Heparanase: A New Metastasis-Associated Antigen Recognized in Breast Cancer Patients by Spontaneously Induced Memory T Lymphocytes

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Abstract

Increased expression and secretion of heparanase (Hpa) by tumor cells promotes tumor invasion through extracellular matrices, tissue destruction, angiogenesis, and metastasis. Here, we show the existence in breast cancer patients of Hpa-specific T lymphocytes by fluorescence-activated cell sorting flow cytometry using Hpa peptide-MHC class I tetramers. We furthermore show memory T-cell responses in a high proportion of breast cancer patients to Hpa-derived HLA-A2-restricted peptides, leading to production of IFN-γ and to generation of antitumor CTLs lysing breast cancer cells. Such CTLs recognized endogenously processed respective Hpa peptides on Hpa-transfected and Hpa-expressing untransfected breast carcinoma cells. According to these results and to the fact that such cells were not found in healthy people, Hpa seems to be an attractive new tumor-associated antigen and its HLA-A2-restricted peptides ought to be good candidates for peptide vaccination to reactivate memory immune responses to invasive and metastatic cancer cells. (Cancer Res 2006; 66(15): 7716-23)

Introduction

Infection, tissue injury, and formation of cancer metastases have in common, the degradation of endogenous macromolecules, which help form physical barriers within tissues. Macromolecules, such as heparan sulfate proteoglycans (HSPGs), are a critical part of basement membranes and other extracellular matrices (ECM; ref. 1). HSPGs are complex molecules composed of a protein core covalently linked to heparan sulfate side chains that interact closely with other ECM components, including growth factors (2) and adhesion molecules. HSPGs also form cell surface barriers, such as the apical lining of blood vessels (3). Malignant tumor cells differ from benign ones in being capable of breaking down such barriers (4) by means of degradative enzymes, such as serine and cysteine proteases and matrix metalloproteinases, and by the recently identified endo-β-D-glucuronidase heparanase (Hpa; refs. 5, 6).

Degradative enzymes of malignant tumors represent interesting cancer therapeutic targets. The search for specific inhibitors of tumor invasion-associated proteases has been complicated by the polymorphism of such enzymes and their heterogeneity of expression. In contrast to proteases, Hpa is monomorphic, consistent with the idea that this enzyme is the dominant heparan sulfate–specific endoglucuronidase in mammalian tissues. One gene encodes a unique protein of 543 amino acids with a putative 56-kDa precursor and a 50-kDa active enzyme (7, 8). Although Hpa is localized within lysosomes of normal resting cells in a stable form (9), tumor cells may secrete Hpa and thereby promote tumor angiogenesis and metastasis (10).

Because specific inhibitors of Hpa are not yet available, we decided in this study to investigate T-cell–mediated immune responses against the enzyme. We were encouraged to do so by a report showing proteinase-3 as a relevant tumor target for T lymphocytes in chronic myelogenous leukemia (CML; ref. 11). There was a strong correlation between the presence of proteinase-3-specific T cells in CML and clinical responses after treatment by IFN-α and allogeneic bone marrow transplantation (11). Hpa expression was found to be associated with the metastatic potential of breast cancer (12). We therefore tested for the presence of Hpa peptide-specific CD8 T cells in breast cancer patients. Hpa peptide-reactive memory T cells were found in ∼40% of breast cancer patients in IFN-γ enzyme-linked immunospot (ELISPOT) assays with frequencies between 1:600 and 1:13,000. Tetramer stainings revealed Hpa8- and Hpa16-specific CD8 T cells in bone marrow and blood of breast cancer patients enriched compared with normal healthy donors. Furthermore, Hpa peptide-stimulated T cells from breast cancer patients were able to recognize and lyse cells from a Hpa-transfected tumor line and from a Hpa-positive nontransfected breast cancer line. Our results furthermore suggest that Hpa induces an equal or even stronger immune response than other breast cancer–associated antigens, such as HER-2/neu or MUC1. Hpa-derived peptides may therefore represent interesting candidates for tumor vaccine antigens for the treatment of patients with breast cancer and possibly also with other malignancies.

Materials and Methods

Patients. Bone marrow and peripheral blood samples were obtained from 54 patients with primary breast cancer and from altogether 28 healthy women. Informed consent was obtained from all participants. The protocol was approved by the Ethical Committee of the University of Heidelberg (Heidelberg, Germany). Bone marrow samples were aspirated from each anterior iliac crest immediately after surgery while the patients were still anesthetized. Bone marrow of healthy donors was obtained using local anesthesia. Heparanized samples were subjected to Ficoll gradient centrifugation (Pharmacia, Upppsala, Sweden), and cells in interphase were collected.

Note: N. Sommerfeldt and P. Beckhove contributed equally to this work.

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**Peptides.** Nonapeptides derived from the amino acid sequence of following tumor-associated antigens (TAA) were synthesized by the institutional peptide core facility [Hpa (Hpa8, amino acids 8-16: ALPPPLMLL; Hpa16, amino acids 16-24: LLPIGLPLG; Hpa183, amino acids 183-191: DLIGFLNIAL), HER-2/neu (amino acids 369-377: KIFGSLAFI; ref. 13), and MUC1 (amino acids 12-20: LILITLYTV; ref. 14) and control antigens [HV (gag, amino acids 77-85: SLXNYVATL; ref. 15), insulin (amino acids 54-62: HVLALYLV; ref. 16), and influenza matrix protein (EMP; amino acids 58-66: GILGFVVTTL)].

**Cell lines.** MCF7, BT-20, BT-549, and HTB-124 are HLA-A0201-expressing breast cancer cell lines. BT-20, BT-549, and HTB-124 naturally express Hpa. Hpa-low/negative MCF7 cells transfected with Hpa (MCF7-Hpa) or with an empty vector (MCF7-mock) were used for analysis of Hpa specificity of cytotoxic T cells. The TAP-deficient cell line T2 expresses HLA-A*0201 and was used for loading with HLA-A0201-restricted peptides.

**HLA stabilization analysis.** The assay was done as described elsewhere (17). Briefly, HLA-A0201-positive TAP-2-deficient T2 cells were kept overnight at 37°C in serum-free X-VIVO 20 medium (Cambrex, Vervier, Belgium). Then, 2 × 10⁵ cells were incubated for 15 hours with respective peptides at concentrations of 25 μg/mL and 1 × 10⁵ cells/mL at 37°C. HLA stabilization was measured afterwards by flow cytometric detection of HLA-A0201 complexes on the cell surface using FITC anti-HLA-A2 monoclonal antibody (mAb) BB7.2 (BD Pharmingen, Heidelberg, Germany). The relative binding affinity of respective peptides was calculated from the mean fluorescence intensities (MFI) as follows: MFI_{peptide} - MFI_{unloaded cells} / MFI_{loaded cells}. Relative binding affinities >0.6 were considered strong (0.3 to 0.6, intermediate; <0.3 to 0.6, low (17).

**Generation of bone marrow–derived T cells and dendritic cells.** To obtain T lymphocytes, we incubated mononuclear cells from HLA-A2-positive patients for 7 days in RPMI 1640 (Life Technologies, Eggenstein, Germany) with 10% human AB serum (PromoCell, Heidelberg, Germany), interleukin (IL)-2 (100 units/mL; Chiron, Ratingen, Germany), and IL-4 (60 units/mL; PromoCell) followed by overnight incubation in the same medium without ILs. Depletion of contaminating B lymphocytes and myeloid precursors was done with magnetic beads conjugated with mAbs against CD19 and CD15 (both from Dynal, Oslo, Norway). Natural killer cells were removed using mAb against CD56 (Beckman Coulter, Krefeld, Germany) and magnetic beads conjugated with mAb against mouse Ig (Dynal). After depletion, the suspension contained 95% to 98% CD3 T cells (~20% were CD8 T cells). For the generation of dendritic cells, mononuclear cells were incubated for 2 hours on Petri dishes at 37°C. Adherent cells were further cultured for 7 days in serum-free X-VIVO 20 medium with human granulocyte macrophage colony-stimulating factor (50 ng/mL; Behringwerke, Marburg, Germany) and IL-4 (1,000 units/mL). Nonadherent dendritic cells were enriched by depletion of contaminating T and B lymphocytes using magnetic beads conjugated with mAbs against CD3 (Dynal) and CD19 and pulsed for 20 hours with tumor-specific nonapeptides derived from Hpa8, Hpa16, Hpa183, HER-2/neu, and MUC1 or with irrelevant peptides derived from HIV and insulin.

**HLA-A2 tetramer analysis.** Tetrameric complexes containing HLA-A*0201 and peptides derived from TAs Hpa (Hpa8 and Hpa16), HER-2/neu, or MUC1 were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility and NIH AIDS Research and Reference Reagent Program (Bethesda, MD). As a control, tetramers containing HLA-A*0201 and HIV-derived peptide SLYNTVATL (Immunomics, Paris, France) were used. Freshly isolated HLA-A2-positive (using FITC anti-HLA-A2 mAb BB7.2) bone marrow and peripheral blood mononuclear cells (PBMC) were incubated for 10 minutes on ice with phycocerythrin (PE)-conjugated tetramers, FITC-CD8 (clone HIT8; BD Biosciences, Heidelberg, Germany), and APC-CD45RA (clone MEM56; Caltag, Burlingame, CA) mAbs followed by four-color flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences). At least 3 × 10⁵ cells were analyzed using Flowjo software (TreeStar Inc., San Carlos, CA). Recordings were made only on propidium iodide–negative cells, and data were expressed as dot plots.

**IFN-γ ELISPOT assays.** The number of IFN-γ-producing T lymphocytes was determined as described previously (16). Briefly, dendritic cells pulsed with nonapeptides derived from respective TAs Hpa, MUC1, or HER-2/neu or from irrelevant control antigens insulin and HIV were cocultured with autologous T cells (dendritic cell/T-cell ratio, 1:5) for 40 hours. The number of IFN-γ spot-forming cells was measured using an Axioplan 2 microscope and KS ELISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany). Spots measured in the presence of dendritic cells pulsed with the insulin-derived peptide were considered nonspecific background (negative control). Samples were considered to contain tumor-reactive T cells when spot numbers in test triplicates significantly exceeded spot numbers in control triplicates (P < 0.05). In these samples, the number of tumor-reactive bone marrow T lymphocytes was calculated as follows: spot numbers in wells with tumor peptide-pulsed dendritic cells minus spot numbers in control wells.

**Intracellular IFN-γ detection.** Purified T cells were stimulated for 6 hours with autologous dendritic cells loaded with Hpa16. After 1 hour of T-cell stimulation, cytokine release was prevented by addition of GolgiStop to the stimulation culture according to the manufacturer's protocol (BD Biosciences). After 6 hours of T-cell stimulation, Hpa-specific CD8 T cells were stained by PE-labeled, Hpa16-loaded HLA-A0201 tetrameric complexes and FITC-conjugated anti-CD8 mAbs as described above, permeabilized using BD Cytofix/Cytoperm fixation kit according to the manufacturer's protocol (BD Biosciences), and stained for intracytoplasmic IFN-γ content using APC-labeled anti-IFN-γ mAb (clone R3-34; BD Biosciences) or respective isotype control (BD Biosciences). Aliquots of T cells were kept unstained and stained likewise as control.

**Expansion of Hpa-specific T cells.** Purified T cells from HLA-A2-positive breast cancer patients were cocultured with autologous dendritic cells pulsed with Hpa16 for 5 days in RPMI 1640 containing 10% AB serum, 200 units/mL IL-2, 60 units/mL IL-4, 20 mg/mL IL-15, 20 mg/mL IL-7, and 5% conditioned medium. Conditioned medium was prepared by stimulation of allogeneic PBMCs at concentrations of 1 × 10⁶ PBMCs/mL RPMI 1640 containing 10% AB serum, 200 units/mL IL-2, 60 units/mL IL-4, and 3 μg/mL phytomammaglutinin (Sigma, St. Louis, MO) for 4 days. The quantification of Hpa16-specific CD8 T cells was done before and after 5 days of stimulation culture using PE-labeled, Hpa16-loaded HLA-A0201 tetrameric complexes and FITC-conjugated anti-CD8 mAbs as described above.

**Generation of CTL and cytotoxicity assay.** The assay was done as described (16). Briefly, dendritic cells were loaded with Hpa peptides (final concentration, 10 μg/mL). The pulsed dendritic cells were coincubated for 7 days with autologous 7-day precultured T cells (dendritic cell/T-cell ratio, 1:5) to generate peptide-specific CTLs. ⁵¹Cr-labeled MCF7 cells transfected with Hpa (MCF7-Hpa) or an empty vector (MCF7-mock) or natural Hpa-expressing breast carcinoma lines were used as targets. In some experiments, HLA-I blocking mAbs (clone W6/32; kindly provided by Dr. G. Moldenhauer, The German Cancer Research Center, Heidelberg, Germany) were added during T-cell-target cell coculture to test for HLA-I restriction of cytolyis.

**Probability estimation for breast cancer–related death.** We used the web-based tool Adviant (http://www.adviantonline.com) to estimate 20 breast cancer patients for their probability to die of breast cancer. Adviant provides an algorithm to integrate major pathobiological variables of breast cancer patients and their tumors that influence their clinical outcome. The algorithm was independently validated by Olivetto et al. (18) and is recommended by the American Society of Clinical Oncology.

**Statistical evaluation.** All statistical analyses were done with two-sided Student's t test.

**Results**

**Characterization of HLA-A2-binding peptides from the Hpa sequence.** We selected from the SYPFETHY algorithm (http://www.uni-tuebingen.de) nonapeptides of the Hpa sequence (7), which should exhibit a high binding capacity to HLA-A*0201, a frequent class I MHC molecule expressed at the cell surface of ~50% of the northern European population. Three nonapeptides with binding scores between 28 and 31 were selected and synthesized within the German Cancer Research Center central
These nonamers were derived from positions 16 (Hpa16), 8 (Hpa8), or 183 (Hpa183) of the amino acid sequence of the molecule. All three peptides had lysine residues within the nonamers at positions (starting from the NH2 terminus) 2, 6, and 9. For comparison, we tested A*0201-binding peptides from known TAAs, such as MUC1 and HER-2/neu, or from self-antigens, such as insulin. Figure 1A and B shows the relative HLA stabilization capacity of the peptides as revealed from fluorescence-activated cell sorting (FACS) analysis with TAP-deficient T2 tumor cells. Although insulin peptide p34 showed the highest relative HLA stabilization capacity, the Hpa peptides had stabilization capacities that were in between those of HER-2/neu (p369) and MUC1 (p12).

Identification of Hpa peptide-binding CD8 T lymphocytes from cancer patients. From two of the three Hpa nonamers, we could obtain HLA-A*0201 tetramers, whereas Hpa183 did not form tetramers because of solubility problems. For control and comparison, we used HLA-A*0201 tetramers with a known peptide epitope of HIV (gag; SLYNTVATL). We analyzed HLA-A2+ T-cell samples from peripheral blood (PBTC) or bone marrow (BMTC) of nine primary operated breast cancer patients (P1-P9) and five healthy donors (HD1-HD5) via FACS cytometry for content of Hpa tetramer-binding CD8 T cells. An example is shown in Fig. 2A. CD8 BMTC (5.36%) bound to HLA-A201 Hpa16 tetramers and 0.36% bound Hpa8 tetramers. The majority of the tetramer-positive cells were CD45RA-negative memory T cells.

Figure 1. A and B, stabilization of HLA-A0201 on T2 cells after peptide loading. A, HLA-A2 expression as quantified by flow cytometry on T2 cells [dark gray histogram; arrow (T2)], T2 cells transfected with HLA-A0201 [light gray histogram; arrow (T2-HLA-A2)], and T2-HLA-A0201 cells pulsed with insulin-derived peptide 34 to 42 [white histogram; arrow (insulin p34)]. One representative example of three. B, mean relative HLA-A0201-binding affinities of different nonameric peptides (Hpa8, Hpa16, Hpa183, MUC1; HER-2/neu369,779; HER-2; and insulin p34), insulin. Columns, mean of at least three different experiments for each peptide.

Figure 2. Specificity for Hpa peptides of bone marrow and peripheral blood-derived CD8 T cells from healthy donors and breast cancer patients. A, flow cytometric analysis of gated viable (propidium iodide negative) CD8 bone marrow T cells from one representative breast cancer patient using PE-labeled HLA-A0201-Hpa peptide tetramers containing Hpa8, Hpa16, or the HIV-derived control peptide (HIVgag) and CD45RA-APC. Bars, numbers, relative percentage of tetramer-binding (top) or tetramer-nonbinding (bottom) cells. B and C, cumulative data of Hpa tetramer-binding viable CD8 T cells from bone marrow and peripheral blood of altogether five healthy donors (B) and nine breast cancer patients (C). Number, tested patient. Zero values mean either negativity or that the frequencies of specific T cells were too low (<1:105) to be detectable.
Control stainings with HIV gag-derived peptide tetramers revealed background values of double-stained CD8 T cells of ≤0.1%. Stainings with Hpa peptide-derived tetramers of cells from normal healthy donors (Fig. 2B) gave maximal values of 0.18%. Breast cancer patients contained higher frequencies of CD8 T cells binding either Hpa8 or Hpa16 tetramers (Fig. 2C). Hpa16-specific T cells were more often observed than Hpa8-specific T cells, and they sometimes could be as high as 3% to 5%. In some patients (P1 and P6), we detected T cells with specificity for both Hpa tetramers. Remarkably, all nine HLA-A2+ breast cancer patients tested contained Hpa tetramer-specific T cells in frequencies above healthy donors.

**Hpa peptide-reactive memory T cells from breast cancer patients producing IFN-γ.** From 20 HLA-A2+ breast carcinoma patients, we tested in ELISPOT assays the capacity of their BMTC to respond specifically to stimulation with autologous dendritic cells pulsed with Hpa peptide 8, 16, or 183. Responses were specific when they differed significantly (P < 0.05) from negative controls, in which we used a HLA-A2-binding peptide from insulin (p34). Figure 3A shows an example of the ELISPOT response of a patient’s BMTC on 48 hours of stimulation with autologous dendritic cells pulsed with the indicated peptides. Only the two Hpa-derived peptides p16 and p183 induced significant memory T-cell responses. As shown in Fig. 3B, BMTC from the patient group reacted six times against Hpa8, eight times against Hpa16, and six times against Hpa183. Nine of 20 (45%) patients showed positive responses to either one or several Hpa peptides. This compares favorably with reactivities to peptides from the known TAAs MUC1 (15%) or HER-2/neu (15%). Figure 3B also shows the results from corresponding ELISPOT tests with eight BMTC samples of HLA-A2+ healthy donors. Compared with insulin p34 as negative control, there was only one significant peptide reactivity and this was directed to Hpa8.

The calculated numbers of TAA peptide-specific ELISPOT-reactive T cells per 10^6 BMTCs are shown in Fig. 3C for patients and healthy donors. Zero values mean that there was no significant difference detectable between test wells and control wells. Individual values are represented by black circles, and mean responses are represented by bars. The highest frequencies were observed in patients with cells responding to Hpa peptides. Thus, the frequency of Hpa-reactive cancer patients (Fig. 3B) and the mean number of specific T cells reacting to any one of the three Hpa peptides (Fig. 3C) were higher than those reacting to the established TAAs MUC1 or HER-2/neu. Because there are several 100 Hpa-derived peptides that fit into HLA-A2 with binding scores >10, the memory repertoire of breast cancer patients may contain a broad spectrum of Hpa-specific CD8 T cells.

**Expansion of tetramer-binding CD8 T cells.** Whereas all previous tests were done with short-term restimulation cultures, we next tested the potential to expand Hpa-specific T cells on 5-day stimulation with peptide-presenting dendritic cells. As shown by the results of Fig. 4A, this was possible with cells from bone marrow and blood. Although there was a low frequency of Hpa-specific cells in the cultures, we obtained on average a 15-fold increase in cell numbers. We next determined whether the stimulated cells were functional. Staining for intracellular IFN-γ revealed that, among tetramer-positive CD8 T cells, stimulated cultures contained significantly more IFN-γ-producing cells than unstimulated cultures. This was true for five samples analyzed 6 hours after stimulation and for three samples analyzed 5 days after stimulation.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Hpa-reactive memory T cells from bone marrow of breast cancer patients. A. Short-term IFN-γ ELISPOT assay of one representative breast cancer patient tested for reactivity Hpa8, Hpa16, Hpa183 (black columns). MUC1-derived peptide MUC1p12. HER-2/neu-derived peptide 369 (light gray columns). IMP-derived peptide p58 (dark gray column), and against HIV-derived peptide gag or insulin-derived peptide p34, which served as negative controls (white columns). Columns, mean of at least three wells per group; bars, SD. *, P < 0.05, significant difference compared with control group stimulated with insulin p34. B, proportions of patients and healthy donors containing T cells reactive against Hpa8, Hpa16, Hpa183, MUC1p12, or HER-2/neu in their bone marrow as revealed by IFN-γ ELISPOT assay. Patients were considered to contain tumor-reactive T cells (black columns, peptide reactive) when spot numbers in test triplicates significantly exceeded spot numbers in control triplicates (P < 0.05); otherwise, these patients were considered nonreactive (white columns). Columns, mean of at least three wells per group; bars, SD. C, frequencies of bone marrow T cells reactive against respective TAAs among total T cells from breast cancer patients and healthy donors. Dot, tested patient. Bars, mean values.

**Generation of Hpa peptide-specific CTLs.** The presence of Hpa peptide-reactive memory T cells in cancer patients suggests processing and presentation of Hpa peptides by host dendritic cells according to the predictions of the SYFPEITHY computer program by which the peptides had been selected. Of further relevance was
the question whether similar peptides are also processed and presented by tumor cells themselves so that they can be recognized as targets by respective specific CTLs. In addition, it was important to find out whether one could generate Hpa peptide-specific CTLs from cancer patients. To investigate these questions, we used as target cells MCF7 breast cancer cells transfected either with Hpa (MCF7-Hpa) or with the empty eukaryotic expression plasmid pcDNA-3 (MCF7-mock). Stainings with a Hpa-specific mAb showed a much stronger cytoplasmic staining with the Hpa transfectant than with the mock transfectant, and results from PCR showed a much stronger band from the Hpa than from the mock transfectant, whereas no differences about viability, chromium uptake, or spontaneous chromium release were detectable (data not shown).

To generate Hpa-specific CTLs, BMTCs from breast cancer patients were cocultured for 7 days with autologous dendritic cells pulsed with respective Hpa peptides. The cytolytic activity of the generated effector cells was then evaluated in a 4-hour $^{51}$Cr release assay using as target cells $^{51}$Cr-labeled MCF7-Hpa or MCF7-mock cells. Figure 5A shows mean CTL reactivities from five tested HLA-A2-positive patients. CTLs reactivated with each Hpa peptide reacted significantly stronger against the Hpa transfectant than against the control cell line. These results thus show (a) that all three Hpa peptides are physiologically processed and presented by MCF7-Hpa cells and (b) that Hpa-specific CTLs can be generated from BMT of breast cancer patients.

Hpa peptide-restimulated BMTC or PBTC from breast cancer patients also reacted significantly stronger against natural Hpa-expressing breast cancer cells than nonstimulated control cultures as shown by the results in Fig. 5B and C. This was true for three different nontransfected HLA-A*0201-positive cell lines. The results were reproduced with effector cells from eight different additional patients. Hpa16 peptide-stimulated test cultures contained significantly higher antitumor cytotoxic activity than control cultures. The CTL activity could be blocked on addition of anti-HLA class I mAb to the 4-hour $^{51}$Cr release assay (Fig. 5C), thus showing MHC I restriction of the peptide-specific CTLs.

Clinical relevance. To get a first idea about a possible relevance of patient’s immune reactivity to Hpa, we used the web-based algorithm Adjuvant! (18), which includes the major established breast cancer risk factors to estimate the probability of patients to die of cancer. In this framework, we compared Hpa-reactive versus Hpa-nonreactive patients as assessed by IFN-γ ELISPOT analysis. Figure 6 shows that the 9 patients with Hpa reactivity had a significantly lower probability to die of cancer than the 11 Hpa-negative patients.

Figure 4. A, frequencies of Hpa16-specific CD8 T cells in unstimulated and Hpa16-stimulated T-cell samples from peripheral blood (black circles) or bone marrow (white and gray circles) of patients 29 (white circles) and 30 (gray and black circles) as analyzed by flow cytometric staining with Hpa16-loaded HLA-A0201 tetrameric complexes at day 0 (unstimulated) and 5 days after stimulation with Hpa16 (stimulated d5). Full lines, connect values before and after stimulation of corresponding samples. B and C, intracellular IFN-γ production in Hpa16-specific CD8 T cells from breast cancer patients before and after stimulation with Hpa16 as detected by flow cytometric analysis of gated CD8+ Hpa16−HLA-A0201 tetramer-binding cells. B, IFN-γ expression by Hpa16-specific CD8 T cells from one representative sample (P31). Filled gray histogram, isotype control; open gray histogram, unstimulated T cells; black open histogram, 6 hours after Hpa16 stimulation. C, proportions of IFN-γ-expressing Hpa16 tetramer-binding CD8+ T cells as mean (columns) and SD (bars) of bone marrow and peripheral blood samples from eight different patients (P31-P38). Hpa16-stimulated T cells were analyzed in five samples after 6 hours and in three samples after 5 days. *, $P < 0.01$, significant difference between IFN-γ content of stimulated and unstimulated T cells.
Discussion
Recent studies indicate that the activity of Hpa, which releases growth factors (19, 20) from HSPGs (12) and generates highly active heparan sulfate fragments (4, 5), promotes growth and metastasis of breast tumors (12). We report here the existence of Hpa peptide-binding and peptide-reactive CD8 memory T cells in a high proportion of breast cancer patients. Their frequencies ranged between 1:600 and 1:13,000. Hpa seems to induce an equal or even stronger immune response in breast cancer patients than the previously described TAAs MUC1 or HER-2/neu. Because Hpa overexpression seems to characterize metastatic cells, Hpa is an interesting new TAA to target cancer cells with metastatic

Figure 5. Cytotoxic activity of bone marrow T cells from breast cancer patients stimulated against Hpa peptides and tested against the indicated target cells. A. BMTCs from breast cancer patients were cocultured for 7 days with autologous dendritic cells pulsed with respective Hpa peptides (Hpa8, Hpa16, or Hpa183). After 7 days of coculture, the cytolytic activity of the cells was evaluated in a 4-hour 51Cr release assay using as target cells 51Cr-labeled MCF7-Hpa or MCF7-mock cells. Points, mean of cytolytic capacity of all five tested patient samples; bars, SD. *, P < 0.05, significant difference between lysis of Hpa-positive target and control. B and C, for generation of CTLs, the BMTCs were cocultured for 7 days with Hpa16-loaded autologous dendritic cells (filled symbols) or they were cultured alone (open symbols, controls). They were then tested for cytolytic activity against 51Cr-labeled natural Hpa-expressing HLA-A0201* breast cancer cell lines BT-549 (B) or HTB-124 or BT-20 (C). Points, mean of cytolytic capacity of four patient samples; bars, SD. **, P = 0.0003, significant difference between all data points from stimulated compared with nonstimulated cultures. The individual Ps were 0.02 (P48), 0.05 (P51), 0.07 (P49), and 0.08 (P50). C, one representative chromium release assay to show HLA-I-restricted lysis of Hpa-positive HTB-124 breast cancer cells by Hpa16-stimulated T cells from three T-cell samples (two bone marrow and one peripheral blood) derived from 2 HLA-A2-positive breast cancer patients and of Hpa-positive BT-20 breast cancer cells by Hpa16-stimulated bone marrow T cells from 1 HLA-A2-positive breast cancer patient. In all tested cases, tumor cell lysis by Hpa16-stimulated T cells (black squares) was significantly enhanced compared with lysis by unstimulated T cells (open triangles) observed and also significantly reduced by anti-HLA-I mAb blocking (black inverted triangles).
potentially. Our data therefore suggest to exploit Hpa-reactive memory T cells in future immunotherapy trials.

A previous study revealed that ~70% of tumor antigen-specific T cells in the bone marrow of breast cancer patients belonged to the memory T-cell population, whereas only 25% were effector T cells. Among the TAA-specific memory T-cell population, 75% were effector memory T cells and 25% were central memory T cells (16, 21). These data are in accordance with our finding that Hpa-specific T cells as determined by HLA tetramer stainings mainly belonged to the CD45RA memory T-cell fraction. The memory T-cell nature of these cells was furthermore corroborated by their capacity to release IFN-γ within 40 hours after antigen stimulation, a feature that we detected only in separated CD45RA+ (naive/effector) T-cell fractions. Such memory T cells could have been generated in the bone marrow where T-cell priming can occur against blood-borne antigens (21). Alternatively, memory T cells could have been generated in true secondary lymphatic organs draining the sites of primary tumor or metastases and may then have homed to the bone marrow as a preferred site for survival.

We were particularly interested in functional activity of Hpa-specific T cells because tetramer analysis does not allow to distinguish between antigen-reactive and anergic T cells. We and others described before the presence of tetramer-positive T cells in the blood of cancer patients, although a functional reactivity could not be shown (21). It is therefore important to point out that this analysis shows functional reactivity of Hpa-specific T cells from breast cancer patients in two distinct assays (IFN-γ production and antitumor cytotoxicity). The memory nature of the Hpa-specific T cells together with their capacity, after specific reactivation, to destroy natural breast cancer cells from patients support the conclusion that Hpa peptides ought to be good candidates to be included in a cancer vaccine.

Recently, it was shown that soluble heparan sulfate chains, as products of the degradation of HSPG, deliver activating signals to macrophages (23) and induce functional maturation in dendritic cells (24), thus contributing to the generation and maintenance of primary immune responses. Heparan sulfate from the cell surface (3, 10) or from the ECM is a chemically distinct stimulator of Toll-like receptor (TLR) 4 (25). Recently, a surveillance has been proposed (26), which suggests that TLRs on immune cells respond (a) to the degradation of endogenous macromolecules in local microenvironments that coincide with infection and injury and (b) to exogenous molecules from microorganisms and perhaps from necrotic cell death. This surveillance model presents a common mechanism for activation of TLRs during both development and immunity (27, 28).

With regard to our findings, we propose that the high frequency of Hpa-specific and Hpa-reactive T cells detected in this study in cancer patients is related to activation of TLRs on dendritic cells in the vicinity of metastases and tissue destruction. Although these dendritic cells might be involved in processing Hpa from metastases, their activation via heparan sulfate interacting with TLR might trigger adaptive immune responses. Hpa activity also plays a role in physiologic processes, including T-cell-mediated immune responses. Because we did not detect Hpa-reactive memory T cells in normal healthy donors, we believe that anti-

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\text{\% probability to die of cancer (IFN-\(\gamma\) ELISPOT)}
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\begin{align*}
\text{HPA-reactivity :} & \\
\text{positive} & (n = 9) \\
\text{negative} & (n = 11)
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! \[\text{Figure 6. Probability to die of cancer in 20 breast cancer patients analyzed for presence of Hpa-reactive bone marrow T cells by IFN-\(\gamma\) ELISPOT assay.}
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Nine patients containing Hpa-reactive bone marrow T cells (light gray column) revealed a significantly \(P < 0.05\), two-sided Student’s t test) reduced probability to die of breast cancer compared with 11 patients lacking such T cells (dark gray column). Column, mean; bars, SE.

Hpa T-cell reactivity is induced only in association with pathologic conditions, such as tissue destruction associated with metastasis formation.

In case of Hpa and also of hyaluronidase (29), it seems that the substrates of the active enzymes activate antigen-presenting cells (APC) via TLR-4 so that the closely associated enzymes themselves become processed and presented to T cells, resulting in immune responses rather than tolerance. This interpretation might perhaps explain why we observed a higher frequency of Hpa memory T cells than of memory T cells to other TAAs where such endogenous activation of APCs is lacking. We only analyzed responses to three Hpa-derived peptides with high binding scores for HLA-A2. We identified altogether ~ 300 to 400 nonapeptides from Hpa, which could also bind with a reasonable affinity to HLA-A2. Our results thus likely underestimate the total Hpa-specific memory pool that might exist in cancer patients. We have to consider, however, that T cells with low avidity and poor tetramer staining may be less effective to provide protective potential than those with intermediate or high affinity.

Several aspects of Hpa suggest that this protein may serve as a new interesting tumor target: (a) there is only one gene in the human genome coding for this enzyme and no polymorphism has been described yet; (b) specific blockade of this enzyme should prevent heparan sulfate degradation from HSPG and thus reduce tumor angiogenesis and invasion; (c) as shown here, the T-cell memory repertoire of breast cancer patients compared with normal healthy persons is enriched with Hpa-reactive CD8 T cells, which can be further activated to become CTLs; and (d) if a tumor develops immune escape variants against such CTLs by down-regulating Hpa expression, such variants might have reduced angiogenic and invasive capacity.

Hpa expression was recently suggested to be associated with the metastatic potential of human tumors, such as pancreatic adenocarcinoma (30, 31), breast cancer (12), and others (32, 33).

\(a\) C. Choi, P. Beckhove, and V. Schirrmacher, unpublished observation.
Hpa expression correlated with a lack of heparan sulfate deposition in the basement membrane and with a more malignant phenotype as revealed by higher invasiveness and metastatic potential (32). Immune responses against Hpa can be expected to reduce its activity and thus to lower the loss of heparan sulfate from basement membrane in invasive breast carcinoma. This could possibly explain why, in our study, the patients with Hpa reactivity were characterized by a significantly lower probability to die of cancer than the Hpa nonreactive patients. In pancreatic cancer, postoperative survival correlated inversely with Hpa expression of the tumor, reflected by a median survival of 34 and 17 months for Hpa-negative and Hpa-positive tumors, respectively (30, 31). Activation of the repertoire of Hpa-reactive memory T cells via specific vaccination could be expected to expand the median survival in patients with Hpa-positive tumors.

Acknowledgments

Received 7/6/2005; revised 5/8/2006; accepted 5/31/2006.

Grant support: German-Israeli Foundation grant CA 90/01682 and Tumorzentrum Heidelberg Mannheim grant FSP III/2 (P. Beckhove).

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We thank the NIH tetrramer facility for the production of tetramers.

References


