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Research Article

WT1 Peptide Vaccine is Unaffected by Airport Screening X-Irradiation

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Abstract

Background: Wilms' Tumour gene 1 (WT1) is a target antigen that has been widely detected in sarcoma, leukaemia, and other cancers. In our clinical trial of a WT1 peptide vaccine including foreign patients from the Middle East, a need has arisen to transport the vaccine abroad. Thus, we assessed the influence of airport X-ray exposure on the structural integrity and biological effect of WT1 peptides.

Methods: WT1 peptides were transferred into three dry shipper containers before being subjected to X-irradiation. The vessels were then exposed to radiation of 0 μGy (control), 24.4 μGy, 198.5 μGy, or 22.0 mGy, which were equivalent to approximately 0, 5, 40, or 4400 rounds, respectively, of airport X-ray scanning. Irradiation-derived fragmentation of WT1 peptides was analyzed by a Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) 5800, and ELISpot functional assays were conducted to evaluate the bioactivity of the tumour-associated antigen X-ray exposure.

Results: There were no significant differences in molecular mass profile or WT1-specific function among the irradiated and non-irradiated samples.

Conclusion: Routine airport X-irradiation has no significant influence on the structural integrity or activity of WT1 peptides when transported in a dry shipper.

Keywords: Airport X-Ray; WT1 Peptide; Cancer vaccination; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Enzyme-Linked immunosorbent assay

Abbreviations

HLA: Human Leukocyte Antigen; WT1: Wilms' Tumour 1; PBMCs: Peripheral Blood Mononuclear Cells; CTLs: Cytotoxic T Lymphocytes; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry; HIV: Human Immunodeficiency Virus

Introduction

With the recent advances in cancer immunology, immunotherapy is rapidly becoming a viable option for personalized cancer treatment [1-4]. Wilms' Tumour gene 1 (WT1), an attractive target antigen in cancer immunotherapy, has been established as a very potent tumour-associated antigen because of it's over expression in leukaemia and solid tumours [5]. WT1 peptide vaccination is a form of immunotherapy that can induce WT1-specific Cytotoxic T Lymphocytes (CTLs). Several clinical trials have demonstrated *in vivo* the safety and efficacy of WT1 peptide vaccination in humans [6-9].

We have been treating patients with cancer from the Middle East for the purpose of international medical aid since 2012 with WT1 peptide-pulsed Dendritic Cell (DC) vaccination therapy. Recently, a female patient with breast cancer who completed DC treatment required a subsequent course of WT1 peptide therapy. To administer the vaccine at her local National Cancer Center, however, it became necessary to fly the drug in a dry shipper container from Japan to Kuwait. Although X-ray inspection of checked baggage at airports is mandatory, there are few reports evaluating the quality of biological samples following airport X-ray screening [10,11]. In this report, we assessed the structural integrity and bioactivity of WT1 peptides following such irradiation procedures.

Materials and Methods

Dry shipper

Model DR-2 dry shipper containers (Taiyo Nippon Sanso Corporation, Tokyo, Japan) (Figure 1) were subjected to the conditions of X-ray inspection that take place during air transport. Prior to X-irradiation, the dry shippers were filled with liquid nitrogen according to the manufacturer's instructions. The internal temperature of each vessel was validated by overnight monitoring to ensure the maintenance of cryogenic temperatures.

WT1 peptide

Good manufacturing practice-grade lyophilized 9 mer WT1*2402 peptide powder (mp235-243: CYTWNQMNL, Peptide Institute Inc., Osaka, Japan) was prepared using cryoserv (20 mg/ml; Nipro, Osaka, Japan), aliquoted at 1mg/50μl into CryoTubes (Thermo Fisher Scientific, Waltham, MA, USA), and then stored at -80 °C before and after X-ray exposure.

X-irradiation

Typical airport scanning protocol presumes a checked item to receive X-radiation doses ranging from a few to several dozen of μGy

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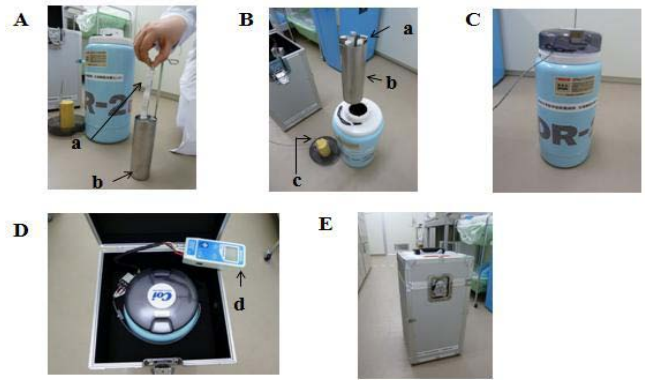

Figure 1: Model DR-2 dry shipper. A: Cryo Tubes containing WT1 peptides were set on a cane (a) and placed into a canister (b). B, C: The assembled unit (a and b) was placed into a liquid nitrogen-filled dry shipper. A thermo sensor was fitted on the inside cover of the vessel (c). D, E: The dry shipper was set in a dedicated case with temperature logger monitor (d).

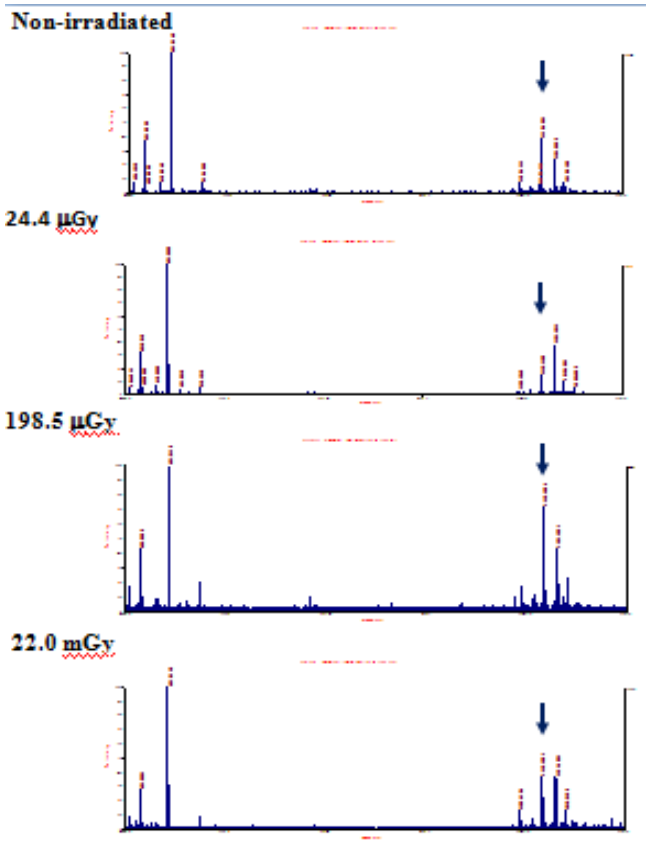

Figure 2: MALDI-TOF MS of irradiated and non-irradiated WT1 peptides. Dry shippers were subjected to 0 μGy, 24.4 μGy, 198.5 μGy, or 22.0 mGy of X-radiation. A MALDI-TOF MS 5800 device was used to assess for pattern changes in irradiated WT1 peptide mass spectrometric profiles. Anon-irradiated peptide was used as a control. Arrows indicate the characteristic mass spectrum peak of the normal intact WT1 peptide. There were no remarkable mass spectrum intensity alterations between 500 and 1200 m/z in irradiated or non-irradiated peptides.

per inspection [12,13]. As the dry shippers would be irradiated more than five times from Japan to Kuwait, the containers were exposed five times to X-rays set at 5 μGy, 50 μGy, or 5 mGy (80 KVp, 20 mA) using a Digital Diagnost system (Royal Philips, Eindhoven, The Netherlands). All measurements were performed with a Radiation Monitor Controller model 9015 X-ray detector (Radcal Corporation, California, USA), which indicated final doses of 24.4 μGy, 198.5 μGy, and 22.0 mGy, respectively, for the above radiation settings.

Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood samples were obtained using BD Vacutainer™ Blood Collection Tubes (Sodium Heparin/Polyester Gel Samples; Franklin Lakes, NJ, USA) from four patients with cancer who had already received WT1 peptide-pulsed DC therapy and in whom the induction of WT1-specific CTLs (WT1-CTLs) had been confirmed. PBMCs were collected according to the manufacturer's instructions and cryopreserved using TC protectors (DS Pharma Biomedical, Osaka, Japan) at temperatures of -80 °C to -180 °C. This trial was approved by the Ethics Committee of Shinshu University School of Medicine (approval numbers 1199 [December 2, 2008] and 2704 [April 8, 2014]).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

A MALDI-TOF MS 5800 device (AB SCIEX, Tokyo, Japan) was used to assess for pattern changes in irradiated WT1 peptide mass spectrometric profiles in a manner directed by the manufacturer. Non-irradiated peptides were adopted as controls. Calibration was carried out using a peptide mass standards kit for calibration of AB SCIEX MALDI-TOF™ instruments (AB SCIEX, Framingham, Massachusetts).

Interferon (IFN)-γ ELISpot assays

To determine the function of X-irradiated peptides as tumour antigens, the enzyme-linked immunosorbent assay was performed using a pre-coated Human IFN-γ ELISpot PLUS Kit (HRP) (Mabtech AB, Nacka Strand, Sweden) according to the manufacturer's instructions. Briefly, cryopreserved PBMCs were thawed and the live cells counted. Next, 1×10^6 live cells/well were resuspended in AIM V medium (Life Technologies, CA, USA) with 10 % fetal bovine serum (Biowest, Nuaillé, France) in the presence of 10 μM irradiated WT1 peptides. As a negative control, 10 μM human leukocyte antigen (HLA)-A*24:02 Human Immunodeficiency Virus (HIV) env (RYLRDQQLL, residue 584–592; MBL, Nagoya, Japan) was used.

After 18-20 hours of incubation at 37 °C under 5 % CO_2, the spots formed by IFN-γ-secreting cells were counted by an automated ELISpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Total WT1 peptide-specific spots were determined by subtraction of the spots for negative controls. Results were expressed as the mean number of peptide-specific spots per 1×10^6 PBMCs from duplicate experiments.

The ratio of WT1-specific spots between non-irradiated and each irradiated peptide sample was calculated as the number of WT1-specific spots stimulated by each irradiated peptide divided by the number of spots stimulated by non-irradiated peptides.

Statistical Analyses

The Friedman test was applied to assess the differences among X-irradiated peptide samples and non-irradiated ones in the IFN-γ ELISpot assays. A p value of <0.05 was set to indicate statistical

significance using IBM SPSS Advanced Statistics ver. 23.0 software (IBM Japan, Tokyo, Japan).

Results

Validation and monitoring of dry shippers

The maintenance of temperatures of lower than -150 °C was confirmed for each dry shipper before irradiation.

MALDI-TOF MS profiling of WT1 peptides

All irradiated and non-irradiated peptides were evaluated by MALDI-TOF MS. The characteristic peak of the intact WT1 peptide at 1172 m/z was clearly visible in all samples, and there were no remarkable mass spectrometry intensity alterations between 500 and 1200 m/z (Figure 2).

Functional assays for WT1 peptide induction of IFN-γ from CTLs

The PBMCs of four cancer patients were employed to evaluate WT1 peptide-induced secretion of IFN-γ from CTLs by ELISpot assays. As evidenced by the representative data in Figure 3, the number of irradiated WT1-specific spots was comparable to that of non-irradiated peptide spots for all dosages.

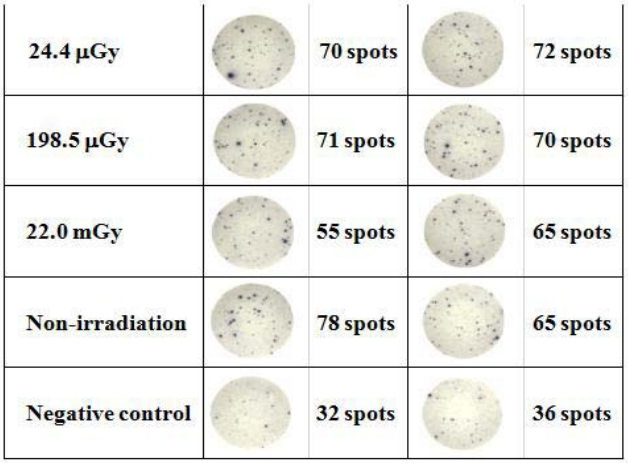

Figure 3: WT1 peptide-specific responses in ELISpot assays. Representative data obtained from patient 1. ELISpot assays were performed in duplicate to assess the function of X-irradiated peptides as tumour antigens. As a negative control, 10 µM HLA-A*24:02 HIV env was used. WT1-specific IFN-γ secretion by PBMCs was detected for each test peptide.

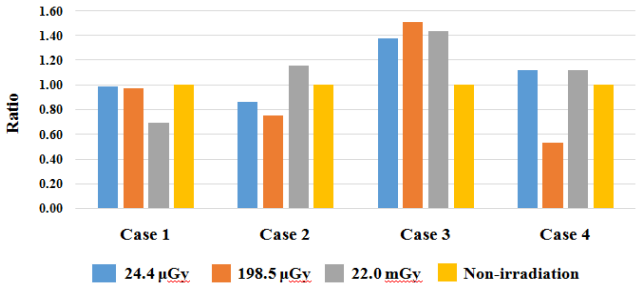

Figure 4: Ratios of WT1-specific spot number between non-irradiated and irradiated WT1 peptides. The ratios of WT1-specific spots between non-irradiated and irradiated WT1 peptides were calculated as the number of WT1-specific spots stimulated with each irradiated peptide divided by the number of specific spots stimulated with non-irradiated peptides. No significant differences were detected at any radiation dose (Friedman test: 0.801).

The mean ratios of the irradiated WT1-specific spots to non-irradiated peptide spots of the 4 patients were 0.99-1.12 (24.4 µGy), 0.53-1.51 (198.5 µGy), and 0.69-1.44 (22.0 mGy). The Friedman test yielded a value of 0.801, indicating no statistical difference between the non-irradiated control and individual irradiated peptide groups (Figure 4).

Discussion

Although the air transport of biological materials requires routine exposure to X-radiation at ports of entry and exit, there are few reports on the influence of radiation on subsequent clinical applicability. It was suspected that the WT1 peptide might become fragmented and loses its property of CTL activation following X-irradiation. As the aim of our peptide vaccination study was to maintain CTL induction in subjects, even while abroad, this investigation was conducted to confirm that X-radiation exerted no remarkable influence on WT1 peptide structure integrity or bioactivity.

Mass spectrometry is a powerful tool that can detect a wide range of protein sizes [14]. We first verified the molecular mass of WT1 peptides using MALDI-TOF MS since the mass of fragmented peptides would be smaller than that of intact ones. MALDI-TOF MS disclosed no fragmentation of WT1 in the 500 to 1200 m/z range, thus indicating no molecular mass alterations in the irradiated peptides.

Next, we confirmed the WT1-specific immunological effect of irradiated peptides on T cells *via* ELIspot assays. The ELISpot assay is a unique method that allows the quantification of actual cytokine secretory activity to evaluate vaccine-induced CTL responses [15,16]. Previous studies showed that specific CTLs induced by WT1 peptide-pulsed DC vaccines *in vivo* potentiated IFN-γ secretory activity under WT1 peptide stimulation in ELISpot assays [17]. Here, the PBMCs obtained from cancer patients having undergone WT1 peptide-pulsed DC vaccination had already been identified as producing of WT1-CTLs. Therefore, the WT1-specific spots witnessed in this study indicated that the irradiated peptides had an affinity to major histocompatibility complex class I and functional activity at levels comparable to non-irradiated peptides. These results were in agreement with the molecular mass profiles revealed by MALDI-TOF MS.

This study clearly demonstrated that airport X-ray inspection had negligible influence on a 9 mer WT1 peptide vaccine in dry shippers up to radiation doses of 22 mGy. However, as other bio samples for immunotherapy may be damaged by such repeated exposure to airport X-radiation, further examination is necessary to confirm the structural integrity and functional activity of RNA, long peptides, whole proteins, and cells. The analysis of other factors threatening the viability of therapeutic agents during air transport, such as mechanical impact forces and air pressure variation, may be warranted as well.

Conclusion

Airport X-ray exposure exerts no remarkable influence on the 9 mer-WT1 peptide vaccine in a dry shipper.

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Author Contributions

Yumiko Higuchi and Shigetaka Shimodaira conceived and designed the study. Masatomo Kawakubo and Kenji Sano analyzed the quantification of the peptides by MALDI-TOF MS. Miki Yuzawa performed the statistical analysis. Yumiko Higuchi analyzed the immune monitoring data and drafted the manuscript.

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