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Acute myeloid leukemia (AML) still has a rather poor prognosis, which is mainly ascribed to relapse in the majority of patients despite achievement of complete remission (CR). To prevent relapse, minimal residual disease (MRD) remaining in CR has to be completely eradicated. The graft-versus-leukemia effect induced by allogeneic hematopoietic stem cell transplantation is the most powerful mechanism for eradication of MRD, thus strongly suggesting the therapeutic potential of immunotherapy.

The Wilms' tumor gene *WT1* is highly expressed in various types of malignancies, and the WT1 protein is one of the most promising leukemia and tumor-associated antigens.^{1–6} Based on findings of pre-clinical studies, we performed a phase I clinical study of WT1 peptide immunotherapy for patients with AML, myelodysplastic syndromes, breast or lung cancer, and were able to confirm the safety and clinical efficacy of this therapy.⁷ In this report, we describe the long-term outcome for AML patients enrolled in this study.

The major inclusion criteria for vaccination were that patients had HLA-A*2402 and that *WT1* mRNA levels, which reflect the amount of MRD,^{8,9} were $>1 \times 10^3$ or $>1 \times 10^2$ copies/ μ g RNA in bone marrow (BM) or peripheral blood (PB), respectively. A natural WT1 peptide (amino acid 235–243 CMTWNQMNL) or the modified WT1 peptide (CYTWNQMNL)¹⁰ emulsified with Montanide ISA51

adjuvant (Freund Corporation, Tokyo, Japan) was i.d. injected, and the vaccination was performed three times at 2-week intervals.

The terms of both clinical and molecular responses were clearly defined as respectively, a decrease in leukemic blast cells in BM and a decrease in WT1 mRNA levels in BM and/or PB. Molecular CR was defined as undetectable levels of WT1 mRNA in PB (<50 copies/ μ g RNA). Molecular relapse was defined as abnormal levels of WT1 mRNA (≥ 50 copies/ μ g RNA in PB and/or ≥ 1000 copies/ μ g RNA in BM). Vaccination was extended for patients who showed clinical response or no progression within 2 weeks from the end of three WT1 vaccinations. As for immunological monitoring, WT1 tetramer⁺ CD8⁺ T cells and IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells were analyzed. Of the eight AML patients with hematological CR (leukemic blast $<5\%$) but with MRD who were WT1-vaccinated, five patients who showed a decrease in *WT1* mRNA levels (molecular response) were repeatedly WT1-vaccinated, but two relapsed later. The remaining three patients received WT1 vaccine for ≥ 8 years and have survived in molecular CR for ≥ 8 years without significant adverse effects except for local erythema at the vaccine injection sites (Table 1).

CASE REPORTS**Case 1**

A 32-year-old male was diagnosed as AML (M4Eo) in January 1997 and achieved hematological CR by induction chemotherapy. After

Table 1. Summary of characteristics and clinical courses of long-term survivors

Case no.	Age/sex	FAB	Karyotype	Prior to WT1 vaccination			WT1 peptide	No. of vaccination	Vaccination periods	Follow-up periods	Current ^a status	Adverse effects
				Status	MRD							
					WT1 mRNA	Other markers						
1	32/M	AML (M4Eo)	Normal	MR	(+)	(-)	Natural 3 mg ↓ modified 3 mg	110	8 yr.	8 yr. 5 m.	CR	Local skin eruption
2	49/F	AML (M1)	Normal	MR	(+)	CD34+ CD56+	Modified 3 mg	113+	8 yr. 3 m.	8 yr. 3 m.	CR	Local skin eruption
3	60/F	AML (Sec)	t(6;11) (q21;q23)	MR	(+)	(-)	Modified 3 mg	157	7 yr. 10 m.	8 yr. 3 m.	CR	Local skin eruption

Abbreviations: AML, acute myeloid leukemia; CR, complete remission; FAB, French American British classification; MR, molecular relapse; MRD, minimal residual disease; Sec, secondary AML. ^aThe present data of white blood cell, hemoglobin and platelet (X104): case 1: 5500, 15.1 and 10.2; case 2: 7200, 15.4 and 14.7; case 3: 4740, 13.0 and 28.0.

recurrence in November 1999, he received chemotherapy again and achieved the second hematological CR. BM examination in February 2003 showed hematological CR, but had MRD of 3.0×10^3 copies of *WT1* mRNA/ μ g RNA (Figure 1a). As his disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of natural WT1 peptide was begun in February 2003. *WT1* mRNA levels in BM decreased to normal levels during administration of three WT1 vaccinations (time point (1)) but increased to abnormal levels after discontinuation of the vaccination (time point (2)). WT1 vaccination was therefore started again, and again *WT1* mRNA levels decreased to normal levels (time point (3)). These findings demonstrated that WT1 vaccination was effective for eradication of MRD. As *WT1* mRNA levels increased after the second discontinuation of WT1 vaccination, the vaccination was initiated once more in June 2003 (time point (4)). The vaccine was changed from a natural to a modified WT1 peptide in September 2004 (Year 1.7) because a phase I/II clinical study of WT1 immunotherapy using a modified WT1 peptide for solid tumors showed the evidence of the modified WT1 peptides' effectiveness. *WT1* mRNA levels decreased to normal levels (<50 copies/ μ gRNA in PB) ~4 years after the start of WT1 vaccination and have been maintained at normal levels until now, although *WT1* mRNA levels remained at abnormal levels during the first 4 years. The WT1 vaccination was continued until February 2011, but stopped at that time point owing to patient's request with our agreement.

Case 2

A 49-year-old woman was diagnosed as AML (M1) in June 2000. Leukemic blast cells were characterized by CD34⁺CD56⁺. The patient achieved molecular CR (*WT1* mRNA: <50 copies/ μ g RNA in PB) after the second induction chemotherapy followed by a multicourse consolidation and maintenance therapy. After molecular CR had been maintained for 2.5 years, however, BM examination showed molecular relapse with 4.5×10^3 copies of *WT1* mRNA/ μ g RNA in February 2003, and fluorescence-activated cell sorting analysis detected a low frequency of CD34⁺CD56⁺ blast cells in BM. The patient refused allogeneic hematopoietic stem cell transplantation and expressed a desire to be treated with WT1 peptide vaccination. The vaccination with 3 mg of modified WT1 peptide was begun in March 2003 (Figure 1b). *WT1* mRNA levels in PB decreased to normal during three WT1 vaccinations (time point (1)). Subsequently, *WT1* mRNA

levels increased or decreased in parallel with the discontinuation or restart of WT1 vaccination, respectively. *WT1* mRNA levels remained abnormal from year 1.5 until year 4.0, but then became normalized and have remained normal until now, while CD34⁺CD56⁺ blast cells have become undetectable.

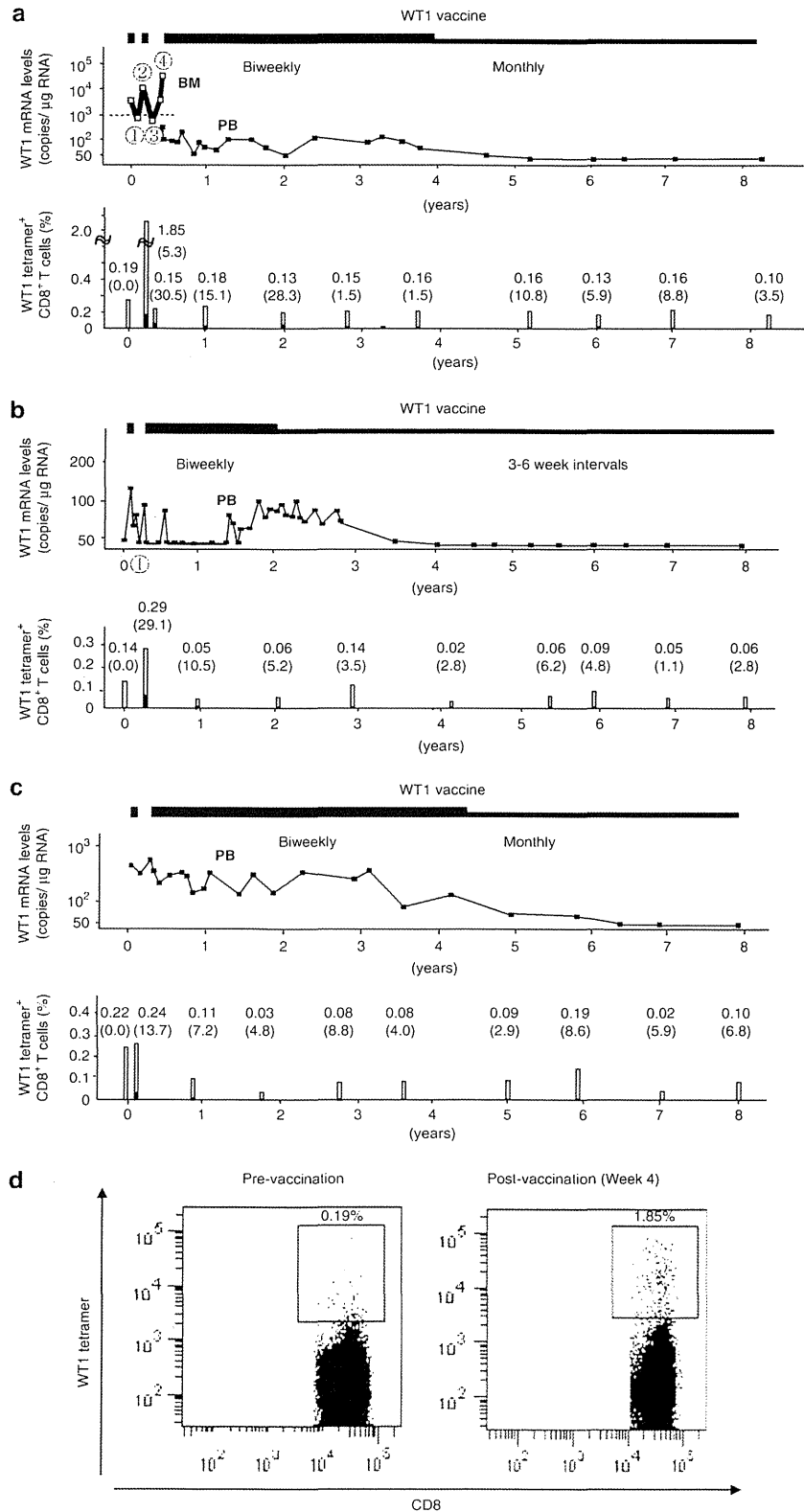
Case 3

A 60-year-old female was diagnosed as AML (M3) with t(15;17) in July 2000 and hematological CR was achieved by induction chemotherapy. The disease recurred in September 2002 in the form of secondary AML with t(6;11)(q21;q23). Induction chemotherapy was administered again and the patient achieved a second CR. BM examination in March 2003 showed hematological CR, but molecular relapse with 4.0×10^3 copies of *WT1* mRNA/ μ g RNA in BM (Figure 1c). As her disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of modified WT1 peptide was initiated in April 2004. *WT1* mRNA levels in PB gradually decreased from 880 copies/ μ g RNA before WT1 vaccination and reached undetectable levels (<50 copies/ μ g RNA) 6.5 years after the vaccination. Cells with t(6;11)(q21;q23) had also remained undetectable. WT1 vaccination was continued until February 2011, but stopped at that time point owing to the patient's request with our agreement.

In all three cases reported here, frequencies of WT1 tetramer⁺CD8⁺T cells that were already high before WT1 vaccination compared with those in healthy donors (HDs) transiently increased further after the vaccination and then decreased, but remained at higher levels during the vaccination. It should be noted that IFN- γ ⁺WT1 tetramer⁺CD8⁺T cells, which are considered to be functionally active, were not detected before WT1 vaccination, but appeared after the vaccination had been initiated and stayed at clearly detectable levels during the vaccination. In case 3, the increase to 0.19% in the frequency of WT1 tetramer⁺CD8⁺T cells including higher frequencies of IFN- γ ⁺CD8⁺T cells in PB around year 6 paralleled the decrease in *WT1* mRNA to undetectable levels.

DISCUSSION

In the three AML who have been treated with WT1 peptide vaccine for ≥ 8 years, *WT1* mRNA levels that reflect the amount of MRD have remained at undetectable levels, suggesting that the vaccination may have resulted in a cure for these patients. On the basis of *WT1* mRNA levels, MRD before WT1 vaccination was



estimated at $\sim 10^{10}$ cells, which may be equivalent to $\sim 1.0\%$ leukemic blast cells in BM. The presence of such high levels of MRD in these patients suggested that clinical relapse was impending.^{8,9} Conventionally, cure-oriented therapy for such

patients comprises only allogeneic hematopoietic stem cell transplantation. As shown by these cases, WT1 immunotherapy may be the first choice of treatment for AML patients who have MRD after induction chemotherapy but cannot tolerate allogeneic

Figure 1. Clinical courses and immunomonitoring. (a) Case 1. (b) Case 2. (c) Case 3. Top: WT1 mRNA levels (copies of WT1 mRNA/ μ g RNA) in BM (white squares) and PB (black squares). Horizontal dotted lines represent upper limits of normal ranges of WT1 mRNA levels in BM. Bottom: Frequencies of WT1 tetramer⁺ CD8⁺ T cells in CD8⁺ T cells in PB (white bars) and frequencies of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells in the WT1 tetramer⁺ CD8⁺ T cells (black bars). Numbers represent the percentages of WT1 tetramer⁺ CD8⁺ T cells and numbers in parentheses show the percentages of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells in WT1 tetramer⁺ CD8⁺ T cells. (d) Representative fluorescence-activated cell sorting data is shown for case 1. WT1 peptide (9-mer natural 235–243 peptide)/ HLA-A*2402 tetramer assay was performed as follows: frozen peripheral blood mononuclear cells (PBMCs) were thawed and stained with phycoerythrin (PE)-conjugated WT1 tetramer, subsequently with allophycocyanin (APC)-anti-CD3 antibody, fluorescein isothiocyanate (FITC)-anti-CD4, -14, -16, -19 and -56 antibodies, and APC-Cy7-anti CD8 antibody, followed by analysis with fluorescence-activated cell sorting instrument. WT1 tetramer-, CD3-, and CD8-positive, and CD4-14-, 16-, 19-, and 56-negative fractions were determined as WT1 tetramer⁺ CD8⁺ T cells. To quantify IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells, PBMCs were stimulated with WT1 peptide or irrelevant modified extracellular signal-regulated kinase (mERK) peptide, and then WT1 tetramer assay was performed for the PBMCs. Frequencies of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells were calculated as (number of IFN- γ ⁺ WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in WT1 peptide-stimulated PBMCs)–(number of IFN- γ ⁺ WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in mERK peptide-stimulated PBMCs)/number of WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in non-peptide-stimulated PBMCs.

hematopoietic stem cell transplantation. The findings of this study suggest that WT1 peptide vaccination merits consideration as an alternative cure-oriented therapy for AML patients showing hematological CR but also MRD, and are at a high risk of clinical relapse.

Frequency of WT1 tetramer⁺ CD8⁺ T cells in the three AML patients was already elevated before WT1 vaccination compared with that usually observed in HDs (<0.08%). These findings indicate that the immune system of these AML patients responded to the endogenous WT1 protein derived from leukemic cells. IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells, an activated type of the WT1 tetramer⁺ CD8⁺ T cells, were not detected before WT1 vaccination, but appeared after WT1 vaccination in all three patients and remained at clearly detectable levels throughout the vaccination. The appearance and continuing presence of these IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells are likely contributors to the eradication of MRD, thus resulting in molecular CR. Furthermore, it was shown that, upon stimulation with WT1 peptide, a fraction of WT1 tetramer⁺ CD8⁺ T cells expressed a CD107 antigen that appeared on surface of CTLs in direct association with secretion of cytotoxic granules that contained perforin and granzyme¹¹ (data not shown), strongly indicating the potential cytotoxicity of WT1 tetramer⁺ CD8⁺ T cells and the relevance of their frequency analysis during the clinical courses.

In conclusion, long-term WT1 peptide vaccination for ≥ 8 years was found to be safe and effective for the eradication of MRD and WT1 immunotherapy merits consideration as a promising cure-oriented therapy for AML patients with MRD who are at high risk of relapse.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A 32-year-old male was diagnosed as AML (M4Eo) in January 1997 and achieved hematological CR by induction chemotherapy. After

Table 1. Summary of characteristics and clinical courses of long-term survivors

Case no.	Age/sex	FAB	Karyotype	Prior to WT1 vaccination			WT1 peptide	No. of vaccination	Vaccination periods	Follow-up periods	Current ^a status	Adverse effects
				Status	MRD							
					WT1 mRNA	Other markers						
1	32/M	AML (M4Eo)	Normal	MR	(+)	(-)	Natural 3 mg ↓ modified 3 mg	110	8 yr.	8 yr. 5 m.	CR	Local skin eruption
2	49/F	AML (M1)	Normal	MR	(+)	CD34+ CD56+	Modified 3 mg	113+	8 yr. 3 m.	8 yr. 3 m.	CR	Local skin eruption
3	60/F	AML (Sec)	t(6;11) (q21;q23)	MR	(+)	(-)	Modified 3 mg	157	7 yr. 10 m.	8 yr. 3 m.	CR	Local skin eruption

Abbreviations: AML, acute myeloid leukemia; CR, complete remission; FAB, French American British classification; MR, molecular relapse; MRD, minimal residual disease; Sec, secondary AML. ^aThe present data of white blood cell, hemoglobin and platelet (X104) : case 1: 5500, 15.1 and 10.2; case 2: 7200, 15.4 and 14.7; case 3: 4740, 13.0 and 28.0.

recurrence in November 1999, he received chemotherapy again and achieved the second hematological CR. BM examination in February 2003 showed hematological CR, but had MRD of 3.0×10^3 copies of *WT1* mRNA/ μ g RNA (Figure 1a). As his disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of natural WT1 peptide was begun in February 2003. *WT1* mRNA levels in BM decreased to normal levels during administration of three WT1 vaccinations (time point (1)) but increased to abnormal levels after discontinuation of the vaccination (time point (2)). WT1 vaccination was therefore started again, and again *WT1* mRNA levels decreased to normal levels (time point (3)). These findings demonstrated that WT1 vaccination was effective for eradication of MRD. As *WT1* mRNA levels increased after the second discontinuation of WT1 vaccination, the vaccination was initiated once more in June 2003 (time point (4)). The vaccine was changed from a natural to a modified WT1 peptide in September 2004 (Year 1.7) because a phase I/II clinical study of WT1 immunotherapy using a modified WT1 peptide for solid tumors showed the evidence of the modified WT1 peptides' effectiveness. *WT1* mRNA levels decreased to normal levels (<50 copies/ μ g RNA in PB) ~4 years after the start of WT1 vaccination and have been maintained at normal levels until now, although *WT1* mRNA levels remained at abnormal levels during the first 4 years. The WT1 vaccination was continued until February 2011, but stopped at that time point owing to patient's request with our agreement.

Case 2

A 49-year-old woman was diagnosed as AML (M1) in June 2000. Leukemic blast cells were characterized by CD34⁺CD56⁺. The patient achieved molecular CR (*WT1* mRNA: <50 copies/ μ g RNA in PB) after the second induction chemotherapy followed by a multicourse consolidation and maintenance therapy. After molecular CR had been maintained for 2.5 years, however, BM examination showed molecular relapse with 4.5×10^3 copies of *WT1* mRNA/ μ g RNA in February 2003, and fluorescence-activated cell sorting analysis detected a low frequency of CD34⁺CD56⁺ blast cells in BM. The patient refused allogeneic hematopoietic stem cell transplantation and expressed a desire to be treated with WT1 peptide vaccination. The vaccination with 3 mg of modified WT1 peptide was begun in March 2003 (Figure 1b). *WT1* mRNA levels in PB decreased to normal during three WT1 vaccinations (time point (1)). Subsequently, *WT1* mRNA

levels increased or decreased in parallel with the discontinuation or restart of WT1 vaccination, respectively. *WT1* mRNA levels remained abnormal from year 1.5 until year 4.0, but then became normalized and have remained normal until now, while CD34⁺CD56⁺ blast cells have become undetectable.

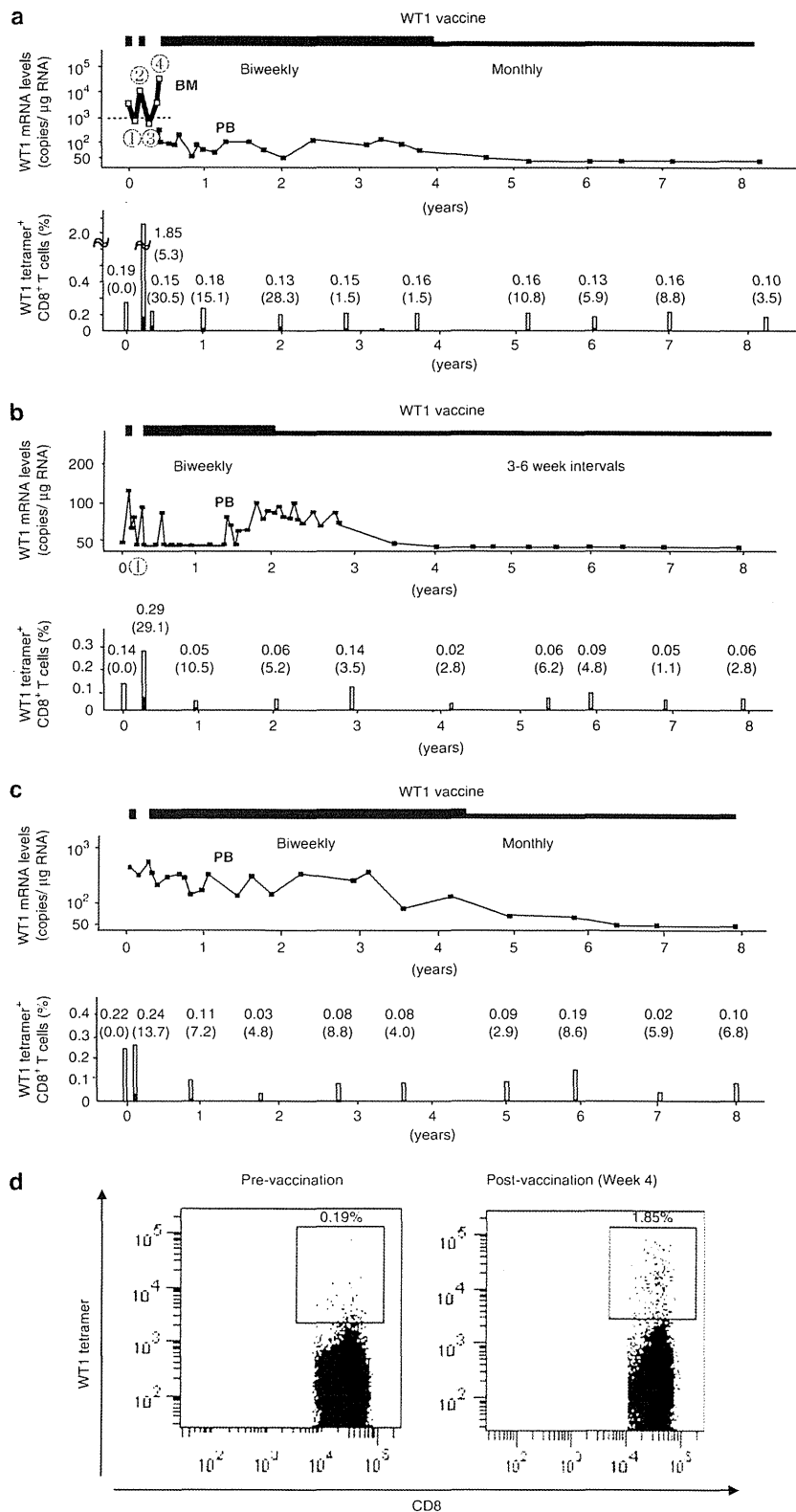
Case 3

A 60-year-old female was diagnosed as AML (M3) with t(15;17) in July 2000 and hematological CR was achieved by induction chemotherapy. The disease recurred in September 2002 in the form of secondary AML with t(6;11)(q21;q23). Induction chemotherapy was administered again and the patient achieved a second CR. BM examination in March 2003 showed hematological CR, but molecular relapse with 4.0×10^3 copies of *WT1* mRNA/ μ g RNA in BM (Figure 1c). As her disease was considered to be at a high risk of hematological relapse, WT1 vaccination with 3 mg of modified WT1 peptide was initiated in April 2004. *WT1* mRNA levels in PB gradually decreased from 880 copies/ μ g RNA before WT1 vaccination and reached undetectable levels (<50 copies/ μ g RNA) 6.5 years after the vaccination. Cells with t(6;11)(q21;q23) had also remained undetectable. WT1 vaccination was continued until February 2011, but stopped at that time point owing to the patient's request with our agreement.

In all three cases reported here, frequencies of WT1 tetramer⁺CD8⁺T cells that were already high before WT1 vaccination compared with those in healthy donors (HDs) transiently increased further after the vaccination and then decreased, but remained at higher levels during the vaccination. It should be noted that IFN- γ ⁺WT1 tetramer⁺CD8⁺T cells, which are considered to be functionally active, were not detected before WT1 vaccination, but appeared after the vaccination had been initiated and stayed at clearly detectable levels during the vaccination. In case 3, the increase to 0.19% in the frequency of WT1 tetramer⁺CD8⁺T cells including higher frequencies of IFN- γ ⁺CD8⁺T cells in PB around year 6 paralleled the decrease in *WT1* mRNA to undetectable levels.

DISCUSSION

In the three AML who have been treated with WT1 peptide vaccine for ≥ 8 years, *WT1* mRNA levels that reflect the amount of MRD have remained at undetectable levels, suggesting that the vaccination may have resulted in a cure for these patients. On the basis of *WT1* mRNA levels, MRD before WT1 vaccination was



estimated at $\sim 10^{10}$ cells, which may be equivalent to $\sim 1.0\%$ leukemic blast cells in BM. The presence of such high levels of MRD in these patients suggested that clinical relapse was impending.^{8,9} Conventionally, cure-oriented therapy for such

patients comprises only allogeneic hematopoietic stem cell transplantation. As shown by these cases, WT1 immunotherapy may be the first choice of treatment for AML patients who have MRD after induction chemotherapy but cannot tolerate allogeneic

Figure 1. Clinical courses and immunomonitoring. (a) Case 1. (b) Case 2. (c) Case 3. Top: WT1 mRNA levels (copies of WT1 mRNA/ μ g RNA) in BM (white squares) and PB (black squares). Horizontal dotted lines represent upper limits of normal ranges of WT1 mRNA levels in BM. Bottom: Frequencies of WT1 tetramer⁺ CD8⁺ T cells in CD8⁺ T cells in PB (white bars) and frequencies of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells in the WT1 tetramer⁺ CD8⁺ T cells (black bars). Numbers represent the percentages of WT1 tetramer⁺ CD8⁺ T cells and numbers in parentheses show the percentages of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells in WT1 tetramer⁺ CD8⁺ T cells. (d) Representative fluorescence-activated cell sorting data is shown for case 1. WT1 peptide (9-mer natural 235–243 peptide)/ HLA-A*2402 tetramer assay was performed as follows: frozen peripheral blood mononuclear cells (PBMCs) were thawed and stained with phycoerythrin (PE)-conjugated WT1 tetramer, subsequently with allophycocyanin (APC)-anti-CD3 antibody, fluorescein isothiocyanate (FITC)-anti-CD4, -14, -16, -19 and -56 antibodies, and APC-Cy7-anti CD8 antibody, followed by analysis with fluorescence-activated cell sorting instrument. WT1 tetramer-, CD3-, and CD8-positive, and CD4-14-, 16-, 19-, and 56-negative fractions were determined as WT1 tetramer⁺ CD8⁺ T cells. To quantify IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells, PBMCs were stimulated with WT1 peptide or irrelevant modified extracellular signal-regulated kinase (mERK) peptide, and then WT1 tetramer assay was performed for the PBMCs. Frequencies of IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells were calculated as (number of IFN- γ ⁺ WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in WT1 peptide-stimulated PBMCs)–(number of IFN- γ ⁺ WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in mERK peptide-stimulated PBMCs)/number of WT1 tetramer⁺ CD3⁺ CD8⁺ T cells in non-peptide-stimulated PBMCs.

hematopoietic stem cell transplantation. The findings of this study suggest that WT1 peptide vaccination merits consideration as an alternative cure-oriented therapy for AML patients showing hematological CR but also MRD, and are at a high risk of clinical relapse.

Frequency of WT1 tetramer⁺ CD8⁺ T cells in the three AML patients was already elevated before WT1 vaccination compared with that usually observed in HDs (<0.08%). These findings indicate that the immune system of these AML patients responded to the endogenous WT1 protein derived from leukemic cells. IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells, an activated type of the WT1 tetramer⁺ CD8⁺ T cells, were not detected before WT1 vaccination, but appeared after WT1 vaccination in all three patients and remained at clearly detectable levels throughout the vaccination. The appearance and continuing presence of these IFN- γ ⁺ WT1 tetramer⁺ CD8⁺ T cells are likely contributors to the eradication of MRD, thus resulting in molecular CR. Furthermore, it was shown that, upon stimulation with WT1 peptide, a fraction of WT1 tetramer⁺ CD8⁺ T cells expressed a CD107 antigen that appeared on surface of CTLs in direct association with secretion of cytotoxic granules that contained perforin and granzyme¹¹ (data not shown), strongly indicating the potential cytotoxicity of WT1 tetramer⁺ CD8⁺ T cells and the relevance of their frequency analysis during the clinical courses.

In conclusion, long-term WT1 peptide vaccination for ≥ 8 years was found to be safe and effective for the eradication of MRD and WT1 immunotherapy merits consideration as a promising cure-oriented therapy for AML patients with MRD who are at high risk of relapse.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CASE REPORT

WT1 peptide vaccine induces reduction in minimal residual disease in an Imatinib-treated CML patient

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Abstract

How to treat CML patients who are resistant to inhibitors of BCR-ABL tyrosine kinase such as Imatinib is a very important and urgent issue in clinical hematology. Here, we report a case of Imatinib-treated CML in which intradermally administered WT1 peptide vaccine elicited WT1-specific immune responses and the resultant reduction in the persistent residual disease in co-administration of Imatinib. BCR-ABL mRNA levels were being maintained under the detection limit for 8 months since week 77 of vaccination. No adverse effects except local erythema at the injection sites were observed. The tetramer assay revealed that the decrease in BCR-ABL mRNA levels was associated with the increase in frequency of WT1-specific cytotoxic T lymphocytes, notably effector-memory type of that, in the patient's peripheral blood. The case presented here indicates that WT1 peptide vaccine may become a safe and cure-oriented therapy for CML patients who have residual disease regardless of the treatment with Imatinib.

Key words WT1 peptide vaccine; CML; immunotherapy; Imatinib

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Accumulating evidence has shown that Imatinib does not eliminate leukemia cells in the most of chronic myelogenous leukemia (CML) patients despite its high effectiveness in the reduction in CML cells (1, 2). Here, we present a case of Imatinib-treated CML in which WT1-peptide vaccine elicited WT1-specific immune responses and the resultant reduction in persistent residual disease.

Case report

A 76-yr-old woman was diagnosed as having chronic-phase CML in April 2005. After treatment with Imatinib, she achieved a complete cytogenetic response (CCyR) in March 2006. However, major BCR-ABL mRNA was persistently detected in peripheral blood

(PB) for 12 months after the achievement of CCyR. She therefore desired to be treated with WT1 peptide vaccine in combination with Imatinib. She had HLA-A*2402 and met the inclusion criteria for the phase I/II clinical study of WT1 peptide-based immunotherapy combined with Imatinib for chronic myelogenous leukemia (#UMIN000003508, <http://www.umin.ac.jp/english/>). In addition to the continued administration of Imatinib, an intradermal injection of 1 mg of 9-mer modified WT1 peptide (p235-243:CYTWNQMNL) (3) emulsified with Montanide ISA51 adjuvant was begun on August 14, 2007, and repeated at 2-wk intervals in co-administration of Imatinib. The levels of major BCR-ABL mRNA in PB decreased to the detection limit at week 13. However, BCR-ABL mRNA levels gradually increased in associa-

tion with weakening of delayed-type hypersensitivity (DTH) to WT1 peptide and reached the maximum at week 38. As these results suggested the reduction in WT1-specific cytotoxic T lymphocyte (CTL) responses because of antigen-induced cell death of the CTLs, intervals of WT1 vaccination were extended from 2 to 3 or 4 wk. Following the extension, major BCR-ABL mRNA levels decreased again and were being maintained under the detection limit for 8 months since week 77 (Fig. 1). No adverse effects except local erythema at the injection sites were observed.

DTH reaction to WT1 peptide became positive after four WT1 vaccinations. The frequency of WT1-specific CD8⁺ T cells (WT1 tetramer⁺ CD8⁺ T cells) among CD8⁺ T cells in PB increased from 0.06% to 0.16% by the beginning of WT1 vaccination but declined to 0.07% in association with the weakening of DTH to WT1 peptide from 8 × 4 to 2 × 1 mm at week 35. However, the frequency of WT1-specific CD8⁺ T cells increased from 0.07% to 0.23% again following the extension of vaccination intervals. It should be noted that after the WT1 vaccination, the frequency of effector-memory type of WT1-specific CD8⁺ T cells, which were considered to be able to differentiate into effector cells and be mounted for cytotoxic activities upon antigen stimulation, markedly increased from 4.5% to 32.6% at week 5 and was kept in more than 30% of the WT1-specific CD8⁺ T cells (Fig. 1).

Discussion

Since stem/progenitor cells of CML cells that played an essential role in relapse and progression of the disease overexpressed the *WT1* gene (4, 5), they could be targeted by WT1-specific CTLs. Therefore, it is reasonable to think that WT1-specific CTLs induced by WT1 peptide vaccine eradicated the residual leukemogenic stem/progenitor cells and induced the reduction in BCR-ABL mRNA which reflected the minimal residual disease in this CML patient.

One important concern about TKIs is their possible mutagenic effects. In fact, although rare, emergence of abnormal Ph-negative clones after the treatment with Imatinib has been reported (6). These results indicate that TKI may have adverse effects on the incidence of secondary myelodysplastic syndrome (MDS) or leukemia (7). Therefore, combination of WT1 peptide vaccine with Imatinib could reduce dose of Imatinib and shorten treatment duration and might contribute to reduction in the risk of Imatinib-induced MDS/leukemia.

Taken together, the results presented here should indicate that WT1 peptide vaccine induced and/or enhanced WT1-specific immune responses, leading to induction of a clinical response. Therefore, the case presented here

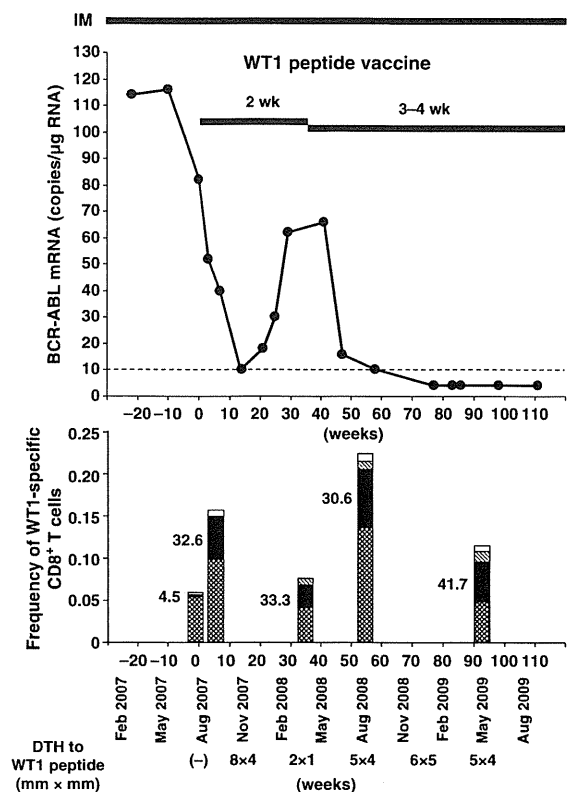


Figure 1 Clinical course of a CML patient who was vaccinated with WT1 peptide. Upper panel, reduction in minimal residual CML cells by WT1 peptide vaccine. WT1 peptide vaccine was intradermally administered initially at 2-wk and later 3–4-wk intervals. Minimal residual disease in the CML patient was detected by the measurement of major BCR-ABL mRNA in peripheral blood. Imatinib was daily administered at the dose of 200 mg because of her intolerance to Imatinib at higher doses. IM, Imatinib. Lower panel, WT1-specific immune responses. WT1-tetramer⁺ CD8⁺ T cells, which were negative for lineage markers such as CD4, CD14, CD16, CD19, and CD56, were considered to represent WT1-specific CD8⁺ T cells. The frequency of WT1-specific CD8⁺ T cells among the CD8⁺ T cells was measured by flowcytometry. The differentiation status of WT1 tetramer⁺ CD8⁺ T cells was analyzed based on CD45RA and CCR7 expression as previously described (8). □, Naive; ▨, central memory; ■, effector memory; ▩, effector. Numbers on left sides of columns show the percentages of E-M type among WT1-specific CD8⁺ T cells. Delayed-type hypersensitivity was measured 48 h after injection of WT1 peptide solution (10 μg/50 μL) without Montanide ISA51 adjuvant.

indicates that WT1 peptide vaccine may become a safe and cure-oriented therapy for CML patients who have residual disease regardless of the treatment with Imatinib. This conclusion should be confirmed by further studies with larger number of patients.

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Conflict of interest

The authors declare no conflict of interest.

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Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination

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Active immunization using tumor antigen-loaded dendritic cells holds promise for the adjuvant treatment of cancer to eradicate or control residual disease, but so far, most dendritic cell trials have been performed in end-stage cancer patients with high tumor loads. Here, in a phase I/II trial, we investigated the effect of autologous dendritic cell vaccination in 10 patients with acute myeloid leukemia (AML). The Wilms' tumor 1 protein (WT1), a nearly universal tumor antigen, was chosen as an immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics. Two patients in partial remission after chemotherapy were brought into complete remission after intradermal administration of full-length *WT1* mRNA-electroporated dendritic cells. In these two patients and three other patients who were in complete remission, the AML-associated tumor marker returned to normal after dendritic cell vaccination, compatible with the induction of molecular remission. Clinical responses were correlated with vaccine-associated increases in WT1-specific CD8⁺ T cell frequencies, as detected by peptide/HLA-A*0201 tetramer staining, and elevated levels of activated natural killer cells postvaccination. Furthermore, vaccinated patients showed increased levels of WT1-specific IFN- γ -producing CD8⁺ T cells and features of general immune activation. These data support the further development of vaccination with *WT1* mRNA-loaded dendritic cells as a postremission treatment to prevent full relapse in AML patients.

cancer vaccine | active specific immunotherapy | phase I clinical trial

Overall, acute myeloid leukemia (AML) has a bad prognosis; indeed, only 23.8% of patients are alive 5 y after diagnosis (1). One of the main reasons for this poor outlook is that the majority of patients will relapse, despite reaching complete remission with classical polychemotherapy (2–4). Relapse is caused by the persistence of malignant cells, also designated as minimal residual disease, during complete remission. Furthermore, most AML patients are more than 60 y old, and the prognosis in this age group is worse because of comorbidity and higher rates of chemotherapy resistance (5).

One treatment with demonstrable effect on residual disease, relapse rate, and survival is allogeneic hematopoietic stem-cell transplantation, in which donor T lymphocytes exert a graft versus leukemia effect. However, allogeneic hematopoietic stem-cell transplantation is still beset by morbidity and mortality issues that preclude its routine use in older AML patients (3, 4). Thus, except for allogeneic hematopoietic stem-cell transplantation in subsets of younger AML patients, there is no consensus as to which postremission treatment should be applied to prevent relapse (3–6).

Recently, immunotherapeutic strategies have been developed to raise autologous antileukemic immunity to control malignant dis-

ease. Vaccination with peptides derived from leukemia-associated antigens has led to clinical and immunological responses in AML (7). These antigens include proteinase 3, from which the immunogenic PR1 peptide is derived (8–10), Wilms' tumor 1 (WT1) protein (8, 10–12), and the receptor for hyaluronic acid-mediated motility (RHAMM/CD168) (13). Although promising, the peptide-vaccination approach has limitations; in particular, the peptides need to be tailored to the MHC antigens of the patient, and not all MHC-restricted peptides are yet defined. This problem can be circumvented by introducing the full-length antigen into professional antigen-presenting cells, such as dendritic cells (DC), which will present multiple epitopes to T lymphocytes in the context of autologous MHC. We have developed a highly efficient, transient, and clinically safe transfection procedure to introduce mRNA encoding an antigen into DC by electroporation (14), a process that results in efficient antigen presentation (15). DC have been intensively investigated as cellular adjuvants for therapeutic cancer vaccination, and since the first reported trial in lymphoma patients in 1996 (16), DC have shown an unsurpassed capacity to induce in vivo antitumor responses. However, the overall clinical response rate, if any, remains very low (17). This poor clinical outcome could be, at least in part, ascribed to the fact that most clinical DC trials to date have been performed in end-stage cancer patients, often with bulky tumor loads and a compromised immune system caused by intensive treatment schedules and/or advanced disease stage (18). These observations plead for a more careful clinical-trial design that selects patients with residual but substantially reduced disease in whom immunotherapeutic interventions could significantly improve the clinical outcome after standard chemotherapy treatment (19). In lymphoma, this strategy used with patient-specific idiosyncratic vaccination led to remarkable complete molecular responses (20). However, to our knowledge, such results have never been reported for DC-based cancer vaccines.

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Here, we present the results of a phase I/II vaccination trial using autologous monocyte-derived DC electroporated with *WT1* mRNA in AML patients who achieved complete or partial remission after polychemotherapy but remained at high risk of full relapse. *WT1* is overexpressed in the vast majority of AML cases (21–25). Furthermore, there is evidence that it plays an important role in the malignant phenotype of AML (24, 26–29). In an immunodeficient mouse model, there is a selective elimination of leukemic stem cells, but not normal human progenitors, and leukemia cells by *WT1*-specific cytotoxic CD8⁺ T cells (30–32). Of note, in a prioritization study carried out by the National Cancer Institute, *WT1* was selected from 75 defined tumor antigens to rank as the most promising cancer vaccine target (33).

The expression of *WT1* mRNA in bone marrow, or preferably, in peripheral blood, has been shown to be a relevant tumor marker in AML (25, 34–38). Especially after treatment, it has a high positive-predictive value as a molecular residual-disease marker (i.e., *WT1* mRNA expression levels above background in peripheral blood always herald clinical relapse) (25, 36, 37, 39). Moreover, failure to reduce *WT1* transcripts below the threshold limits after chemotherapy invariably predicts relapse in patients with complete remission, which enables the early prediction of treatment outcome and the distinction of patients with continuous complete remission from those with only apparent complete remission (25, 39).

In this study, we show the immunogenic and antileukemic activity of a *WT1*-targeted DC vaccine in AML patients, evidenced by the conversion of partial to complete remission and the induction of molecular remission. Importantly, we found *WT1*-specific and non-specific immunological correlates of these clinical responses.

Results

Clinical Results. The clinical details of the 10 AML patients recruited into this study are summarized in Table S1. Successful vaccine production was obtained in all patients from a single apheresis procedure (10–15 L), and DC vaccination was well-tolerated. In all patients, there was local erythema and induration at the site of injection, starting from the second vaccination. Patient with unique patient number (UPN)09 reported pain at the level of the draining axillary lymph nodes after DC vaccination. In patient UPN016, the platelet count dropped after the first DC injection and normalized 5 wk after the fourth vaccination (Fig. 1); she also experienced a mild flare-up of a preexisting inflammation of the Achilles and foot tendons, which started around the period of the fourth DC vaccination.

The most striking and demonstrable clinical effects were observed in patients UPN08 and UPN16, who were both in partial remission after chemotherapy and reached complete remission after DC vaccination. Before DC vaccination, these two patients were refractory to chemotherapy, and AML disease was not controlled according to classical morphological criteria [i.e., the percentage of myeloblasts in the bone marrow was increased above normal and was higher after the last (consolidation) chemotherapy than after the first (induction) chemotherapy]. After DC vaccination, the myeloblast percentage decreased to normal, from 6% to 1% in UPN08 (Fig. 1A) and from 9% to 0.3% in UPN16 (Fig. 1B). This was confirmed by flow-cytometric and histological examinations of the bone marrow. Concomitant with these changes, there was normalization of elevated *WT1* mRNA levels in peripheral blood after DC vaccination (Fig. 1). Patient UPN16 has relapsed in the bone marrow 9 mo after the start of DC vaccination, and this relapse was preceded by an increase in *WT1* mRNA expression levels above normal (Fig. 1B).

The return to normal values of the *WT1* tumor marker not only confirmed the antileukemic effect of DC vaccination in patients in partial remission, but it also revealed efficacy in some patients in complete remission (UPN01 and UPN06). In these latter subjects, *WT1* mRNA expression levels also illustrated the dynamics of minimal residual disease and the temporary nature

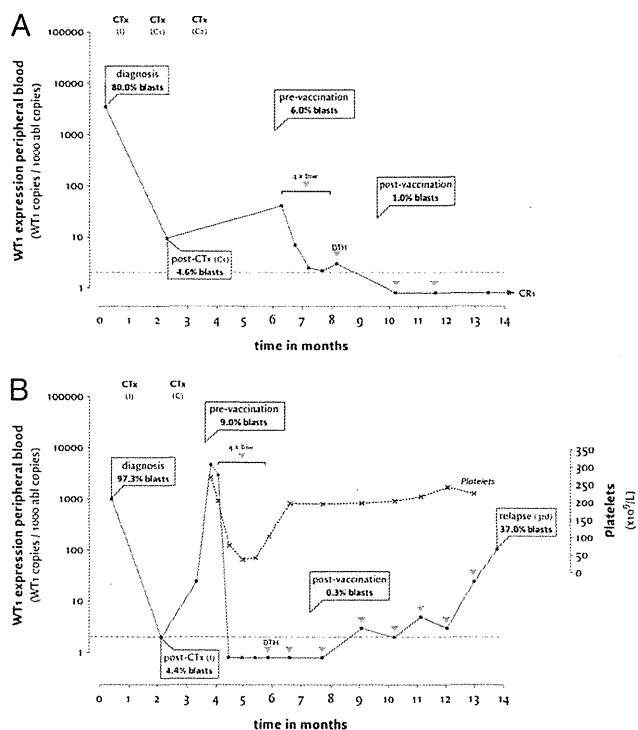


Fig. 1. Induction of complete remission by DC vaccination in patients UPN08 (A) and UPN16 (B). The gray-striped bars indicate the periods of chemotherapy (CTx) administration with subsequent hematological recovery from bone-marrow aplasia (I, induction chemotherapy; C, consolidation chemotherapy; C1, first cycle; C2, second cycle). A detailed description of the administered chemotherapeutic regimens is provided in Table S1. The brown arrowheads indicate the time points of DC immunization; the first cycle consisted of four biweekly (biw) injections (4 × biw) followed by DTH immunomonitoring testing. Both patients achieved a complete remission after four biweekly DC vaccinations, as evidenced by normalization of the myeloblast percentage in the bone marrow (inserts) and *WT1* mRNA expression levels (blue line). The horizontal dashed line represents the upper normal limit of *WT1* mRNA expression in peripheral blood. In patient UPN16, DC therapy was accompanied by a transient thrombocytopenia (dotted green line). Note the refractoriness to chemotherapy with abnormally increased bone-marrow blast cell percentage and *WT1* expression after the last chemotherapy course before DC vaccination. Patient UPN16 eventually relapsed in the bone marrow, and this relapse was preceded by molecular relapse as indicated by the loss of control of *WT1* expression levels.

of DC vaccine-induced control. After normalization of *WT1* mRNA expression associated with the initial round of DC vaccinations, this tumor marker increased on different occasions, compatible with molecular relapse. This was reversed by additional rounds of DC vaccination, which were administered usually on a bimonthly basis (Fig. 2). Patient UPN01 relapsed almost 4 y after starting DC vaccination, and elevated levels of *WT1* mRNA in peripheral blood were observed 3 mo before relapse. Thus, as in patient UPN16, molecular relapse preceded morphological and clinical relapse.

As of May 2010, three patients are in continuous complete remission with a normal peripheral blood picture. Of those three patients, two had increased *WT1* mRNA levels in blood that normalized post-DC vaccination; *WT1* expression normalized after the second DC vaccination in patient UPN08 (Fig. 1A) and after an additional fifth DC vaccine administered 2 mo after the initial cycle of four biweekly DC vaccinations (UPN06) (Fig. 2). In the third patient (UPN10), we observed a normalization of bone-marrow *WT1* mRNA expression from an increased value before DC vaccination to background levels postvaccination. This normalization

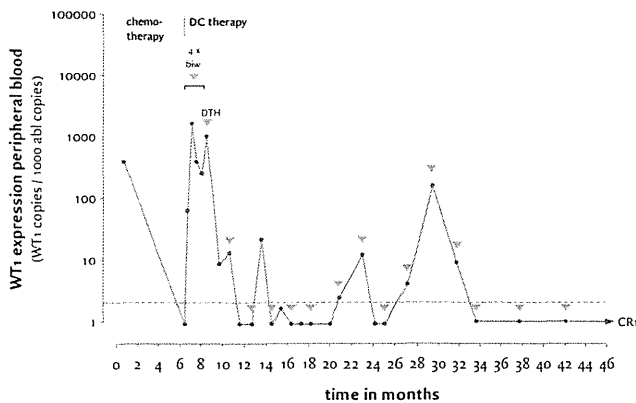


Fig. 2. Longitudinal control of AML minimal residual disease with repetitive DC vaccinations in patient UPN06. The gray-striped bar indicates the time period for induction and consolidation chemotherapy (CTx; details in Table S1) and subsequent recovery from bone-marrow aplasia. The brown arrowheads indicate the time points of DC vaccination; the first cycle consisted of four biweekly injections ($4 \times \text{biw}$) followed by DTH immunomonitoring. The kinetics of *WT1* mRNA expression levels in peripheral blood are represented by the blue line, showing a normalization below the background threshold (horizontal dashed line) after chemotherapy and a first molecular relapse that was reversed with five successive DC vaccinations. Increases in *WT1* mRNA expression were observed on several occasions and were consistently controlled by maintenance DC vaccine administrations on a bimonthly basis. This graph is also representative of similar observations in patient UPN01, with the difference that patient UPN01 relapsed molecularly (i.e., loss of control of *WT1* mRNA expression levels) and subsequently, morphologically in the bone marrow.

of the *WT1* mRNA tumor marker seen after DC vaccination is indicative of molecular remission. In the two other patients who responded to DC vaccination but eventually relapsed, *WT1* mRNA expression in blood initially normalized after the first DC vaccination (UPN16) (Fig. 1B) and after the second DC vaccination (UPN01). As of now, 5 of 10 patients can be considered as clinical responders (UPN01, UPN06, UPN08, UPN10, and UPN16) and 3 of them as long-term responders (UPN01, UPN06, and UPN08), based on a complete remission status lasting for at least 3 y. Seven patients died, six because of relapse of AML, and the seventh had a progressively deteriorating clinical condition, presumably caused by adenocarcinoma of the lung (UPN07). In these seven patients, *WT1* mRNA expression either did not normalize postvaccination in blood (UPN03, UPN07, and UPN09) or bone marrow (UPN02 and UPN05) or returned to pathologically increased values after initial normalization (UPN01 and UPN16).

Immunomonitoring. Immunomonitoring was performed on peripheral blood mononuclear cells (PBMC) and plasma samples obtained before and after DC vaccination. Immunophenotypic analyses revealed no significant changes in the circulating frequencies or

absolute numbers of lymphocyte subsets [CD4^+ and CD8^+ T cells, B cells, and natural killer (NK) cells]. Notably, DC vaccination did not affect the relative frequencies of regulatory CD4^+ T (Treg) cell subsets, including naturally occurring $\text{CD25}^+\text{FoxP3}^+$ Treg and induced IL-10^+ and/or $\text{TGF-}\beta^+$ Treg. Furthermore, the relative frequencies of naive ($\text{CD45RA}^+\text{CD62L}^+$), terminally differentiated effector ($\text{CD45RA}^+\text{CD62L}^-$), effector memory ($\text{CD45RA}^-\text{CD62L}^-$), and central memory ($\text{CD45RA}^-\text{CD62L}^+$) subsets within the $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ T cell compartments remained unchanged. However, a significant increase in circulating levels of plasma IL-2 and activated $\text{HLA-DR}^+\text{CD4}^+$ T cells was observed postvaccination, irrespective of clinical response (Fig. S1). More importantly, we found a significant correlation ($P = 0.01$) between clinical responses and the presence of high numbers of activated NK cells postvaccination (i.e., more than 40% HLA-DR^+ cells within the total NK cell population in four of five clinical responders and zero of five nonresponders). Furthermore, the level of HLA-DR^+ NK cells in all evaluable patients postvaccination was significantly higher compared with healthy volunteers [$32.25 \pm 16.8\%$ vs. $19.9 \pm 10.9\%$, respectively (mean \pm SD; $n = 10$); $P = 0.04$], whereas the prevaccination levels were not significantly increased [$26.8 \pm 17.7\%$ vs. $19.9 \pm 10.9\%$ (mean \pm SD; $n = 10$); $P = 0.25$].

WT1-specific immune responses were evaluated using anti-*WT1* antibody analysis, *WT1* peptide/HLA-A*0201 (pHLA-A*0201) tetramer staining, and functional intracellular cytokine assays. Analysis of humoral anti-*WT1* responses showed relatively low, but not significantly different, concentrations of *WT1* antibodies in the plasma before and after vaccination. Subclass analysis of *WT1* antibodies showed a predominant T helper 1-associated IgG2 subtype.

Because patient accrual in our trial was independent of HLA haplotype caused by the polypeptide full-length antigen strategy, only five patients (UPN01, UPN05, UPN08, UPN09, and UPN16) were evaluable by pHLA-A*0201 tetramer analysis. Four pHLA-A*0201 tetramers specific for different HLA-A*0201-restricted *WT1* epitopes were used: WT1_{37-45} , $\text{WT1}_{126-134}$, $\text{WT1}_{187-195}$, and $\text{WT1}_{235-243}$. Increased (>1.5 -fold) frequencies of *WT1*-specific tetramer $^+\text{CD8}^+$ T cells (11) were observed in UPN01 and UPN08 compared with UPN05, UPN09, and UPN16 (Table 1). A significant positive correlation ($P = 0.025$) was found between long-term responders (UPN01 and UPN08) and an increase in *WT1*-specific tetramer $^+\text{CD8}^+$ T cells. Interestingly, this association was observed for more than one of the epitopes studied (i.e., $\text{WT1}_{187-195}$ and $\text{WT1}_{235-243}$ in UPN01 and all four epitopes examined in UPN08), suggesting a clinically relevant polypeptide-specific T cell response (Table 1). In patient UPN16, who responded to DC vaccination acutely but not in the long term, there was no increase postvaccination in the frequency of *WT1*-specific CD8^+ T cells, but the frequency of HLA-DR^+ NK cells nearly doubled compared with prevaccination levels (from 29% to 57%); this observation suggests that the short-term antileukemic effect seen in patient UPN16 was mediated, at least in part, by NK cells rather than by CD8^+ T cells.

Table 1. Overview of *WT1*-specific tetramer $^+\text{CD8}^+$ T cell frequencies pre- and post-DC vaccination in the peripheral blood of HLA-A*0201 $^+$ AML patients

	Percent WT1_{37-45}		Percent $\text{WT1}_{126-134}$		Percent $\text{WT1}_{187-195}$		Percent $\text{WT1}_{235-243}$	
	Pre-DC	Post-DC	Pre-DC	Post-DC	Pre-DC	Post-DC	Pre-DC	Post-DC
UPN01*	0.016	0.002	0.006	0.006	0.006	0.011	0.004	0.009
UPN05	ND	ND	0.215	0.157	0.174	0.064	ND	ND
UPN08*	0.015	0.097	0.017	0.187	0.010	0.086	0.026	0.065
UPN09	0.078	0.003	0.102	0.002	0.036	0.004	0.036	0.005
UPN16	0.212	0.040	0.204	0.007	0.253	0.009	0.029	0.034

ND, not determined.

*Long-term responders.

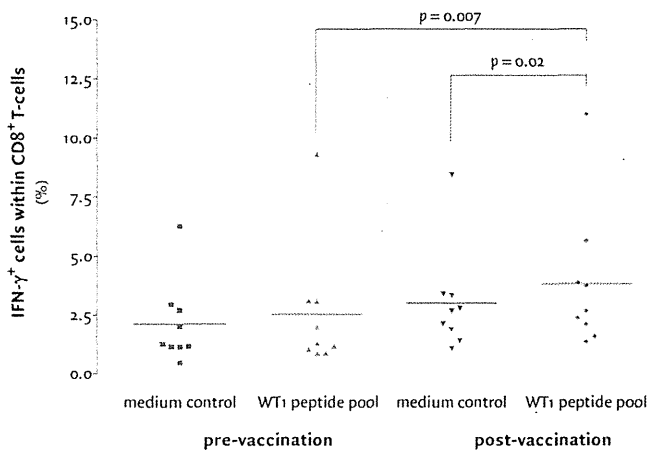


Fig. 3. Increase in WT1-specific IFN- γ -producing CD8⁺ T cells postvaccination. PBMC were restimulated using *WT1* mRNA-electroporated mature DC for 1 wk. After 7 d, cultured PBMC were rechallenged using a WT1 peptide pool and assayed for intracellular IFN- γ production. Rechallenge with medium served as the negative control in all cases. Intracellular cytokine staining showed significantly higher percentages of IFN- γ -producing CD8⁺ T cells postvaccination compared with antigen stimulation of PBMC obtained prevaccination ($P = 0.007$) and compared with a medium control ($P = 0.02$; $n = 9$; insufficient cell numbers were available from patient UPN05 for culture and subsequent analysis).

Detection of functional WT1-specific T cells was performed using a 1-wk in vitro antigen rechallenge protocol followed by intracellular cytokine staining. This approach revealed a significant increase in WT1-specific IFN- γ -producing CD8⁺ T cells postvaccination (Fig. 3) compared with both prevaccination and medium controls. However, there was no correlation between antileukemic clinical responses and the frequency of WT1-specific IFN- γ ⁺CD8⁺ T cells. Moreover, by assaying simultaneously for CD107a mobilization and intracellular IL-2, IFN- γ , TNF- α , and IL-10 production on a single-cell level after in vitro restimulation with *WT1* mRNA-electroporated or WT1 protein-loaded DC, polyfunctional WT1-specific T cells were not identified in any substantial numbers. Keyhole limpet hemocyanin (KLH)-specific T cell responses were readily detected in a 3-d in vitro restimulation assay, revealing a partially T helper 2 (Th2)-skewed cytokine profile characterized by secretion of IL-5, IL-10, and TNF- β .

In addition to these in vitro T cell responses, all patients exhibited in vivo delayed-type hypersensitivity (DTH) skin reactions (indurations of ≥ 2 mm) after four vaccinations to the vaccine (i.e., KLH-exposed *WT1* mRNA-electroporated DC) and to KLH separately as well as to nontransfected or *WT1* mRNA-transfected DC not exposed to KLH.

Discussion

In this phase I/II DC vaccination study, we have shown that *WT1* mRNA-electroporated autologous DC are immunogenic and that they induce a measurable antileukemic effect in AML patients treated with polychemotherapy but at high risk of full relapse. DC vaccination was associated with the achievement of molecular remission in 5 of 10 patients who exhibited elevated *WT1* mRNA expression levels before therapy. Moreover, in two of these patients, partial remission with demonstrable AML disease was converted into complete remission by the four biweekly DC vaccinations. Three of these five patients, including patient UPN08 who was in partial remission before vaccination, are currently alive and well with normal blood counts and normal blood *WT1* mRNA levels. It should be noted that all of the five responding patients were expected to experience a full-blown relapse based on partial

remission status indicating morphologically demonstrable AML disease (3) and/or increased *WT1* mRNA levels after chemotherapy (25, 36, 37, 39). Of note, two of five responding patients eventually relapsed. These relapses were preceded by loss of control of *WT1* mRNA expression levels, confirming the value of this parameter as a predictive tumor marker for AML relapse. Loss of clinical and molecular response has also been described in WT1 peptide-vaccination studies (10–12).

The close temporal relationship between DC vaccination and the antileukemic effect is a strong argument for a causal association. More importantly, clinical responses were correlated with both innate (i.e., superior levels of activated NK cells in responders) and adaptive (i.e., increased WT1 tetramer⁺CD8⁺T cell frequencies in long-term responders only) immune responses postvaccination, indicating that the vaccine elicited clinically relevant and vaccine-specific immunity. To date, only a few DC vaccine studies have attempted to evaluate the nonspecific, yet potentially clinically relevant, NK cell response to immunization (40), but there is an increasing body of evidence that DC–NK interactions during the early phase of innate immunity can impact the quality and magnitude of the subsequent adaptive immune response (41). In our trial, it can be hypothesized that the effect of DC vaccination in clinical responders is, at least in part, mediated initially by a (nonspecific) innate immune response but that a subsequent adaptive WT1-specific T cell response is necessary to mediate a long-lasting (defined as a remission period of more than 3 y) (42) clinical benefit, as observed in UPN01 and UPN08. This hypothesis fits with the observation that patient UPN16, who showed a strong initial clinical response immediately after vaccination associated with extremely high levels of activated NK cells (57% of all NK cells) yet without any evidence of increased WT1-specific CD8⁺ T cell immunity, relapsed 9 mo after the start of DC vaccination and succumbed 3 mo later. Moreover, we observed that WT1-specific CD8⁺ T cell populations in these long-term clinical responders targeted multiple HLA-A*0201-restricted WT1 epitopes (43), providing in vivo human proof of concept that single-antigen mRNA-based loading of DC results in polyepitope-specific T cell responses. The ex vivo peripheral blood WT1-specific tetramer⁺CD8⁺ T cell populations identified in the current trial were small but clearly identifiable and are consistent with the data of a recent study showing a lower level of WT1-specific CD8⁺ T cells in blood compared with bone marrow (44).

Notably, we also consistently observed signs of general immune stimulation postvaccination in the whole patient population, regardless of clinical outcome, as evidenced by significant increases in plasma IL-2 levels and frequencies of circulating activated CD4⁺ T cells. Notwithstanding the fact that these observations were based on direct ex vivo blood analyses, in vitro antigen rechallenge assays failed to reveal significant increases in either WT1- or KLH-specific IL-2-secreting CD4⁺ T cells postvaccination. However, there was a significant increase in the peripheral blood postvaccination of WT1-specific IFN- γ ⁺CD8⁺ T cells in the patient population as a whole, but this was not correlated with clinical benefit. This lack of correlation has been reported previously in a WT1 peptide-vaccine trial in cancer patients (11). Thus, whereas numerous reports already indicated that T cell responses after DC vaccination in humans can be more complex than initially expected (45–48), our findings underscore the need for more appropriate functional T cell assays that provide a more global view of the vaccine-specific T cell repertoire (e.g., by detection of transient CD137 expression on CD8⁺ T cells on antigen (re) challenge, which is independent of cytokine profile) (49). Furthermore, our data reiterate the importance of a comprehensive immunomonitoring approach that includes disease-localized tissue sampling. The positive DTH reaction to the vaccine and its single antigenic components, indicative of an in vivo cell-mediated immune response, indirectly points to a migration of at least

part of the DC vaccine to the lymph nodes, where it presumably elicited antivaccine T cell responses. However, further investigation is warranted, because it is possible that the inclusion of KLH as a noncognate CD4⁺ T helper antigen might have negatively influenced the immunological and clinical outcome of the WT1-targeted DC vaccination in some patients, which a partially Th2-skewed KLH-specific T cell response might suggest.

The antileukemic effect of the first round of four biweekly DC vaccinations was transient in some patients, and additional bi-monthly DC administrations were needed to induce repeated normalizations of the *WT1* mRNA tumor marker. Similar booster effects of DC vaccinations have been observed in melanoma patients (45). The reason for the occasionally transient effect of DC vaccination, which is also observed in WT1 peptide-vaccine trials, may be the lack of cognate CD4⁺ T cell stimulation, which is otherwise considered to be necessary for the maintenance of long-term CD8⁺ T cell memory. The inefficient WT1-specific CD4⁺ T cell stimulation postvaccination was most probably caused by cytoplasmic antigen expression after mRNA electroporation, leading to predominant MHC class I antigen presentation. One way to tackle this limitation and potentially improve the efficacy of DC vaccination is to introduce MHC class II-skewing signals into the mRNA construct [e.g., dendritic cell lysosome-associated membrane glycoprotein (DC-LAMP)] (50).

In contrast to peptide-vaccination studies, which have shown clinical activity in AML (9–13), DC vaccination in AML patients to date has been ineffective or inconclusive (51–53). The clinical effectiveness of our DC approach may be caused by several factors. First and similar to analyses conducted in WT1 peptide-vaccination studies (10–12), the use of the sensitive *WT1* mRNA residual disease marker revealed a clinical effect in some complete remission patients with increased *WT1* mRNA expression levels indicative of ongoing leukemic activity (54). Second, we were able to obtain DC from blood CD14⁺ monocytes in all cases from AML patients in remission, in contrast to the generation of DC from primary AML cells, which was only successful in a minority of patients tested (53). Third, the choice of the WT1 antigen (33), rather than leukemic cell lysates or AML-derived DC (51–53), may also be advantageous, because WT1 is already recognized immunologically in AML patients (8, 55, 56); furthermore, WT1-targeted vaccine approaches have been successful in increasing the specific immune response in such a way that they can control AML, at least in some patients, as evidenced here and in other studies (10–12). Fourth, our unique clinical trial design (57), which is in stark contrast to current DC trials focusing on end-stage cancer patients, may have contributed to the demonstrable clinical effects observed (58).

In conclusion, vaccination with *WT1* mRNA-electroporated DC exhibits antileukemic activity in AML patients and elicits both innate and adaptive immune responses correlated with clinical benefit. These findings support the further development of vaccination with WT1-loaded DC as an immunotherapeutic strategy to prevent relapse in AML. Finally, WT1 is overexpressed in a majority of malignancies (24, 56), and WT1 peptide vaccination has led to clinical responses in patients with various solid tumors (11, 56). Thus, the promising data presented here suggest that *WT1* mRNA-electroporated autologous DC might serve as a platform model for the development of a universal cancer vaccine in the adjuvant setting.

Materials and Methods

Patients. After informed written consent was obtained, patients with AML (except acute promyelocytic leukemia) diagnosed according to World Health Organization (WHO) criteria were enrolled in a phase I/II trial approved by the Ethics Committee of Antwerp University Hospital (ClinicalTrials.gov number NCT00834002). All patients were in hematological remission after at least one prior antileukemic chemotherapeutic regimen and were not enrolled until 1 mo after polychemotherapy. Complete remission was defined by the absence of blasts in blood and by less than 5% blasts in marrow. Partial

remission was defined as a $\geq 50\%$ decrease in marrow blasts with normalization of blood counts. Inclusion criteria were the absence of a matched sibling donor for allogeneic hematopoietic stem cell transplantation (allo-HSCT) (if ≤ 60 y) and high risk of full-blown relapse as defined by (i) insufficient disease control as in partial remission status (more than 5% blasts in the bone marrow) (3), and/or (ii) *WT1* mRNA levels increased above background after chemotherapy (35, 39), and/or (iii) age ≥ 61 y (3–5), and/or (iv) previous relapse (3), and/or (v) hyperleukocytosis at presentation (white blood cell count $> 20,000/\mu\text{L}$) (2), and/or (vi) poor risk cytogenetic or molecular markers at presentation (2–4) (Table S1).

DC Vaccination. Clinical grade DC vaccines were prepared after leukapheresis of nonmobilized blood (Cobe Spectra) and immunomagnetic selection using CliniMACS (Miltenyi Biotec) (57). Monocyte-derived *WT1* mRNA-loaded DC vaccines were generated as described previously (57). DC vaccines were administered intradermally at biweekly intervals as previously reported (57).

Immunomonitoring. Detection and subtyping of anti-WT1 antibodies in pre- and postvaccination plasma samples were performed as previously described (59) (more details in *SI Materials and Methods*). Cytokine plasma levels were determined with the Th1/Th2 multiplex immunoassay (BenderMed Systems). Direct ex vivo analysis for lymphocyte subsets and activation markers was performed by flow cytometry using directly conjugated monoclonal antibodies (BD Biosciences). In vitro T cell restimulation assays and detection of antigen-specific cytokine responses were performed as described in *SI Materials and Methods*.

For detection of circulating WT1-specific CD8⁺ T cells, pHLA-A*0201 tetramers were used. To minimize inter- and intrapatient variability, all tetramer analyses were performed on the same day by the same operator on a validated flow cytometer using identical reagents and instrument settings. Thawed peripheral blood mononuclear cells (PBMC) ($1\text{--}2 \times 10^6$ per experimental condition) obtained before vaccination and after the fourth vaccination were washed and stained for 15 min at 37 °C with phycoerythrin (PE)-labeled pHLA-A*0201 tetramers refolded with the following WT1 peptides: WT_{37–45}, WT_{126–134}, WT_{187–195}, and WT_{235–243} (43). Cells were then washed and stained with the following mAbs: anti-CD8-Pacific Blue (Dako), anti-CD3-allophycocyanin (APC), anti-CD14-FITC, and anti-CD19-FITC (BD Biosciences). Dead cells were excluded using propidium-iodide staining (1 $\mu\text{g}/\text{mL}$; Sigma-Aldrich), and the FITC channel was used to exclude B cells and monocytes. For each sample, at least 10^4 viable CD3⁺CD8⁺ T cells within a standard lymphocyte gate were acquired using a CyFlow ML flow cytometer (Partec), and data were analyzed with FlowJo software (Tree Star). Because of the low frequencies of WT1-specific tetramer⁺CD8⁺ events detected in peripheral blood (Table 1), all tetramer data were reanalyzed in an independent and blinded fashion to ensure consistent interpretation as described previously (44).

To assess cell-mediated immunity in vivo, DTH skin testing was performed 2 wk after the fourth DC vaccination by intradermal injection, and measurement of induration was 48 h later.

Molecular Tumor-Marker Monitoring. Longitudinal tumor-marker monitoring in blood and bone marrow was performed by qRT-PCR for *WT1* gene expression as described previously (25, 35, 39). Values above 2 and 25 copies of *WT1* mRNA per 1,000 *ABL* copies in blood and marrow, respectively, were considered to be above normal background and thus, indicative of residual disease. More details are in *SI Materials and Methods*.

Data Mining and Statistical Analysis. Flow-cytometric data analysis was performed using FlowJo version 8.4.4 (TreeStar). GraphPad Prism 4.0 software (GraphPad Software) was used for graphical data representations and statistical computations. Statistical analysis was performed using Student *t* test or one-way ANOVA, where appropriate. Correlations between immunological and clinical responses were examined with a two-sided χ^2 test. Any *P* value < 0.05 was considered statistically significant.

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Stereotactic Body Radiotherapy for Early Stage Lung Cancer

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Stereotactic body radiation therapy (SBRT) is a newly developed technique currently in clinical use. SBRT originated from stereotactic radiosurgery for intracranial tumors. SBRT has been widely used clinically for lung cancer. The practice of SBRT demands different kinds of patient fixation, breathing control, target determination, treatment planning, and verifications. The history and current standard technique are reviewed. Clinical studies of lung cancer showed high local control rates with acceptable toxicities. Past and on-going clinical trials are reviewed.

Key words

Radiosurgery, Early stage lung cancer,
Stereotactic body radiation therapy,
Stereotactic ablative radiotherapy,
Stereotactic radiotherapy, Lung neoplasms,
Conformal radiotherapy

Introduction

Stereotactic radiotherapy (SRT) is method to deliver highly precise radiation to intracranial tumors that can maintain fixed precision within 1-2 mm. This treatment method aims to minimize the radiation dose administered to normal tissues and increase the radiation administered to the tumor by accurately focusing radiation onto the target lesion (hereafter "target"). SRT was originally developed for clinical

application in the 1960s as "gamma knife radiosurgery," most commonly employing single high dose radiation, and evolved to the current form after linear accelerator (LINAC)-based radiosurgery was introduced by 1983. Both techniques initially were developed for treating intracranial brain tumors. The application of the stereotactic technique on the trunk of the body was initiated in the 1990s. In the USA, this is known as stereotactic body radiation therapy (SBRT) or stereotactic ablative external radiotherapy, while in Europe, it is known as extracranial stereotactic radiotherapy. In Japan

it is commonly known as pin-pointed radiotherapy. Its first recognized use was by Blomgren et al. [1] of the Karolinska Institute in 1991, while in Japan, it was implemented by Uematsu et al. [2] in 1994. However, the main problem with performing SBRT on tumors of the trunk as opposed to brain tumors was tumor movement caused by bodily and respiratory movements. Therefore, when performing SBRT for tumors of the body trunk, it is extremely important to establish accurate patient fixation, breathing control systems, and verification of the irradiation field prior to each treatment.

Practice of SBRT

1. Patient fixation

To restrain the bodies of patients during treatment, it is important to have suitable fixation methods. Various vacuum contact-type braces are currently available for use in SBRT that involve styrofoam in a plastic body frame.

2. Breathing control

In lung tumors, tumor movement during respiration cannot be ignored. Methods that deal with the respiratory movement of patients can be broadly divided into the breath holding technique, the restricted respiration technique (abdominal compression), and respiratory gating technique. Efforts to reduce tumor movement during respiration (internal margin) are required.

The breath holding technique is a voice or light-gated intermittent irradiation system in which the patient temporarily suspends his/her breathing following an audible or light signal, allowing for irradiation. In this technique, breath is generally held at the resting expiratory level, and theoretically internal tumor volume, a radiation volume including tumor motion, is minimized, making it possible to set the minimal irradiated volume. In addition, devices can be used to help suspend breathing [3].

An alternative to this is the restricted respiration technique in which major movements of the patient's diaphragm are restricted by compressing the upper abdomen using either a belt or plate-like brace. Tumor movement is ascertained by fluoroscopy, and if found to be greater than 8-10 mm, it is common to restrict movement [4]. In practice, restriction of respiration is required owing to tumor movement greater than 8-10 mm in less than 25% of all patients.

In contrast to these methods, a respiratory gating technique has been developed that synchronizes irradiation with

the respiratory phase (mainly the expiratory phase) while the patient breathes freely but periodically. This is a frequently used technique in which sensors are attached to the thoracic wall of the patient, or gold markers are introduced into the tumor [5], and irradiation is performed while sensing the patient's breathing.

3. Target determination and computed tomography (CT) imaging

In highly accurate treatment planning, using CT images usually taken at 1-3-mm intervals, radiation oncologists delineate the contour of the tumor and the organs at risk. The breath synchronization and breath holding methods mentioned above are a prerequisite of treatment, and CT images are obtained in accordance with these techniques. When the restricted respiration technique is employed, a prolonged or slow scan CT imaging technique is used in which images are obtained slowly, with a scan time of >4 seconds taken per slice to improve the accuracy of irradiation. It is important to be aware that the target definition differs according to these CT imaging techniques.

In recent years, 4-dimensional (4D) CT imaging technology has emerged, which involves placing infrared markers on the body surface of the patient to obtain a respiratory signal. Using this technique, target information (such as maximum intensity projection image) for the entire respiratory phase can be obtained. It is extremely useful in SBRT.

4. Treatment planning

Once the target is delineated using reconstructed 3-dimensional (3D) images such as Beam's eye view or Room's eye view, the radiation field is determined by combining various factors, including the direction and energy of radiation. Non-coplanar 3D conformal multiport irradiation and static multiple arc radiotherapy are often used. In these methods, using either fixed multiport irradiation with >6 ports or rotatory irradiation of >400°, an approximate equivalent dose distribution can be achieved. Target dose in treatment planning include homogeneous radiation dose within the target (within 10%) and reduction (<15%) of lung capacity (V20) of >20 Gy of radiation. It is possible to achieve this by administering 3D irradiation from a total of 6-8 fields, including irradiation at an angle of +/- 20-40° non-coplanar beams as well as coplanar beams to the body's axis (Fig. 1). Calculating the correct 3D radiation dose allowing for inhomogeneity correction is essential, as is the correction of the radiation dose due to the frame. Furthermore, attention should be paid to the radiation dose prescription used. In Japan, the isocenter is most often the radiation dose prescrip-