suggest that each of these two types of Mϕ has its own distinct function; R-Mϕ with high icGSH induces the polarization to the Th1 response and O-Mϕ with reduced levels of icGSH induces the Th2 response. This means that Th1/Th2 balance is regulated by the balance between R-Mo/Mϕ and O-Mo/Mϕ. The balance between these two classes of Mϕ is associated with the skewed production levels of IL-12 and IL-10, which further affects the development of the tumor stroma area, possibly due to the skewed production of IFN-γ. Peterson et al also demonstrated that GSH levels in antigen-presenting cells modulate Th1 and Th2 response patterns.[12]

Recently, analogous to the Th1/Th2 nomenclature, a dichotomy of polarized M1- and M2-Mϕ has been proposed.[13,14] The M1-Mϕ are characterized as producing high levels of IL-12 and low levels of IL-10, while M2-Mϕ produce low levels IL-12 and have the tendency to shift from arginine metabolism to the production of ornithine and polyamines via arginase, as was previously shown by Hamuro et al[11] and Murata et al.[15,16] Many circumstantial evidence have suggested that M1-Mϕ closely corresponds to R-Mo/Mϕ and M2 Mϕ to O-Mϕ.

While the classification of the propensities of Mϕ as R-Mϕ and O-Mϕ based on the intracellular content of GSH may not be absolute but rather relative, we adopted this method as we think it is useful in conceptualizing the convergence of diversified immunological outcomes initiated by a variety of stimuli. The R-Mϕ and O-Mϕ classification simply refers to distinct metabolic states or to the intracellular redox status of Mϕ; however, this status may actually exist on a continuum and be distinctly separable. The R-Mo/O-Mo paradigm is summarized in Figure 1.

Mϕ/Mo REDOX STATUS AND THE ROLE OF icGSH

The GSH is a nonprotein tripeptide that contains thiol. It is abundant in virtually all cells, playing significant roles in many biological processes. GSH also constitutes the first line of cellular defense mechanisms against oxidative injury and is the major intracellular redox buffer in ubiquitous cell types. In this way icGSH/GSSG dominates the generation of diverse cytokines and inflammatory mediators. Hamuro et al[11] have shown that GSH redox status plays a central role in determining which of the R- and O-Mϕ/antigen presenting cells (APC) are dominant during a variety of immune responses. They demonstrated that ic-GSH/GSSG dominates the generation of diverse cytokines and inflammatory mediators. Redox status in cytosole, such as changes in the amount of GSH (or the GSH/GSSG ratio), affects NF-ϕB/IϕB complex leading to its activation, NF-ϕB then translocates into the nucleus, resulting in DNA binding and transactivation.

Utsugi et al[17,18] revealed the molecular mechanism by which GSH redox status affects IL-12 production in human APC. They demonstrated that p38 mitogen-activated protein kinase (MAPK) positively, and c-jun N-turminal kinase (JNK) negatively regulates LPS-induced IL-12 production and, as a result, the GSH/GSSG ratio induced by GSH precursor enhances IL-12 production.

In a recent report Alam et al[19] demonstrated that an altered cellular redox plays a profound role in inflammation by activating various kinases and redox-sensitive transcription factors such as NF-κB rel proteins. NF-κB rel proteins differentially regulate the genes encoding various proinflammatory cytokines. The GSH/GSSG balance modulates IκBα signaling and the levels of CaM expression in Mϕ, which subsequently influences nuclear c-rel translocation, and thereby leads to the regulation of IL-12 production levels.

Further, low icGSH levels in APC are correlated with defective processing of antigen with disulfide bonds, indicating that this thiol may be a critical factor in regulating antigen processing.[20,21]

RELATIONSHIP BETWEEN R-Mo/O-Mo STATUS AND ANTITUMOR IMMUNITY

In a prior report we applied the R-Mo/O-Mo parameter to clinical specimens and determined the GSH status in Mo from 30 colon/rectal cancer-bearing patients (Stage O-IV) prior to surgery. The GSH index is useful for measuring individual patient's icGSH as it requires a relatively small amount of Mo compared with other biochemical measurements. The Mo were stained with monochlorobimane and observed with a fluorescence microscope.[8] Each patient's Mo image was analyzed using image software and quantified as total GSH in the $CD14^+$ Mo (designated as GSH index). The Cox's proportional analysis of the 30 patients showed that GSH index and TIL presence/absence were significant independent factors that could predict survival. These results indicate that knowing R-Mo or O-Mo status is as equally predictive of patient survival as knowing whether TIL is present or absent in tumor parenchyma. Therefore determining R-Mo or O-Mo status is a useful biomarker to understand and predict the antitumor status of solid tumor bearing patients.

The evidence from histology and survival of colorectal patients described above suggest that antitumor responses occur at a high frequency in patients with R-Mo. In a previous paper, we demonstrated that IL-12 responsiveness was a significant factor that predicts survival and is a good indicator that allowed us to predict the existence of sensitized T-cells as part of an ongoing antitumor immune response.[8] When comparing R-Mo colorectal cancer patients, patients showing TIL presence, and those with IL-12 responsiveness, the

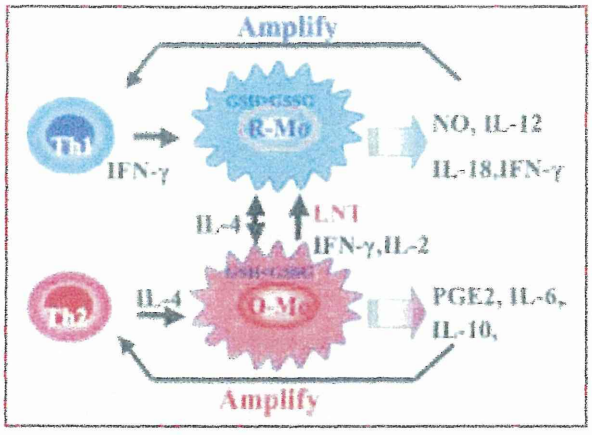

Figure 1. This diagram shows the phase transitions of O-Mo to R-Mo and vice versa. Mo/Mφ were divided into two activated status based on the intracellular content of GSH. IcGSH in Mo/Mφ is critical for the secretion of not only various cytokines but also various effecter molecules. These factors further indicate a crucial role of intracellular GSH in Mo/Mφ in determining whether Th1 or Th2 response is dominant. It is possible to transform O-Mo to R-Mo by administering OK-432 and LNT.

relationship is such that the R-Mo group and TIL presence group mostly overlap but not completely, whereas the IL-12 responsiveness group is contained within the range of the other two groups (Figure 2).

These results demonstrate that the intracellular redox status monitored by icGSH levels in Mo/Mφ is pivotal for the development of antitumor response (presumably Th1 and wound healing status); therefore, determining icGSH has great potential as a favorable prognostic biomarker in predicting survival. Additionally, there seems to be a significant advantage in using GSH index to classify cancer stage to predict overall survival. Recently, Ma et al[22] demonstrated that the presence of the M1 form of tumor-associated Mφ in resected tumor specimens was positively associated with the survival of lung cancer patients. These results confirm the idea that the microenvironment supported by R-Mφ /M1-Mφ, lead to patients' Th1 antitumor immune response and affect their prognosis.

There are several reports that activated Mφ/Mo icGSH levels are higher than resident Mφ/Mo.[23] Furthermore, the capacity of allo-stimulatory and IFN-γ production correlated with icGSH levels in Mo-derived dendritic cells.[24] Low icGSH levels in antigen-presenting cells correlated with defective processing of antigens.[21] Furthermore, GSH and IL-2 are involved in the growth and replication of activated lymphocytes.[25] These data suggest that the icGSH levels of Mo/Mφ/DC affect not only antitumor activity, but also the antigen-presenting activities of Mo/DC. Additionally, it is easy to conclude that the icGSH of Mo influences lymphocyte proliferation, differentiation, movement, and chemokine/cytokine production.[26–28]

PHASE TRANSITION OF Mφ STATUS TO INDUCE ANTITUMOR IMMUNE RESPONSE

As we have prior stated, intracellular redox status of Mφ is critical for inducing antitumor responses. Functional plasticity of Mφ offers hope that phase transition of Mφ status from O-Mφ to R-Mφ may act positively to induce antitumor immune response. Murata et al[15] confirmed that the GSH content in Mφ could be modulated easily by the application of N-acetyl cystein (NAC), glutathione monoester (GSH-OEt), buthionin sulfoximine (BSO), and maleic acid diethyl ester (DEM) in vitro and in vivo. As a result, GSH deprived Mφ (O-Mφ) showed elevated IL-6 and IL-10 production. On the

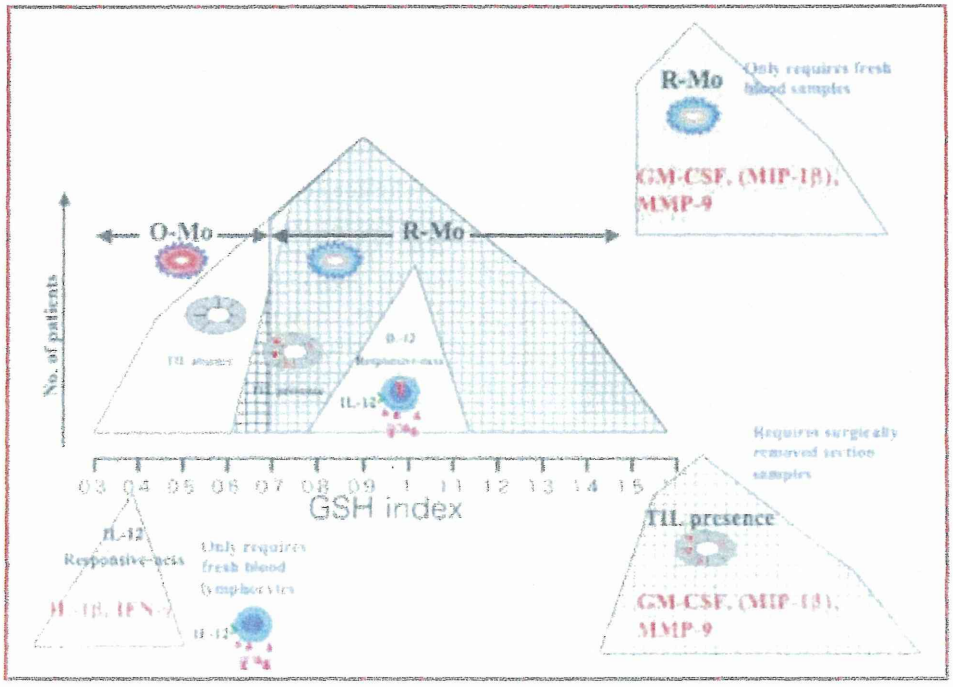

Figure 2. Schematic diagram of the relationship among Mo status based on the GSH index, TIL presence/absence in tumor parenchyma, and IL-12 responsiveness of CD4T and/or CD8T-cells.

other hand, GSH-OEt primed R-Mϕ produced IL-12 upon IFN-γ and LPS stimulation, whereas DEM elicited O-Mϕ did not. These results reveal that various appropriate treatments enable Mϕ phase transition and induce Th1 polarization.

Lentinan and OK432 are commonly used clinically in Japan, and we believe their effects are partly dependent on the transition of O-Mo to R-Mo and determine the Mo status of BRM treated patients. As shown in Figure 3, it is possible for O-Mo to transit to R-Mo by clinical treatments using LNT or OK432 agents.

The LNT is a β-(1–3)-D-glucan extracted from edible mushroom Lentinus edodes (Berk.) Sing and then purified.[4] β-(1–3)-D-glucans, a physiological molecule ubiquitously present in the cell walls of fungi, are well known as being critical for the host defense system.[29] Pre- and postoperative administration of β-D-(1–3) glucan, LNT, in combination with interleukin-2 (IL-2) was reported to completely cure murine tumor models due to the synergistically augmented tumor tissue stromal reaction. LNT-induced R-Mϕ induce Th1 response by producing IL-12, and reducing O-Mϕ induced Th2 thereby reducing the production of IL-6, IL-10, and prostaglandin (PG) E2. In patients with inoperable recurrent gastric cancer undergoing chemotherapy, intravenously injected LNT, because of its ability to induce Th1 response through its action on monocytes, elicits a remarkable life-prolonging effect and is now widely used as an immunotherapeutic drug.[30]

Our previous reports showed that OK-432 administration increased the GSH indices of Mo, and these Mo stimulated CD4T cells to produce more IFN-ϕ than O-Mo.[8] Streptococcal preparation OK-432, a penicillin-killed and lyophilized preparation from the low-virlence strain (Su) of *Streptococcus pyogenes* was developed by Okamoto et al[31] and is commonly used as a BRM for immunotherapy in malignant tumors. It has been shown that the lipoteichoic-acid-related molecule (OK-PSA), extracted from OK-432, induced Th1-type cytokines, such as IFN-γ, TNF-α, IL-2, IL-12, and IL-18 and elicited a marked antitumor effect.[32] Additionally, in vitro culture of immature DC generated from adherent peripheral blood mononuclear cells using GM-CSF and IL-4 with OK432 resulted in increased cell surface expression of matured DC marker (OK-DC). Prior reports have demonstrated that OK432, a strong inducer of IL-12 and IFN-γ. efficiently augment the primary allogeneic T-cell responses and cytotoxic T lymphocytes (CTL) response by OK-DC. These findings strongly suggest that for cancer patients with a Th2-dominant state, OK-432 is a useful immunotherapeutic agent, since it acts as a potent Th1 response inducer by converting Mo status.

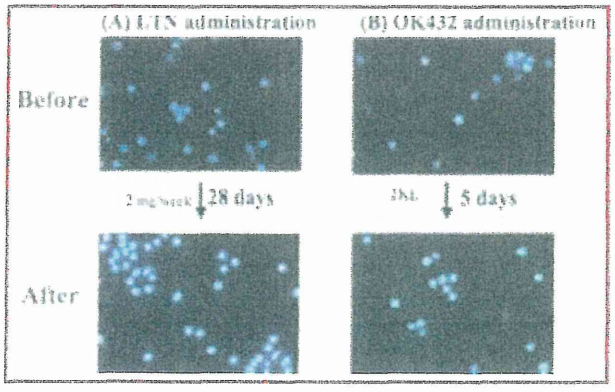

Figure 3. Conversion of O-Mo to R-Mo after BRM treatment. MCB staining in two cancer patients treated with LTN or OK-432. Left panels: Mo from lung cancer patient before and after treatment with LTN. Right panel: Mo from breast cancer patient before and after treatment with OK432.

ALTERNATIVE BIOMARKER TO GSH MO

We have demonstrated previously that GSH index obtained from peripheral blood specimen is an alternative biomarker to confirm TIL existence in tumor parenchyma. IL-12 responsiveness using purified blood CD4/CD8 T lymphocytes from colon/rectal cancer patients, suggests the existence of sensitized CD4/CD8 T-cells.[8,33] Albeit being good biomarkers to predict antitumor immune-reactions in solid tumor bearing patients without the need of surgery, both GSH index and IL-12 responsiveness require fresh blood lymphocytes/Mo. Additionally, in our prior experiments, we pursued alternative biomarkers using plasma cytokine/chemokine analysis with a suspension array system using the original 30 colon/rectal cancer patients. We found that several cytokine/chemokine, and MMP levels in the preoperative plasma from colorectal cancer patients correlated well with Mo redox status, TIL, and overall survival (Table 1). Higher GM-CSF was also observed in the plasma of the R-Mo and TIL presence group, and ultimately these groups experienced good survival outcome. Although the difference was marginal, higher IL-1β and MIP-1β levels were found in the R-Mo or TIL presence group and this led to good survival outcome. Higher plasma MMP-9 was also observed in the R-Mo and TIL presence group. Additionally, higher plasma IL-1β and IFN-γ correlated with positive IL-12 responsiveness of CD4/CD8 T-cells and also led to good survival outcome. As shown in Table 1, these results indicate that GM-CSF, MIP-1β, and MMP-9 are biomarkers for R-Mo and TIL existence, and IL-1β and IFN-ϕ are related to antitumor T-cell responses.

Why does higher plasma levels of GM-CSF, IL-1β, and MIP-1β correlate with R-Mo/TIL existence in tumor parenchyma? GM-CSF is well known as a key cytokine for the maturation of DC derived Mo. MIP-1β is a chemokine, which acts as a potent chemoattractant for immune cells such as CD4T, CD8T, NK, and DC cells. It has been demonstrated that intratumoral expression of MIP-1ϕ induces strong antitumor responses.[34,35] Besides its central role in inflammation, IL-1β has also been recognized as a powerful player in tumor progression, angiogenesis, and invasiveness. On the other hand, IL-1β also has been used as an adjuvant to mount antitumor immunity.[36,37] Although the role of MMP-9, a member of gelatinase subgroup, in cancer is controversial, a report has suggested that high MMP-9 in epithelial ovarian cancer cells was associated with long-term survival.[38]

When R-Mϕ is dominant in tumor tissue stromal inflammation, tissues are remodeled after extravasation occurs,

Table 1. Relationship Among Mo Status, TIL Existence, IL-12 Responsiveness and Overall Survival

Plasma Cytokine log (pg/ml)	Categories: Pre-operative Mo Status			TIL in Tumor Islets			IL-12 Responsiveness			Cox Hazard Analysis: Overall Survival	
	O-Mo (n=6)	R-Mo (n=14)	p	absence (n=7)	existance (n=23)	p	positive (n=7)	negative (n=23)	p	HR (95% CI)	p
O-Mo/R-Mo	—	—	—	—	—	—	—	—	—	0.194 (0.038–0.887)	0.0352*
absence/existance TIL	—	—	—	—	—	—	—	—	—	0.194 (0.038–0.887)	0.0352*
IL-12 responsiveness	—	—	—	—	—	—	—	—	—	$1.86e^{-7}$ (−0,898)	0.04*
IL-1b	−0.92±0.24	−0.57±0.13	0.22	−.0±0.24	−0.54±0.13	0.10	−0.98±0.22	−0.82±0.12	0.0078**	0 (−0,638)	0.016*
GM-CSF	0.75±0.17	1.17±0.09	0.034*	0.74±0.16	1.17±0.009	0.030*	1.36±0.16	0.98±0.09	0.062	0.219 (0.054–0.973)	0.046*
IFN-γ	1.53±0.18	1.66±0.10	0.73	1.49±0.18	1.69±0.10	0.35	1.98±0.17	1.54±0.09	0.03*	0.036 (0.001–0.455)	0.0014**
MIP-1b	1.41±0.14	1.72±0.08	0.081	1.39±0.14	1.73±0.08	0.055	1.84±0.15	1.59±0.08	0.16	0.282 (0.113–0.875)	0.031*
MMP-9	4.89±0.15	5.50±0.08	0.0023**	4.98±0.16	5.54±0.09	0.016*	5.61±0.17	5.28±0.09	0.11	0.317 (0.109–1.099)	0.068

Abbreviations:
GSH: glutathione, GSSG: oxidized glutathione, Mo: monocyte, Mϕ: macrophage, R-Mo: reductive monocyte, O-Mo: oxidative monocyte, TIL: tumor infiltrating T lymphocyte, BRM: biological response modifier, IcGSH: intracellular glutathione APC: antigen presenting cell, CTL: cytotoxic T lymphocyte, LNT: Lentinan, MMP: matrix metalloproteinase, MCB: monochlorobimane, DC: dendritic cell, PGE2: prostaglandin E2

immune cells are then infiltrated into tumor tissue and, as a result, this chain reaction induces and keeps specific Th1 immune responses working efficiently. As GM-CSF and IL-1ϕ, are known ?to induce Th1 responses, it is believed that these cytokines/chemokines will create a suitable inflammatory environment to boost antitumor immunity. IFN-γ is a typical Th1 cytokine and its presence reflects the occurrence of antitumor immune response. In this context, it is noteworthy that IFN-γ is a potent inducer of Mϕ transition to R-Mϕ, while Th2 cytokine IL-4 induces a transition to O-Mϕ.[15] It is interesting to note that GM-CSF tends to induce R-Mϕ, while M-CSF induces O-Mϕ (Hamuro, unpublished data).

Although further consideration is required of the plasma biomarkers for various tumors presented in this review before they can be used clinically, published results indicate that several cytokine/chemokine /MMP from frozen plasma samples can reliably be used as alternative biomarkers.

CONCLUSION

In this review, we discussed the notion that patients with poor antitumor immune responses have lower survival rates, and have introduced a method of ascertaining antitumor immune status using peripheral blood specimens before surgery. Predictions using peripheral blood markers will be helpful in determining therapeutic options for patients with solid tumors and more importantly allow for the optimal administration of presurgery patient-oriented treatments. These predictions are based on the idea that GSH status in Mo affect the clinical outcome of solid tumor bearing patients, and that knowing Mo status using easily obtainable plasma samples, will improve the prognosis of patients who may require treatment to boost their immune response prior to surgery. To induce this immune response we believe that BRM such as LTN and OK-432 are suitable options.

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