Correlations between Serum Monocyte Chemotactic Protein-1 Levels, Clinical Prognostic Factors, and HER-2/*neu* Vaccine-Related Immunity in Breast Cancer Patients

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Abstract

Purpose: We studied serum monocyte chemotactic protein-1 (MCP-1) levels in breast cancer patients in relationship to their clinicopathologic variables and immune response to a HER-2/*neu* E75 vaccine.

Experimental Design: We measured MCP-1 levels in 32 HER-2/*neu*$^+$ breast cancer patients before and after vaccination with a HER-2/*neu* E75 peptide + granulocyte macrophage colony-stimulating factor vaccine. Clinical prognostic variables were collected. Vaccine-specific immunologic responses were monitored.

Results: Serum MCP-1 levels >250 pg/mL (MCP-high) correlated with favorable prognostic variables. MCP-high patients compared with MCP-low (<250 pg/mL) patients showed statistically significant later onset of disease, earlier stage of disease, fewer nodal metastasis, and less chemotherapy. MCP-high patients had increased levels of preexisting HER-2 immunity when compared with MCP-low patients (69% versus 21%; $P = 0.02$). However, MCP-low patients showed higher inducible levels of MCP-1 compared with MCP-high patients (median increase, 41% versus 0%; $P = 0.001$) after vaccination. Moreover, MCP-low patients with >50% increase in MCP-1 levels (response-high) had worse clinical prognostic variables compared with patients with <50% increase (response-low). Response-high patients had statistically significant more poorly differentiated tumors, later stage of disease, and higher percentage of large tumors. Patients with >30% postvaccination MCP-1 increase also showed significant increases in E75-specific CD8$^+$ T-cells (0.05% versus 0.38%; $P = 0.03$) in response to vaccination.

Conclusions: High serum MCP-1 levels in breast cancer patients correlate with favorable prognostic variables and increased preexisting HER-2/*neu* immunity. E75 vaccination induces the largest MCP-1 response in patients with unfavorable clinicopathologic variables. Therefore, low serum MCP-1 levels may identify patients with worse prognosis and those most likely to benefit from this vaccination.

In 1863, Rudolph Virchow was the first to observe the link between malignancy and chronic inflammation (1, 2). In 1909, Paul Ehrlich postulated the role of the immune system in the repression of the subclinical carcinomas (3). However, the theory of immune control of subclinical tumors was ahead of its time and had to await the field of immunology. In 1957, Sir Macfarlane Burnet and Lewis Thomas described the concept of "immunosurveillance," which credits the immune system with constant surveillance and deletion of preneoplastic cells that arise due to natural instability (4, 5). With the advent of clinical transplantation, the immunosuppressed as well as the immunodeficient individual showed an increased incidence of malignancies, thereby establishing the physiologic role of the immune system in the suppression of neoplasms and paving the way for the development of the field of immunotherapy.

Given the limited effectiveness of current treatments for metastatic breast cancer, numerous investigators have attempted various modalities of immunotherapy to target established disease. Although results from monoclonal antibody treatment in conjunction with adjuvant therapy have shown promise in the treatment of metastatic breast cancer (6, 7), cancer vaccines have not shown the same desired effect (8, 9). This is not altogether surprising considering that the natural history of metastatic disease is based on cancer cells becoming resistant to both

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immunoregulation and conventional therapies (10, 11). Such observations emphasize the need to use cancer vaccine therapies in patients who are disease-free following conventional therapies or as part of a multimodality adjuvant therapy (10–12).

We are currently conducting phase I/II studies investigating a HER-2/*neu* immunogenic peptide (E75) with granulocyte macrophage colony-stimulating factor (GM-CSF) as a simple vaccine strategy for breast cancer. In our clinical trials, we are monitoring the safety and assessing the optimal dosing of this vaccine required to produce a peptide-specific immunologic response. Most importantly, we are vaccinating immunocompetent patients with breast cancer who are disease-free after standard conventional therapies but who are at high risk for recurrence (12). By studying these patients, we are determining if induced E75-specific immunity conveys clinical benefit by preventing recurrence. Furthermore, we are investigating novel methods for monitoring these and future vaccine trials (13).

We have done previously a preliminary analysis of 22 different cytokines in the serum of breast cancer patients from our clinical trials and compared them with healthy female controls (14). Furthermore, we compared the serum cytokine profiles between node-negative and node-positive patients and analyzed cytokine levels in the same patients before and after vaccination. This analysis revealed significant differences in the serum cytokine profiles in these patients, the most striking of which was exhibited by the preexisting levels and vaccine-inducible levels of the chemokine monocyte chemotactic protein-1 (MCP-1).

MCP-1 is a 76–amino acid protein that was originally purified and cloned from human gliomas and myelomonocytic cells in 1989 (15). It is the first chemokine discovered in the C-C subfamily of chemokines and is produced by a variety of cells, including monocytes, smooth muscle cells, fibroblasts, and endothelial cells, and several malignant tumors. As originally described, its main function is chemotaxis to monocytic cells. However, subsequent research has implicated MCP-1 as an active participant in the tumor microenvironment, influencing factors, such as tumor-associated macrophages, angiogenesis, and metastasis (16–18). Despite these studies of MCP-1 in the tumor microenvironment, no consensus exists as to the cellular origin that results in serum levels of this chemokine and the current status is that both the immune system and the tumor microenvironment components may contribute to its circulating levels.

In this study, we have compared the serum MCP-1 levels in 32 HER-2/*neu*$^+$ breast cancer patients before initiating vaccinations with their known clinical prognostic variables as well as with available immunologic evidence of their preexisting antitumor immunity. Given the described role of MCP-1 as a proinflammatory mediator, we have also investigated the ability of our vaccine to induce MCP-1 levels and correlated the extent of induction with the prognostic variables of patients as well as other immunologic evidence of response to the E75 peptide vaccine.

Materials and Methods

Patient characteristics and clinical protocols. The Department of Clinical Investigation, Walter Reed Army Medical Center, approved these clinical protocols. These clinical trials are conducted under an Investigational New Drug Application (IND#9187) approved by the Food and Drug Administration. All patients had histologically confirmed breast cancer that expressed HER-2/*neu* by standard immunohistochemistry. All breast cancer patients had completed a standard course of surgery, chemotherapy, and/or radiation therapy, as required, before enrollment, and those patients on chemoprevention were continued on their specific regimen. After screening for eligibility criteria and proper counseling and consenting, these patients were enrolled into the studies and then HLA typed to determine their HLA-A2 status because E75 binds this specific HLA allele found in ~40% to 50% of the general population (19). HLA-A2$^+$ patients were vaccinated, whereas HLA-A2$^-$ patients were followed prospectively as matched controls for clinical recurrence. Before vaccination, patients were skin tested with a panel of recall antigens (Mantoux test = mumps, tetanus, and *Candida*). Patients were considered immunocompetent if they reacted (>5 mm) to two or more antigens.

Vaccine. The E75 peptide was commercially produced in good manufacturing practices grade by Multiple Peptide Systems (San Diego, CA). The peptide was purified to >95%. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in sterile saline at the following concentrations: 100 μg in 0.5 mL, 500 μg in 0.5 mL, and 1 mg in 0.5 mL. The peptide was mixed with GM-CSF (Berlex, Seattle, WA) at 250 μg in 0.5 mL, and the 1.0 mL inoculation was split and given intradermally at two sites within 5 cm of each other. All inoculations were given in the same extremity.

Node-positive vaccination series. The study was done as a two-stage safety trial (12). In the first stage, three patients were assigned to each dose/schedule group receiving six monthly inoculations: 100 μg (100.6), 500 μg (500.6), or 1,000 μg (1,000.6) of E75 peptide + GM-CSF. A fourth group received 500 μg peptide + GM-CSF but only four inoculations (500.4), omitting the fourth and fifth vaccinations. In the second stage as shown in Table 1, four additional groups with six patients each were vaccinated as follows: 500.4, 500.6, 1,000.6, and 1,000.4. All of the node-positive vaccinated patients discussed in this article belonged to the second stage of this trial and were a part of the 500.6 (3 patients), 500.4 (3 patients), 1,000.6 (2 patients), and 1,000.4 (2 patients) categories, all receiving 250 μg GM-CSF. The choice of the patients from the second stage of the trial was due to the implementation of a serum banking strategy that was activated only during the second stage of the trial. As such, the earlier groups of patients did not have serum samples available for analysis. For the 10 node-positive patients used in this analysis, the mean age was 54 years and 90% of the patients had received chemotherapy. The mean time elapsed from completion of chemotherapy to enrollment and MCP-1 level determination was 14.2 months. All patients were found to be immunologically competent by Mantoux testing before enrollment in the clinical trial.

Node-negative vaccination series. Similar to the node-positive patients, node-negative patients have also undergone primary surgical and medical therapies and were considered to be without evidence of disease at the time of enrollment into the trial. The purpose of this ongoing trial is to determine the optimal dose of the immunoadjuvant, GM-CSF, and the optimal schedule of inoculations for the E75 + GM-CSF vaccine. Based on the study design as shown in Table 1, patients are vaccinated according to a dose and schedule escalation scheme (five groups with 10 patients in each). The first group of 10 patients in the trial was vaccinated with 500 μg E75 peptide and 125 μg GM-CSF under schedule 1 (0, 1, and 5 months for a total of three doses), and the second group was vaccinated with the same dosing but under schedule 2 (0, 1, 2, and 5 months for a total of four doses). The third group of 10 patients are receiving 500 μg E75 peptide vaccine in addition of 250 μg GM-CSF under schedule 2. The two remaining groups of the five total groups are to be vaccinated with escalating doses of the E75 peptide and more frequent schedules. All of the 22 node-negative patients used in this analysis were from the first three groups, and the mean age was 53 years. Of these node-negative patients, 40% had received chemotherapy, and the mean time elapsed from completion of chemotherapy to enrollment and MCP-1 level determination was

25.2 months. All patients were found to be immunologically competent by Mantoux testing before enrollment in the clinical trial.

Peripheral blood collection and preparation of serum. Peripheral blood was drawn from patients before receiving each inoculation and at 1 and 6 months after completing the series for the isolation of peripheral blood mononuclear cells used in immunologic monitoring assays of the clinical trials (13). For the preparation of serum samples, peripheral blood (10 mL) was drawn into a Vacutainer Gel & Clot Activator tube (Becton Dickinson, Franklin Lakes, NJ) and centrifuged. The serum was then aspirated and aliquoted into Nunc cryovial tubes and placed in a −84°C freezer. The serum samples were thawed immediately before their use for the measurement of cytokine levels. The procedures for collection, preparation, freezing, and thawing of all the serum samples used in this study were done in an identical and consistent manner. None of the serum samples had been thawed previously before thawing for the Luminex assay.

HLA-A2:Ig dimer assay. The presence of $CD8^+$ E75-specific cells in freshly isolated peripheral blood mononuclear cells from patients was directly assessed using the dimer assay as described previously (13). Briefly, the HLA-A2:Ig dimer (PharMingen, San Diego, CA) was loaded with the E75 or control peptide (E37) by incubating 1 μg dimer with an excess (5 μg) of peptide and 0.5 μg β_2-microglobulin (Sigma, St. Louis, MO) at 37°C overnight and then stored at 4°C until used. Freshly isolated peripheral blood mononuclear cells were plated at 5×10^5 per well in round-bottomed 96-well plates (Becton Dickinson) and washed twice with stain buffer (PharMingen). Human γ-globulin (Sigma) was added and the samples were incubated for 5 minutes before adding the dimer preparations. The cells were incubated with the peptide-loaded dimer (at 1 μg dimer/well) for 45 minutes and washed once in PBS. Cells were then stained with rat anti-mouse IgG1-phycoerythrin (clone A85-1), CD8-FITC, and CD3-APC (PharMingen). All incubations were done at 4°C. Two-color fluorometric analysis was carried out on a BD FACSCalibur analyzer (Becton Dickinson). The data were analyzed using the CellQuest software and displayed as a dual-variable density plot correlating CD8-FITC and IgG1-phycoerythrin fluorescence. Quadrants were set based on staining obtained using irrelevant peptide (E37)–loaded dimers as a negative control. Results are expressed as the percent of E75-specific CTL (control E37 dimer results subtracted) of the total $CD8^+$ population.

Serum cytokine measurement by Luminex technology. We used the Luminex 100 system (Luminex Corp., Austin, TX) to evaluate the sera from a total of 32 breast cancer patients (22 node-negative and 10 node-positive) who were deemed without evidence of disease following standard therapies. Levels of 22 cytokines, including MCP-1, were assessed. The assays were repeated and our data were replicated. We used the Lincoplex kit (Linco Research, St. Charles, MO). Briefly, 25 μL diluent and 25 μL serum were added to each well. Mixed microbeads (25 μL) were added. The plate was incubated and agitated for 1 hour, washed, and reincubated with 25 μL detection antibody for 30 minutes. The plate was then washed again and incubated with 25 μL streptavidin-phycoerythrin for 30 minutes. The plate was then washed twice and the beads were resuspended in the plate with 100 μL sheath fluid. The plates were then analyzed using the Luminex 100 system. The readout for the concentration of each cytokine was detected as mean fluorescence intensity by the instrument. These values were subsequently converted to picogram per milliliter of cytokine based on the mean fluorescence intensity values from a set of standards that were run simultaneously in the assay.

Statistics. Summary statistics were obtained using established methods. Associations between nonparametric categorical variables were evaluated using Wilcoxon signed rank test for the related data and the Wilcoxon rank sum test for unrelated data. χ^2 and unpaired t tests were used when applicable. $P < 0.05$ was considered significant.

Results

Correlation of serum MCP-1 levels and prognostic variables. We have shown previously increased serum MCP-1 levels in breast cancer patients compared with healthy, female controls (14); however, there was substantial variability in levels among the patients. Therefore, we have further analyzed the prevaccination MCP-1 levels in 32 breast cancer patients, including 22 node-negative and 10 node-positive patients, who have enrolled into clinical trials of the E75 HER-2/*neu* peptide vaccine (see Materials and Methods). These patients were sorted according to increasing serum MCP-1 levels (Table 2). An arbitrary cutoff of 250 pg/mL was identified based on the prevalence of nodal status and stage of disease (solid line; Table 1). Patients with serum MCP-1 levels below this cutoff amount were labeled MCP-low, and those with levels above this threshold were labeled MCP-high. The MCP-low group consisted of 22 patients, whereas the MCP-high group consisted of 10 patients. The average serum MCP-1 level for MCP-high group was 373.4 pg/mL compared with MCP-low serum MCP-1 levels of 118.1 pg/mL (P = 0.00002). The MCP-high group consisted of node-negative patients exclusively. Further analysis showed the MCP-high group to be associated with better prognostic variables compared with MCP-low group (Table 3). Age at onset of disease was significantly younger in MCP-low group compared with MCP-high group (53.8 versus 63.9, respectively; P = 0.01). The percentage of MCP-low patients with stage II or worse disease based on the American Joint Commission on Cancer 2002 classification was 55% compared with 0% for the MCP-high group (P = 0.003). Likewise, the percentage of patients in the MCP-low group with metastasis to the axillary lymph nodes was 45% compared with 0% for

Table 1. Vaccination trial groups

Trial	Group	Peptide dose (μg)	GM-CSF dose (μg)	Inoculations (total no.)	Schedule (mo)
Node-positive	500.4	500	250	4	0, 1, 2, 5
	500.6	500	250	6	0, 1, 2, 3, 4, 5
	1,000.4	1,000	250	4	0, 1, 2, 5
	1,000.6	1,000	250	6	0, 1, 2, 3, 4, 5
Node-negative	500.3	500	125	3	0, 1, 5
	500.4	500	125	4	0, 1, 2, 5
	500.4	500	250	4	0, 1, 2, 5
	500.6	500	250	6	0, 1, 2, 3, 4, 5
	1,000.6	1,000	250	6	0, 1, 2, 3, 4, 5

Table 2. Correlation of serum MCP-1 levels with known clinical prognostic variables in breast cancer patients

Study no.	Serum MCP-1 level	Age	Race	Grade of tumor	Stage 2 or above	Tumor T_2 or above	Node-positive	No. nodes	Estrogen/progesterone receptor +/+	HER-2/*neu* oncogene 3+ by IHC	Chemotherapy
MCP-low											
NNV15	28.8	58	White	—	No	No	No	0	Yes	No	No
B54	40.8	45	White	Poor	Yes	Yes	Yes	4	Yes	Yes	Yes
B57	47.8	62	White	Poor	Yes	No	Yes	1	No	Yes	Yes
NNV11	50.19	45	Other	Poor	No	No	No	0	No	No	Yes
NNV22	59.37	53	White	—	No	No	No	0	—	—	No
NNV23	62.7	46	White	Poor	Yes	Yes	No	0	Yes	No	Yes
B46	70.7	52	White	Mod	Yes	No	Yes	2	No	Yes	Yes
NNV6	80.35	46	White	Mod	No	No	No	0	Yes	No	Yes
NNV4	85.9	53	White	Mod	No	No	No	0	Yes	No	Yes
NNV19	86.4	50	Other	Mod	No	No	No	0	Yes	No	Yes
B41	93	49	White	Well	Yes	No	Yes	2	Yes	No	Yes
B56	97.27	43	White	Poor	Yes	No	Yes	6	No	Yes	Yes
NNV14	123.33	74	White	Mod	No	No	No	0	Yes	—	No
B53	135	50	White	Mod	Yes	No	Yes	2	No	No	Yes
B50	136.33	51	White	Poor	Yes	Yes	Yes	5	No	No	Yes
B44	155.32	62	Other	Poor	Yes	Yes	Yes	3	No	No	Yes
NNV20	157.18	55	White	Mod	No	No	No	0	Yes	No	No
NNV13	178.08	52	White	Poor	Yes	Yes	No	0	Yes	Yes	Yes
NNV10	196.7	57	White	Well	No	No	No	0	Yes	No	—
NNV5	230.16	59	Other	Mod	No	No	No	0	No	Yes	Yes
B51	237.6	73	White	Well	Yes	No	Yes	1	Yes	No	No
B55	244.6	48	White	Poor	Yes	Yes	Yes	8	Yes	Yes	Yes
MCP-high											
NNV12	266.23	61	White	—	No	No	No	0	No	Yes	No
NNV3	281.18	77	Other	Well	No	No	No	0	Yes	No	No
NNV8	285.21	62	White	Well	No	No	No	0	Yes	No	No
NN21	287.6	58	White	Mod	No	No	No	0	Yes	Yes	Yes
NNV24	337.6	56	White	Well	No	No	No	0	Yes	No	No
NNV18	357.7	77	White	Poor	No	No	No	0	Yes	No	No
NNV17	419.5	62	Other	Poor	No	No	No	0	Yes	No	No
NNV7	427.38	50	White	Mod	No	No	No	0	No	No	No
NNV9	453.8	74	White	Mod	No	No	No	0	Yes	No	No
NNV16	617.32	62	White	—	No	No	No	0	Yes	—	No

NOTE: An arbitrary cutoff (250 pg/mL) in the serum MCP-1 levels was found to generally divide breast cancer patients into those with favorable and unfavorable clinical prognostic variables. NNV, node-negative patients; B, node-positive patients; Poor, poorly differentiated tumor; Mod, moderately differentiated tumor; Well, well-differentiated tumor; IHC, immunohistochemistry.

MCP-high group (P = 0.01). As a result, the MCP-low group patients were more likely to receive postoperative chemotherapy compared with MCP-high group (76% versus 10%; P = 0.002). Although a comparison of the two groups showed that the MCP-low group harbors larger tumors, higher expression of HER-2/*neu* protein, and less differentiated tumors as described by poorer grade and lack of estrogen receptor or progesterone receptor, they did not reach statistical significance.

Correlation of serum MCP-1 levels and preexisting HER-2/neu immunity. Having observed the strong correlation of serum MCP-1 with known clinical prognostic variables, we compared the serum MCP-1 levels of patients with any evidence of preexisting antitumor immunity. The latter was assessed using the HLA-A2:Ig dimer assay to show the presence of E75 peptide-specific $CD8^+$ T cells before vaccination. We have shown previously that many patients with HER-2/*neu*-expressing cancers have significant levels of these E75-specific CTLs, which are capable of recognizing and lysing HER-2/*neu*-expressing tumor cells (12, 13). Using the same ordering of patients by increasing serum MCP-1 levels and the previously defined cutoff amount, we compared the percentage of patients with >0.3% E75-specific $CD8^+$ T cells in the MCP-low and MCP-high groups. The level of 0.3% E75-specific CTL has been established previously as the threshold value for detecting the presence of preexisting immunity (based on our 4 years of experience with the HLA-A2:Ig dimer assay for the measurement of E75-specific CTL in cancer patients and normal individuals; ref. 20). This analysis revealed that only 14% of the MCP-low group had

significant levels of preexisting E75-specific CD8$^+$ T cells compared with 60% of the MCP-high group (P = 0.02; Fig. 1).

Effects of vaccination on serum MCP-1 levels and correlation with clinical variables. Our previous analysis of serum cytokine profiles in patients vaccinated with the E75 peptide showed a statistically significant increase in serum MCP-1 levels following vaccination with the E75 peptide (14). When analyzing the previously defined MCP-high group, no substantial enhancement was noted after vaccination. Having observed a spectrum of serum MCP-1 response to vaccination in the MCP-low cohort, we chose to analyze this subgroup further. We calculated the percentage change of serum MCP-1 levels following vaccination. The formula used was [(Post − Pre) / Pre] × 100, where Post refers to serum obtained after two monthly vaccinations and Pre refers to serum samples obtained before initiation of the vaccination series. The patients were then sorted according to increasing percentage change in postvaccination serum MCP-1 levels (Table 4). An arbitrary break of 50% increase postvaccination in serum MCP-1 level was noted in the MCP-low group (dashed line; Table 4). The subgroup of MCP-low patients who showed increased serum MCP-1 levels ≥50% of their prevaccination value was labeled the response-high group. Those below the 50% cutoff were labeled response-low. The average percentage increase postvaccination in response-high group was 90% compared with 27% in response-low group (P = 0.003). Of the 22 patients in the MCP-low group, 15 patients belonged in the response-low, whereas 7 patients were in the response-high group.

We next analyzed the response-low and response-high groups for the same previously used clinical prognostic variables (Table 5). The response-low group showed 40% stage II or worse (American Joint Commission on Cancer 2002) disease compared with 86% in the response-high group (P = 0.04). This trend continued to the tumor size as well with 7% of the response-low group patients showing a T_2 (American Joint Commission on Cancer 2002) or larger tumor compared with 71% of response-high group (P = 0.01). Moreover, the percentages of poorly differentiated tumors in the response-low group compared with the response-high group were 33% and 86%, respectively (P = 0.03). Although nodal involvement, hormone receptor expression, and HER-2/*neu* expression all followed the same trend of worse prognostic variables in the response-high group, these differences did not reach statistical significance (Table 5). Overall, the patients with the worse prognostic variables showed the greatest MCP-1 level increases in response to the vaccine.

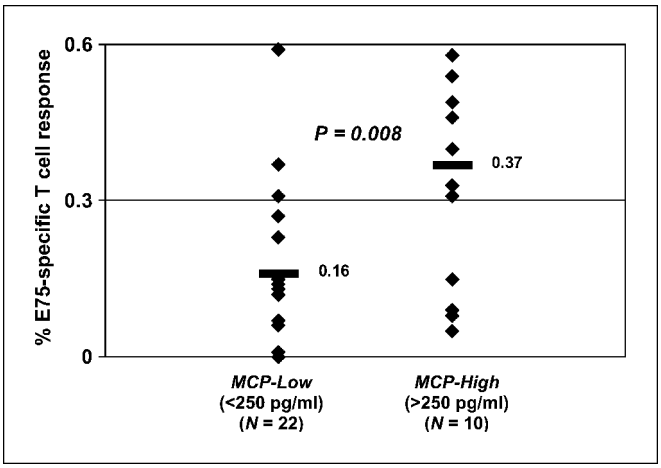

Fig. 1. Preexisting E75-specific CD8$^+$ T cells in breast cancer patients with low and high MCP-1 levels. Bar, mean value. Solid line, threshold above which all dimer values are considered positive.

Correlation of postvaccination serum MCP-1 levels and induced HER-2/neu immunity. Next, we compared the percentage change in serum MCP-1 level of each patient following vaccination with their respective vaccine-specific immunologic response as measured by clonal expansion of E75-specific CD8$^+$ T cells using the dimer assay. The same MCP-low and MCP-high classification used previously was retained for this analysis. Percentage increase in vaccinated serum MCP-1 level was calculated as described previously. Phenotypic dimer assay results for prevaccination and postvaccination values were available. The difference in postvaccination percentage of E75-specific CD8$^+$ T cells was calculated by simple subtraction of prevaccination value from the postvaccination value. Patients were again sorted according to percentage increase of serum MCP-1 level following vaccination. A distinct cutoff based on the dimer difference between prevaccination and postvaccination values was observed at 30% increase in postvaccination serum MCP-1 levels in the MCP-low group. This arbitrary line divided the MCP-low group into dimer-low

Table 3. Correlation of serum MCP-1 levels with known clinical prognostic variables in breast cancer patients

	MCP-low (‹250 pg/mL), n = 22	MCP-high (›250 pg/mL), n = 10	P
Age (average)	53.8	63.9	0.01
Race (% not Caucasian)	18	20	0.71
Grade (% poorly differentiated)	41	25	0.42
% Stage 2 or higher	55	0	0.003
% T_2 or larger tumors	27	0	0.07
% Node positive	45	0	0.01
Average nodes positive	1.5	0.0	0.005
% Estrogen/progesterone receptor negative	33	10	0.17
% HER-2/*neu*$^+$	35	22	0.80
% Received chemotherapy	76	10	0.002
Average MCP-1 levels	118.1	373.4	0.00002

and dimer-high groups with mean dimer values of 0.05% and 0.38%, respectively (P = 0.03; Fig. 2). Therefore, a correlation was found between induction of serum MCP-1 levels and vaccine-induced, peptide-specific immunity.

Discussion

In this study, we have shown that breast cancer patients who show elevated levels of serum MCP-1 have more favorable clinical prognostic variables as well as evidence of preexisting antitumor immunity in their peripheral blood T cells. In contrast, patients with low serum levels of MCP-1 exhibit an association with poor clinical prognostic variables and undetectable endogenous antitumor immunity. Following repeated inoculation with the E75 + GM-CSF vaccine, we discovered that serum MCP-1 levels could be induced; interestingly, the patients showing the most significant increase in MCP-1 levels in response to vaccination were those with the worst prognostic variables. This enhancement of serum MCP-1 levels also correlated with vaccine-induced, peptide-specific immunity. These results suggest that MCP-1 may play a role in endogenous as well as induced antitumor immunity. Therefore, our initial findings from this study may have significant clinical relevance in that low serum MCP-1 levels may serve to identify patients with a worse prognosis as well as those most likely to benefit from this vaccination strategy.

The origin of serum levels of MCP-1 is unclear; however, the two most probable sources are the immune system and the tumor microenvironment. It is generally accepted that MCP-1 is a potent proinflammatory mediator produced by several cells in the immune system, including monocytes (21, 22). Alternatively, the tumor cells themselves and/or peritumoral components can produce cytokines (23–25). The secreted cytokines result in chemotaxis of monocytes from the circulation into the periphery where they differentiate into macrophages or tumor-associated macrophages. A delicate balance of inflammatory cell infiltration and cytokine expression seems to influence the degree of preneoplastic or antineoplastic environment (26, 27). One study investigating the MCP-1 production in a melanoma cell line showed that increased MCP-1 production by the melanoma cells resulted in increased tumor-associated macrophage infiltration and significantly increased destruction of the tumor cells (28). Currently, only one analysis of serum MCP-1 level in the context of breast cancer has been published, and in that study of patients with invasive breast cancer, ductal carcinoma *in situ*, benign breast lesions, and healthy women, Lebrecht et al. failed to show any significant differences in the serum cytokine levels of these four groups (29). However, a

Table 4. Following vaccination, the MCP-low patients (serum MCP-1 level <250 pg/mL) can be further characterized into the response-low (<50% increase in serum MCP-1 levels postvaccination) and response-high (>50% increase in serum MCP-1 levels postvaccination)

Study no.	Serum MCP-1		MCP-1 % ↑	Age	Race	Grade	Stage 2 or above	Tumor T_2 or above	Node-positive	No. node	Estrogen/progesterone receptor +/+	HER-2/*neu* oncogene 3+ by IHC	Chemotherapy
	Pre	Post											
Response-low													
B54	40.8	35.9	−12	45	White	Poor	Yes	Yes	Yes	4	Yes	Yes	Yes
B53	135	130.9	−3	50	White	Mod	Yes	No	Yes	2	No	No	Yes
NNV5	230.16	255.44	11	59	Other	Mod	No	No	No	0	No	Yes	Yes
NNV4	85.9	98.5	15	53	White	Mod	No	No	No	0	Yes	No	Yes
B51	237.6	277.6	17	73	White	Well	Yes	No	Yes	1	Yes	No	No
NNV15	28.8	36.7	27	58	White	—	No	No	No	0	Yes	No	No
NNV10	196.7	251.6	28	57	White	Well	No	No	No	0	Yes	No	—
NNV22	59.37	79.6	34	53	White	—	No	No	No	0	—	—	No
NNV20	157.18	212.12	35	55	White	Mod	No	No	No	0	Yes	No	No
B56	97.27	131.38	35	43	White	Poor	Yes	No	Yes	6	No	Yes	Yes
NNV11	50.19	70.23	40	45	Other	Poor	No	No	No	0	No	No	Yes
NNV19	86.4	122.16	41	50	Other	Mod	No	No	No	0	Yes	No	Yes
B46	70.7	102	44	52	White	Mod	Yes	No	Yes	2	No	Yes	Yes
NNV14	123.33	182.1	48	74	White	Mod	No	No	No	0	Yes	—	No
B41	93	138.6	49	49	White	Well	Yes	No	Yes	2	Yes	No	Yes
Response-high													
B44	155.32	239.44	54	62	Other	Poor	Yes	Yes	Yes	3	No	No	Yes
B55	244.6	381.5	56	48	White	Poor	Yes	Yes	Yes	8	Yes	Yes	Yes
NNV13	178.08	319.04	79	52	White	Poor	Yes	Yes	No	0	Yes	Yes	Yes
B57	47.8	89	86	62	White	Poor	Yes	No	Yes	1	No	Yes	Yes
B50	136.33	257.6	89	51	White	Poor	Yes	Yes	Yes	5	No	No	Yes
NNV23	62.7	130.08	107	46	White	Poor	Yes	Yes	No	0	Yes	No	Yes
NNV6	80.35	208.7	160	46	White	Mod	No	No	No	0	Yes	No	Yes

NOTE: Response-high patients show worse clinical prognostic variables. NNV, node-negative patients; B, node-positive breast cancer patients; Poor, poorly differentiated (grade 3); Mod, moderately differentiated (grade 2); Well, well-differentiated (grade 1); IHC, immunohistochemistry.

trend for increasing serum MCP-1 levels in cancer patients based on extent of disease compared with healthy patients was noted. The lack of statistical difference is most likely due to the different patient populations used for their studies compared with ours. Our patients have been treated by standard of care modalities and have been rendered disease-free before vaccination and/or serum collection. In contrast, Lebrecht et al. studied and collected serum specimens from patient with active disease. It is possible that patients with established disease burdens have relative immunologic tolerance toward their tumors and, therefore, a lack of an inflammatory process; however, they would be more likely to have tumor production of cytokines contributing to the levels measured in the serum. On the other hand, patients with treated disease and decreased tumor burden would be more likely to have the capacity to mount an inflammatory response and hence generate the associated serum MCP-1 levels. Further studies of serum cytokine levels before and after breast cancer treatment would be of value to clarify this question. To that end, however, others have shown statistically significant increases in serum MCP-1 levels in patients with ovarian (23) as well as pancreatic cancer (30) compared with healthy controls.

One of the major findings in our study was that of a strong correlation between serum MCP-1 levels and known clinical prognostic variables. This finding may have significant clinical relevance. It must be kept in mind, however, that these patients have completed their primary treatment regimen; therefore, it will be necessary to validate this aspect of our study with a larger sample size of patients to more clearly define the true diagnostic and/or prognostic usefulness of this correlation in post-treatment patients. In our preliminary results, we have shown that patients with a high serum MCP-1 level have disease onset at a later age and harbor less aggressive disease. Interestingly, the MCP-high group was completely composed of node-negative patients. This is in sharp contrast to the findings of Lebrecht et al. who reported a statistically significant correlation between higher serum MCP-1 levels and both larger tumors (>2 cm) and presence of axillary lymph node metastasis, but again it must be remembered that those patients had active disease. In support of our findings, however, Tonouchi et al. showed a statistically significant correlation of increased serum MCP-1 levels in gastric cancer patients and lack of lymph node metastasis as well as smaller tumors (24).

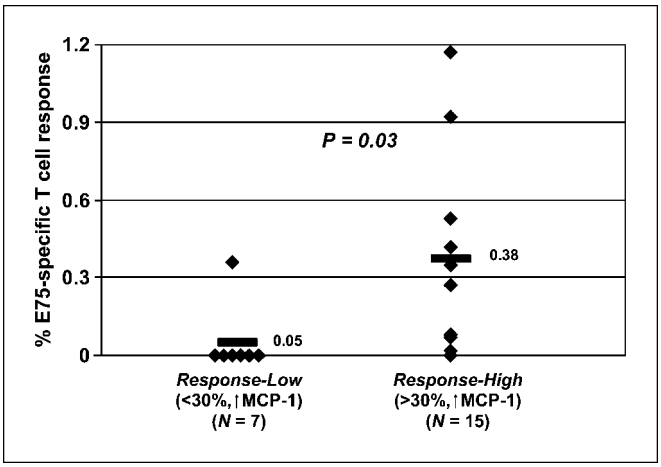

Fig. 2. Comparison of E75-specific $CD8^+$ T-cell response to vaccination in MCP-low breast cancer patients with low (‹30%) and high (›30%) increases in serum MCP-1 level postvaccination.

In a subsequent report, they studied patients undergoing gastric or colon surgery with preoperative and postoperative serum MCP-1 levels and found that patients with increased levels of preoperative serum MCP-1 showed significantly smaller tumors than their low MCP-1 counterparts (31). Although it did not reach statistical significance, they also showed that well-differentiated tumors were associated with increased serum MCP-1 levels. A similar observation was made in a study of frozen sections of breast cancer tissue, where the authors found a correlation between increased parenchymal MCP-1 expression and well-differentiated tumors (25).

Moving beyond an analysis of the clinical variables and probing for a possible link to endogenous antitumor activity, we have also shown that the MCP-high cohort of patients showed higher levels of preexisting E75-specific $CD8^+$ T cells as well as better clinical prognostic variables. Taken collectively, the above observations along with the known proinflammatory characteristics of MCP-1 suggest an endogenous antitumor immune response in patients with high serum MCP-1 levels. It is conceivable that such preexisting antitumor immunity would help contain the developing tumor and would allow the patient

Table 5. Correlation of postvaccination percentage increase in serum MCP-1 levels with known clinical prognostic variables in MCP-low breast cancer patients

	Response-low (‹50%, ↑ MCP-1), *n* = 15	Response-high (›50%, ↑ MCP-1), *n* = 7	*P*
Age (average)	54.4	52.4	0.58
Race (% not Caucasian)	20	14	0.79
Grade (% poorly differentiated)	33	86	0.03
% Stage 2 or higher	40	86	0.04
% T_2 or larger	7	71	0.01
% Node-positive	40	57	0.77
Average nodes positive	1.1	2.4	0.33
% Estrogen/progesterone receptor negative	29	43	0.87
% HER-2/*neu*$^+$	31	43	0.79
% Received chemotherapy	64	100	0.20
Average MCP-1 response	27%	90%	0.003

to present with less aggressive disease and be older at onset of disease. In contrast, the MCP-low group was associated with worse disease and younger age at diagnosis. It is possible that the reduced serum MCP-1 level along with decreased antigen-specific T-cell response suggests a more tolerant immune response in patients resulting in more aggressive disease.

We have shown previously an increase in serum MCP-1 level in breast cancer patients vaccinated with the HER-2/*neu* E75 peptide vaccine (14). Because we observed a wide spectrum of MCP-1 levels in these patients, we investigated the correlation of serum MCP-1 induction with known clinical prognostic variables as well evidence of vaccine-specific clonal expansion. Interestingly, we observed that the MCP-low group showed a remarkable inducible level of serum MCP-1 compared with the MCP-high group following vaccination, suggesting a clinically applicable role of vaccination in patients with low baseline MCP-1 levels. Furthermore, when we assessed which patients responded best to the vaccine based on induced MCP-1 levels, we were able to distinguish two distinct subgroups in the MCP-low group. We observed that the response-high group had the worse clinical prognostic variables, which was surprising. However, this group showed a significant increase in E75-specific $CD8^+$ T cells in response to vaccination as well. Taken collectively, we observed that the patients who responded the best to our vaccination strategy were patients who had the worst clinical prognostic variables, possessed evidence of immunologic tolerance, and had low serum MCP-1 levels at baseline.

This latter observation has two significant clinical implications. First, our data suggest that the HER-2/*neu* E75 peptide vaccination strategy is very effective. In this article, we have presented data confirming vaccination efficacy in patients with both clinical and immunologic evidence of tolerance. Following vaccination, these patients respond not only by increasing the serum levels of MCP-1 but also by clonal expansion of vaccine-specific CTL. We have reported previously preliminary data suggesting that this vaccine-induced immunity may result in improved disease-free survival in vaccinated node-positive breast cancer patients compared with control group of 85.7% and 59.8% at 22 months median follow-up with a recurrence of 8% compared with 21%, respectively ($P < 0.19$; ref. 12). Second, the clinical and immunologic variables associated with the MCP-low group allow for predictability as well individualization of treatment to these patients. We have shown data implicating this group of patients with worse prognosis as shown by the extent of their disease burden as well as the early age of disease onset. It is conceivable that patients could have their serum MCP-1 level tested on diagnosis with breast cancer and treated per standard of care. Postoperatively, they could be considered for adjuvant therapy, including vaccination based on their predictive factors, such as HER-2/*neu* status and serum MCP-1 level. Those HER-2/neu^+ patients with more aggressive tumors could then be vaccinated if they showed low baseline serum MCP-1 levels. However, MCP-high patients might be less likely to benefit from vaccination and may be best served by other adjuvant modalities.

We acknowledge certain variables in our vaccination scheme within the trial. For example, there was a lack of uniformity in vaccine dosing to our patient population. The prevaccination time point, however, would not have been affected by this discrepancy; therefore, our findings on the association between serum MCP-1 levels and clinical disease are clearly valid. The postvaccination time point analysis of patients based on the different doses of the E75 peptide and GM-CSF certainly could have been affected but did not reveal any significant changes in serum cytokine profiles (data not shown). Furthermore, the role and influence of GM-CSF in this study certainly did not escape our concern. All of our node-positive patients received 250 μg GM-CSF in contradistinction of 125 μg GM-CSF given to most of the node-negative cohort. If the GM-CSF dosing affected serum cytokine levels to any significant amount, one would expect that change to become apparent in the node-positive group due the higher dose of GM-CSF received. This, however, was not our observation. In fact, the node-negative cohort had the higher levels of serum MCP-1 levels when compared with node-positive patients (14). Finally, because all of the MCP-1 levels were drawn on patients post-treatment, we cannot discount the effect that surgery, chemotherapy, and radiation might have on the MCP-1 levels. We have attempted to minimize this potential effect by including patients in this analysis that were on average 1 year post-treatment. The patients were also proven to be immunocompetent before enrollment. Finally, we have assessed whether MCP-1 levels were significantly different based on time since completion of treatment. We found no correlation between MCP-1 levels and time since last chemotherapy. Although reassuring, the only way to completely control for treatment effects would be to perform these analyses on pretreatment samples as we suggested earlier. We are in the process of obtaining these samples for evaluation.

In conclusion, our data suggest that patients with increased serum MCP-1 levels probably display a more robust immune response to their tumor, whereas those with decreased serum levels of MCP-1 may be plagued by a tolerant immune system and more aggressive disease. Vaccination with the E75 peptide seems to be effective in the highest-risk patients with the worse disease. Therefore, serum MCP-1 screening might not only be beneficial for predicting associated clinical prognostic variables but also may help identify vaccine eligible patients. Additionally, MCP-1 levels may be useful for immunologic monitoring of the response to vaccination in the immunotherapy of breast cancer patients.

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