Quantification of the CD8+ T cell response against a mucin epitope in patients with breast cancer

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Abstract

Introduction: Mucin 1, encoded by the MUC1 gene, is a tumor-associated antigen expressed on the surface of breast cancer cells. It would be of interest to see whether there is a naturally existing T cell immune response against mucin epitopes in cancer patients.
Materials and Methods: Using tetramer and interferon-γ assays, the immune response to one MUC1 peptide epitope in the peripheral blood of breast cancer patients was quantified. The data were compared with the clinical course of the patients.
Results: CD8+ T cells capable of recognizing the HLA-A*0201-restricted STAPPVHNV epitope were detected in 9 of 19 patients with a frequency ranging 0.01–0.082%. No significant difference was found between the occurrence of epitope-specific CD8+ T cells of patients with progressive disease and disease-free patients. However, all patients with stable disease showed a specific immune response, including both patients with the highest frequency.
Conclusions: The results of this study provide further evidence that a natural specific cellular immune response against this mucin epitope exists in breast cancer patients.

Key words: breast cancer, mucin, T cells, tetramer, IFN-γ assay.

Abbreviations: TAA – tumor-associated antigens, mAb – monoclonal antibody, PBMC – peripheral blood mononuclear cell, TCR – T cell receptor.

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INTRODUCTION

Breast cancer does not induce significant immune responses that effectively destroy malignant cells [19]. However, given the evidence of T cell-mediated immunosurveillance [22] based on the recognition of epitopes from tumor-associated antigens (TAAs) [21], it is of interest to quantify the immune responses that appear in cancer patients. As both spontaneous and induced T cell responses may influence the clinical course and the outcome of the patient [14], this evaluation might contribute to a better assessment of the prognosis of the disease. It also provides further insight into ways to optimize the design of immunotherapeutic strategies [1].

The tumor antigen mucin 1 (MUC1, CD 227), encoded by the MUC1 gene, is a large, heavily glycosylated, transmembrane protein expressed on the apical surface of mucosal epithelial cells [9]. MUC1 has been considered as a potential target for immunotherapy as its expression is changed, with the glycoprotein becoming shorter and less branched, in cells that have undergone malignant transformation, leading to the exposure of previously masked epitopes [4]. One of the MUC1-derived epitopes, peptide $MUC1_{950-958}$ (STAPPVHNV), has been proven to induce a T cell response *in vitro* and subsequently to trigger *ex vivo* lysis of tumor cell lines [3]. The peptide is localized in the tandem repeat region of MUC1 and is presented on the cell surface in an HLA-A*0201--restricted manner [3]. In cancer patients, cellular immune responses against several other MUC1-derived peptides have been reported [6], but the precursor frequencies of the CD8+ T cells recognizing the $MUC1_{950-958}$ epitope circulating in the peripheral blood have so far not been extensively evaluated in breast cancer patients.

Several assays have been developed to monitor

immune response against TAAs. The tetramer assay allows the quantification of antigen-specific cells independently of their functional properties with a sensitivity of 1:50,000, whereas the interferon (IFN)-γ secretion assay quantifies a specific cytokine response to TAAs. Both methods combined provide a comprehensive picture of the frequency and function of tumor-specific T cells. We examined the immunogenicity of the MUC1$_{950-958}$ peptide in breast cancer patients by quantifying the epitope-specific CD8$^+$ T cells.

MATERIALS AND METHODS

Patients

Breast cancer patients referred to the outpatient's unit of the Medical Clinic for Hematology and Oncology, Charité Berlin, were randomly screened for the presence of HLA-A2 following informed consent. From a cohort of 19 HLA-A2-positive patients, blood samples were obtained for the evaluation of immune response. Tumor grade, axillary node status, and previous treatment of the patients were recorded over a time period of 30 months (Table 1). Most of the patients had been pretreated by surgery, radiation, or multiple cycles of chemotherapy. None of the patients had received chemotherapy or radiotherapy within the four weeks prior to sample collection.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using Lymphoprep™ (1.077 g/ml; Biochrom, Berlin, Germany), washed, and cultured overnight in RPMI medium (Biowhittaker, Verviers, Belgium) supplemented with 10% FCS, 2 mM L-glutamin, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2-cell counting and the tetramer and IFN-γ assays were performed the next day.

Tetramer assay

Phycoerythrin (PE)-conjugated tetrameric complexes consisting of HLA-A*0201 and STAPPVHNV peptide were purchased commercially (Proimmune, Oxford, UK). Additionally, HIV gag-derived SLYNTVATL peptide (Proimmune, Oxford, UK) was used as a negative control. Approximately 1–2 million PBMCs from each patient were stained with tetramers for 30 min at room temperature followed by staining with an FITC-conjugated antihuman CD8 monoclonal antibody (mAb; Becton Dickinson, Heidelberg, Germany). Quadrant analysis was applied to determine the percentage of tetramer-positive CD8$^+$ T cells.

IFN-γ assay

PBMCs were stimulated for 5 h with 10 μg/ml of the mucin peptide containing the STAPPVHNV sequence (Biosyntan, Berlin, Germany) in RPMI-1640 medium supplemented with 10% AB serum and cultured at 37°C in 5% CO_2. An unstimulated sample containing the same amount of cells was used as a negative control. At least one million PBMCs were washed and resuspended in 90 μl of cold medium. Ten μl of the IFN-γ-

Table 1. Clinical features of the breast cancer patients

Patient number	Age/sex	Stadium at diagnosis	Prior treatment	Metastases	Clinical course
1	77/f	pT1pN1M1 G3	S	M (L, B) †	PD
2	35/f	cT2cN1M0 G2	C+S	free	CR
3	82/f	pT1pNxM1 G2	C+S+R	M (L)	SD
4	76/m	cT2cN1M1 Gx	ST	M (B)	SD
5	53/f	pT1pN1M1 Gx	C	M (P) †	PD
6	63/f	pT3pN1M1 G3	C+S	M (L, B, P)	PD
7	68/f	pT1pN0M1 G3	C+S+R	M (brain) †	PD
8	60/f	pT4pN1M1 G3	C+S	M (P)	SD
9	75/f	pT3pN1M1 G2	C+S	M (LN)	PD
10	45/f	cT3cN1M0 G3	C+S	NA	NA
11	33/f	cT3cN0M0 G2	C+S+R	free	CR
12	76/f	pT1pN0M1 G2	C	M (L, B, S) †	PD
13	50/f	pT2pN0M1 G3	S+R	M (P)	PD
14	71/f	pT4pN1M1 G2	C+S+R	M (B)	SD
15	60/f	pT2pN1M1 G2	C+S	M (B) †	PD
16	52/f	pT1pN1M1 G3	C+S	M (L, P, B)	PD
17	39/f	cT2cN0M0 G1	C+S	free	CR
18	44/f	pT2pN2M0 G3	C+S	free	CR
19	66/m	cT4cNxM1 Gx	C+R	M (P, B)	PD

Gx – grade unknown, C – chemotherapy, R – radiation, S – surgery, ST – supportive therapy, NA – not available, M – metastases, L – liver, B – bone, P – lung, S skin, LN – lymph node, † – death, PD – progressive disease, CR – complete remission, SD – stable disease, f – female, m – male.

-catch reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and incubated for 5 min on ice. Subsequently, 10 ml of warm medium were added and the IFN-γ secretion assay was performed by incubation at 37° for 45 min. Sedimentation of the cells was prevented by inverting the tubes every 5 min. After 45 min, 5 ml of cold PBS buffer (GIBCO BRL, Life Island, USA) containing 0.5% BSA (Sigma, Munich, Germany) and 2 mM of EDTA (Sigma) was added to each tube and the cells were centrifuged for 15 min at 300×g and 4°C. The supernatants were completely removed and the cells were resuspended in 80 μl of ice-cold buffer. Ten μl of the PE-labeled anti-IFN-γ mAb (Miltenyi Biotec) and 10 μl of the FITC-labeled mAb specific to CD8 (Becton Dickinson, Heidelberg, Germany) were added and incubated for 15 min on ice. Finally, 10 ml of cold buffer were added and the cells were centrifuged for 15 min at 300×g and 4°C. Propidiumiodid (Sigma) was added to each sample to a final concentration of 1 μg/ml to exclude dead cells from the analysis.

Samples from both the tetramer and IFN-γ assays were subsequently analyzed by flow cytometry using a FACS-Calibur (Becton Dickinson) and CellQuest software. Background values from unstimulated control cells were subtracted.

RESULTS

Tetramer assay

The CD8$^+$ T cell-frequency against specific peptides was evaluated using tetramer analysis of the PBMCs from cancer patients. Frequencies ranging from 0 to 9 CD8$^+$ T cells per 10^5 PBMCs were assessed as the background (Table 2). CD8$^+$ T cells capable of recognizing the MUC1$_{950-958}$ peptide were identified in 9 of the 19 patients (Table 2). In the positive samples, the frequency of HLA-A2-MUC1-specific CD8$^+$ cells in the PBMCs ranged from 0.01 to 0.082%. Figure 1 shows the results of the patient with the strongest cellular response specific to MUC1$_{950-958}$.

Table 2. Number of tetramer-binding CD8$^+$ T cells

Patient number	Number of tetramer-positive CD8$^+$ cells/10^5 PBMCs	
	HIV	MUC1$_{950-958}$
1	4	**26**
2	4	6
3	**10**	**15**
4	0	**40**
5	5	5
6	**10**	7
7	ND	4
8	**13**	**10**
9	0	**10**
10	0	0
11	2	**30**
12	7	4
13	3	6
14	**16**	**82**
15	1	1
16	2	**29**
17	0	3
18	7	3
19	7	**15**

The numbers in bold represent specific frequencies.
ND – not determined.

HLA-A2-HIV-specific CD8$^+$ T cells were not detectable in 14 of 18 patients. However, four patients revealed frequencies of HIV-epitope-specific T cells ranging from 0.01 to 0.016%.

IFN-γ assay

The amount of released IFN-γ from the PBMCs of the five patients who showed significant responses in the tetramer assay (patients 1, 3, 4, 11, 14) was smaller than 0.01%, which was defined as the background (Table 3).

Clinical course

Ten of the 19 patients progressed in their tumor disease, four patients remained stable, in four patients the
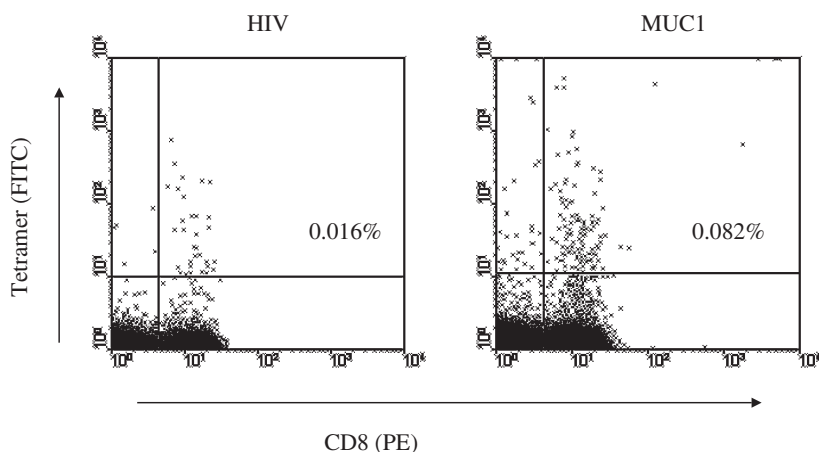

Fig. 1. Example depicting the strongest immune response against MUC1 which could be detected in the breast cancer patients. The frequency of specific T cells was evaluated using the tetramer assay. PBMCs were stained with HIV- or MUC1$_{950-958}$-loaded HLA--A*0201-specific tetramers. The cells were then stained with an FITC-labeled anti-CD8 mAb. An HIV-derived peptide served as the negative control.

Table 3. IFN-γ release by CD8+ T cells

Patient number	Percentage of IFN-γ-releasing CD8+ cells/10^5 PBMCs
8	0
16	0.002
17	0.001
18	0

disease was not detectable, and in one patient the clinical course could not be followed (Table 1).

Four patients with progressive disease had detectable MUC1-specific CD8+ T cells and six patients did not (Tables 1 and 2). All four patients with stable disease stained tetramer positive, including both the patients with the highest frequency. Three of the four disease-free patients had no tumor-specific T cells. The patients with stable disease showed a significantly higher MUC1-specific CD8+ T cell frequency than the patients with progressive disease (p=0.046) as detected by two-sided Student's *t*-test. There was no significant difference in MUC1-specific frequency between the disease-free patients and the patients with stable or progressive disease.

DISCUSSION

The data presented in this report demonstrate the existence of CD8+ T cells against $MUC1_{950-958}$ peptide in 46% of the analyzed breast cancer patients as detected by tetramer assays. This result is in accordance with the findings of Choi et al. [5] in patients with multiple myeloma and Rentzsch et al. [20], who were able to detect $MUC1_{950-958}$-specific T cell responses in a similar percentage of breast cancer patients by quantifying the amount of IFN-γ-specific mRNA after peptide stimulation. Recently, Gûckel et al. [11] observed $MUC1_{950-958}$--specific T cells to a similar extent, mainly in patients in an early stage of disease. The CD8+ T cell frequencies are lower than previously described for other MUC1 epitopes [6], where frequencies were between 0.2% and 1%. In our study, the presence of a specific T cell population which includes CD8+ NK- and NK-T cells in a frequency ranging from 0.01 to 0.082% against the $MUC1_{950-958}$ peptide suggests that the investigated peptide can be recognized on the surface of breast cancer cells. However, although 92% of the breast cancer cases expressed MUC1, more than half of the patients failed to generate a spontaneous T cell response against the $MUC1_{950-958}$ peptide [7, 10]. The peptide presentation may vary considerably among patients [15]. Insufficient peptide presentation due to a reduction or absence of the MHC I presentation pathway or the downregulation of the transporter protein associated with antigen presentation in the HLA complex in breast cancer cells, which are frequently observed in breast cancer, may result in weak T cell responses [2, 12]. Furthermore, low

avidity of the T cell receptor (TCR), decreased expression of the TCR ζ-chain, or the suppression of T cells either by previous therapy or by cytokines secreted by tumor cells may account for a weak or absent immune response [19, 24]. Moreover, it is possible that the frequency of naturally existing $MUC1_{950-958}$-specific T cells lies below the sensitivity of the tetramer assay.

Interestingly, four patients showed a specific T cell response against the HIV gag epitope. However, a study by Kan-Mitchell et al. [13] showed that uninfected individuals also possess T cells which are able to recognize this peptide.

In our experiments, the T cells were not able to produce IFN-γ after stimulation with the $MUC1_{950-958}$ peptide. This finding is consistent with the results of previous studies in which the existence of peripherally circulating, tumor-reactive cells has been reported, but the use of functional assays such as ELISPOT often failed to detect TAA-specific responses [8, 16, 17]. Presumably, in cancer patients functional CD8+ T cells could be localized in the lymph nodes and not in the peripheral blood. Furthermore, the loss of effector function could be due to anergy of specific T cells.

We evaluated further whether the frequencies of $MUC1_{950-958}$-specific T cells measured by the tetramer assay influenced the clinical course of the patients. No significant difference was found between the occurrence of epitope-specific CD8+ T cells of patients with progressive disease and disease-free patients. However, all patients with stable disease showed an immune response. Similar results were reported for the evaluation of a pre-existing T cell immune response in patients with breast cancer or other tumor types [18, 20, 23].

In summary, there were specific T cells against the $MUC1_{950-958}$ peptide detectable in nearly the half of the investigated breast cancer patients.

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