Significance of Macrophage Chemoattractant Protein-1 in Macrophage Recruitment, Angiogenesis, and Survival in Human Breast Cancer

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ABSTRACT

Tumor cells stimulate the formation of stroma that secretes various mediators pivotal for tumor growth, including growth factors, cytokines, and proteases. However, little is known about the local regulation of these soluble mediators in the human tumor microenvironment. In this study, the local expression of cytokines, chemokines, and angiogenic factors was investigated in primary breast cancer tissue. The concentrations of interleukin (IL)-1, IL-4, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α, IFN-γ, IL-8, macrophage chemoattractant protein (MCP)-1, epithelial-neutrophil activating peptide-78, vascular endothelial growth factor, and thymidine phosphorylase (TP) were measured in 151 primary breast cancer extracts by ELISA. Tumor-associated macrophages (TAMs) were also examined by immunohistochemistry with anti-CD68 antibodies. The correlation between soluble mediators and the relationship between TAM count and soluble mediators were evaluated. MCP-1 concentration was correlated significantly with the level of vascular endothelial growth factor, TP, TNF-α, and IL-8, which are potent angiogenic factors. IL-4 concentration was correlated significantly with IL-8 and IL-10. On the other hand, an inverse association was observed between TP and IL-12. The level of MCP-1 was associated significantly with TAM accumulation. In the immunohistochemical analysis, MCP-1 expression was observed in both infiltrating macrophages and tumor cells. Prognostic analysis revealed that high expression of MCP-1, as well as of VEGF, was a significant indicator of early relapse. These findings indicate that interaction between the immune network system and angiogenesis is important for progression of human breast cancer, and that MCP-1 may play an important role in the regulation of angiogenesis and the immune system.

INTRODUCTION

Interaction between tumor cells and stroma is essential for tumor growth. Tumor cells stimulate the formation of stroma that excretes a variety of growth factors, cytokines, and proteases. TAMs[2] are one of the major components of tumor stroma and are capable of eliciting diverse aspects to tumor growth as a positive or negative regulator (1). Macrophage infiltration into tumors is regulated by a number of cytokines and chemokines, in particular MCP-1. MCP-1 is a member of the C-C chemokine family and possesses chemotaxic activity for monocytes and T lymphocytes (2–5). MCP-1 is produced not only by tumor cells but also by stromal cells such as fibroblasts, endothelial cells, and monocytes. *MCP-1* gene transfer enhances the metastatic potential of cancer cells with increased neovascularization, whereas MCP-1 itself activates monocyte cytostatic function against tumor cells (6, 7).

T cells play an essential role in the immune reaction to tumors and have the potential to prevent tumor spread (8). Activated CD4+ T cells differentiate into at least two functionally distinct subsets, Th1 and Th2 (9). The Th1 subset, stimulated by IL-12, produces IL-2 and IFN-γ, which activate cell-mediated immune responses, whereas the Th2 subset produces IL-4, IL-5, IL-6, IL-10, and IL-13, which encourage humoral immunity (9–11). Cell-mediated immune responses supported by Th1-type cells are thought to be optimal in suppression of tumor development, and tumors are capable of producing several mediators such as IL-4 and IL-10, which inhibit cell-mediated responses (12, 13). Recent studies indicate that interaction between chemokines and their receptors is involved in the positioning of T cells (14). For example, MCP-1 has been demonstrated to affect the differentiation of T cells (15, 16) by enhancing IL-4 production in T cells (15). Neutralization of MCP-1 by antibodies resulted in enhanced production of IFN-γ

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[2] The abbreviations used are: TAM, tumor-associated macrophage; MCP-1, macrophage chemoattractant protein-1; Th1 and Th2, T helper 1 and 2, respectively; VEGF, vascular endothelial growth factor; TP, thymidine phosphorylase; IL, interleukin; TNF, tumor necrosis factor; ENA-78, epithelial-neutrophil activating peptide-78; IP-10, IFN-γ inducible protein-10; ER, estrogen receptor; PgR, progesterone receptor; FU, 5-fluorouracil.

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in T cells on recognition of the tumor (16). These studies suggest that MCP-1 regulates T-cell development.

Angiogenesis is a multistep cascade involving various soluble mediators. There is increasing evidence to support the important role of the immune system in angiogenesis. TAM content is reported to have a close correlation with microvessel density and prognosis in breast cancer (17–19). Notably, the accumulation of TAMs with positive TP is a potent prognostic indicator of early relapse in primary breast cancer (20). Transfection of tumor cells with the *MCP-1* gene promotes angiogenesis in a murine model, and MCP-1 implants induce angiogenesis in rabbit cornea, indicating the important function of MCP-1 in angiogenesis (6, 21). IL-4 has a profile as a potent angiogenesis factor (22, 23). IL-8, a member of the ELR-CXC chemokine family, was initially identified as a chemoattractant for neutrophils and is now known as a potent endothelial mitogen (24–28). ENA-78, a member of the non-ELR-CXC chemokine family, was also reported to be an angiogenic factor (29). On the other hand, IL-12 has been reported to inhibit angiogenesis through induction of IP-10, which is known to be a negative regulator of angiogenesis (30, 31). One of the major angiogenic factors, VEGF, which has been reported to be associated with microvessel density and poor prognosis in various solid tumors, inhibits the development of dendritic cells (32–39). These findings indicate that angiogenesis, either directly or indirectly, interacts closely with the immune system and prompted us to investigate the local regulation of these mediators in view of the balance between immune cytokines and angiogenesis factors. In the present study, the local expression of a variety of soluble factors in primary breast cancer tissue was investigated, and their correlation and clinical significance as prognostic factors was determined.

MATERIALS AND METHODS

Breast Cancer Tissue. One-hundred and fifty-one unselected tissue samples from primary invasive ductal carcinoma of the breast, resected surgically at Tokyo Metropolitan Komagome Hospital between 1991–1997, were used in this investigation. Representative parts of the specimens were frozen in liquid nitrogen immediately after surgical resection and stored at −70°C until preparation of tissue extracts. The main characteristics of the patients are described in Table 1.

Sample Preparation for ELISA. Tumor tissues were homogenized in 10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$, and 50 μM potassium phosphate and then centrifuged at 10,000 × *g* for 15 min. The supernatant was stored at −80°C until use. A portion of the supernatant was dialyzed overnight at 4°C in a buffer containing 20 mM potassium phosphate (pH 7.4) and 1 mM 2-mercaptoethanol and then used for ELISA. The protein concentration of the supernatants extracted from tumor tissue was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Measurement of Cytokines, Chemokines, and Angiogenesis Factors. The concentrations of cytokines (IL-1, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ), chemokines (IL-8, MCP-1, and ENA-78), and angiogenesis regulators (VEGF and TP) in the tumor extracts were measured by ELISA (R&D systems, Minneapolis, MN). The measurements were performed according to the manufacturer's instructions. The minimal detection limit for each factor was as follows: IL-1, 0.1 pg/ml; IL-4, 0.13 pg/ml; IL-6, 0.094 pg/ml; IL-10, 0.5 pg/ml; IL-12, 0.5 pg/ml; TNF-α, 0.18 pg/ml; IFN-γ, 3 pg/ml; IL-8, 10 pg/ml; MCP-1, 5 pg/ml; and ENA-78, 15 pg/ml. Measurement of VEGF and TP concentrations was performed as described previously (40). Briefly, VEGF concentrations were measured by a colorimetric ELISA using a polyclonal antibody to human VEGF121 (091895; Toagosei, Ibaraki, Japan), which reacts specifically with the soluble isoforms of the peptide. The minimal detectable level was 5 pg/mg protein. TP levels were also determined by a colorimetric ELISA. This sandwich immunoassay used two anti-human TP monoclonal antibodies (Nippon Roche Research Center, Kamakura, Japan; 104B and 232-2). The minimal detectable concentration was 2 units/mg. One unit of TP is equivalent to the enzymatic activity that generates 1 ng of 5-fluorouracil from 5-deoxy-5-fluorouridine/h. The numbers of cases examined are listed in Table 2.

Table 1 Patients' backgrounds

Characteristics	No. of patients (%)
Patients enrolled	151
Median age	55 (range, 20–86)
Menopausal status	
Pre-	62 (41.1)
Post-	89 (58.9)
Tumor size	
<2 cm	6 (4.0)
2–5 cm	97 (64.2)
>5 cm	48 (31.8)
Number of nodes involved	
0	54 (35.8)
1–3	37 (24.5)
≥4	60 (39.7)
Adjuvant therapy[a]	
CAF(CEF)	−, 6 (4.0)
	+, 38 (25.2)
CMF(CF)	−, 5 (3.3)
	+, 23 (15.2)
FU derivatives	−, 12 (7.9)
	+, 17 (11.3)
Tamoxifen	−, 28 (18.5)
	+, 72 (47.7)

[a] CAF(CEF), cyclophosphamide, Adriamycin or epirubicin and 5-FU; CMF(CF), cyclophosphamide, methotrexate, and 5-FU; −, negative for lymph node metastasis; +, positive for lymph node metastasis.

Determination of Conventional Biological Factors. All surgical specimens were examined by conventional procedures to obtain a histological diagnosis. ER and PgR levels were determined with ELISA systems from Otsuka Assay Institute (Tokushima, Japan) as reported previously (20). The cutoff value for both ER and PgR was 5 fmol/mg protein.

Immunohistochemistry of TAM and MCP-1. TAM count was determined for 50 of the 151 cases. Sections (3–5 μm) of paraffin-embedded tumor tissues were used in an indirect anti-peroxidase immunohistochemical assay (Dako, Carpinteria, CA). TAMs were stained with an anti-CD68 monoclonal antibody (PG-M1; Dako A/S, Copenhagen, Denmark), and pos-

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Table 2 Correlation between biological markers and nodal stratification

Biological markers	Case no.	Median (range)	Median		P^a (− *vs.* +)
			Node negative	Node positive	
ER	148	4.80 fmol/mg (0–870.00)	7.00	3.60	0.14
PgR	148	11.10 fmol/mg (0–3090.00)	13.50	10.90	0.38
VEGF	151	162.60 pg/mg (9.25–2957.64)	164.60	196.13	0.28
IP	151	162.47 units/mg (6.74–1125.71)	169.56	160.81	0.10
INF-α	151	0.69 pg/mg (0–23.50)	0.51	0.76	0.67
IL-1	135	3.48 pg/mg (0.01–9.07)	3.62	3.46	0.86
IL-4	135	3.07 pg/mg (0–53.263)	0.00	1.94	0.012
IL-6	135	5.31 pg/mg (0–24.66)	3.91	5.53	0.61
IL-8	151	3.58 pg/mg (0–952.40)	0.00	3.58	0.39
IL-10	135	0.00 pg/mg (0–20.99)	0.00	0.00	0.16
IL-12	135	0.05 pg/mg (0–2.97)	0.00	0.06	0.60
MCP-1	135	0.00 pg/mg (0–434.26)	0.00	2.82	0.93
ENA-78	74	0.00 pg/mg (0–824.86)	0.00	0.00	0.11

[a] The statistical test used is the Student *t* test.

itively stained cells were counted by eye in the five most confluent microscopic fields (per mm^2; Ref. 20). The mean count was determined from the highest three counts of the five and considered as the TAM count. MCP-1 expression was examined by the same indirect immunohistochemical method using an anti-MCP-1 monoclonal antibody as described previously (41).

Adjuvant Therapy and Patient Follow-Up. The schedule for adjuvant treatment was based on each patient's individual characteristics including axillary node involvement, tumor size, age, and ER. Poly-chemotherapy including six cycles of cyclophosphamide, Adriamycin/epirubicin, and FU was given to node-positive patients under the age of 55, and FU derivatives were given to the other remaining node-positive and high-risk node-negative patients. Tamoxifen was given to hormone receptor-positive patients for at least 2 years (5 years to node-positive patients). The patients were followed-up every 3 months, and recurrence was confirmed by histological examination or image examination.

Statistical Analysis. The correlation between two factors was evaluated by the Spearman's rank correlation coefficient. Unpaired groups were compared by the Student's *t* test. Kaplan-Meier survival curves were plotted, and the difference in prognosis between the two groups was analyzed by the log-rank test. Multivariate analyses were performed using the Cox proportional hazards model. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The median value and the range of each biological marker are shown in Table 2. Only two cases had measurable levels of IFN-γ, and this factor was therefore excluded from the analysis. A significant correlation between axillary node status and the level of IL-4 was detected. The level of IL-4 was significantly higher in node-positive than in node-negative patients ($P = 0.012$). However, other molecules were not associated with node status.

Relationship between Macrophage Accumulation and Soluble Mediators. TAM counts varied from 5 to 480 counts/mm^2 (median, 125 counts/mm^2). The levels of MCP-1 and TP were correlated significantly with macrophage accumulation ($P = 0.0197$ and $P = 0.036$, respectively; Table 3). The levels of TNF-α and IL-8 also tended to be correlated with TAM counts ($P = 0.0514$ and $P = 0.095$, respectively).

Table 3 Relationship between biological markers and macrophage accumulation

Biological markers	Spearman's analysis ($n = 50$)	
	r	*P*
ER	−0.161	0.171
PgR	−0.128	0.288
VEGF	0.052	0.659
TP	0.246	0.036
TNF-α	0.230	0.0514
IL-1	−0.018	0.885
IL-4	−0.041	0.762
IL-6	0.088	0.532
IL-8	0.229	0.095
IL-10	0.093	0.485
IL-12	−0.003	0.981
MCP-1	0.333	0.0197
ENA-78	0.022	0.865

Correlation Analysis between Biological Markers. According to Spearman's nonparametric rank correlation test, there were several correlations between those markers involved in angiogenesis and the immune reaction (Table 4). Expression of MCP-1 had a significant positive correlation with expression of VEGF, TP, TNF-α, and IL-8 ($P = 0.0003$, $P < 0.0001$, $P < 0.0001$, and $P = 0.007$, respectively) and had a similar relationship with expression of IL-6 and IL-10 ($P = 0.0584$ and $P = 0.0747$, respectively). Expression of VEGF was also found to be positively associated with expression of TP, TNF-α, and IL-6 ($P = 0.0004$, $P = 0.0002$, and $P = 0.012$, respectively). TP expression was associated significantly with the expression of TNF-α and IL-1 ($P < 0.0001$ and $P < 0.0001$, respectively). Among the cytokines, IL-4 expression was strongly correlated with expression of IL-8 and IL-10 ($P = 0.0069$ and $P = 0.0005$, respectively). An inverse association was observed between TP and IL-12 ($P = 0.0266$).

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Table 4 Interfactorial analysis of the factors by Spearman's method[a]

ER	—											
PgR	0.605	—										
	<0.001[b]											
VEGF	0.036	−0.033	—									
	0.6287	0.661										
TP	−0.209	0.064	0.290	—								
	0.0552	0.398	0.0004[b]									
TNF-α	−0.014	0.010	0.306	0.426	—							
	0.915	0.892	0.0002[b]	<0.0001[b]								
IL-1	0.078	0.073	0.077	0.528	0.280	—						
	0.521	0.560	0.528	<0.0001[b]	0.033[b]							
IL-4	−0.223	−0.241	−0.150	−0.131	−0.012	0.137	—					
	0.030[b]	0.0206	0.125	0.184	0.9003	0.3476						
IL-6	−0.148	−0.320	0.273	−0.050	0.204	0.115	0.235	—				
	0.145	0.004[b]	0.012[b]	0.631	0.0619	0.252	0.0389[b]					
IL-8	−0.066	−0.002	0.146	−0.066	0.080	−0.226	0.276	0.233				
	0.412	0.981	0.092	0.414	0.3236	0.107	0.0069[b]	0.0399[b]	—			
IL-10	−0.154	−0.155	0.056	−0.032	0.093	−0.071	0.484	0.327	0.208			
	0.130	0.130	0.689	0.809	0.480	0.630	0.0005[b]	0.0252[b]	0.0565			
IL-12	−0.176	−0.160	−0.043	−0.189	−0.079	−0.124	0.040	0.046	0.014	0.125	—	
	0.066	0.091	0.609	0.0266[b]	0.353	0.3083	0.7114	0.691	0.878	0.209		
MCP-1	−0.015	−0.052	0.343	0.374	0.448	0.097	0.056	0.216	0.240	0.201	−0.092	—
	0.863	0.556	0.0003[b]	<0.0001[b]	<0.0001[b]	0.521	0.5866	0.0584	0.0070[b]	0.0747	0.3691	
ENA78	−0.101	0.011	0.018	−0.006	0.098	0.101	0.034	0.146	0.134	−0.797	0.078	0.124
	0.356	0.919	0.871	0.956	0.3660	0.378	0.8192	0.373	0.218	0.425	0.573	0.288
	ER	PgR	VEGF	TP	TNF-α	IL-1	IL-4	IL-6	IL-8	IL-10	IL-12	MCP-1

[a] Upper column, ρ; lower column, *P*.
[b] Statistically significant.

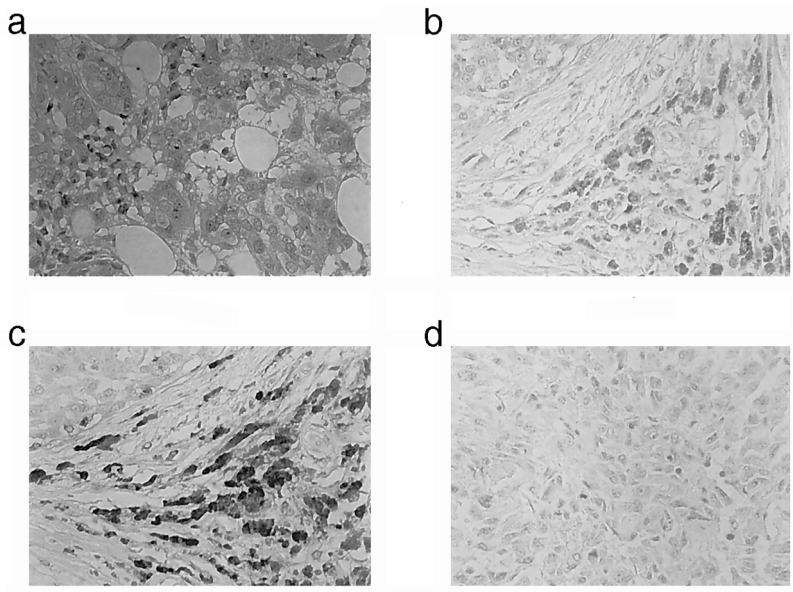

Fig. 1 Immunohistochemical analysis of MCP-1 in invasive ductal carcinomas. *a*, positive staining of MCP-1 was seen in the cytoplasm of tumor cells. *b*, infiltrated tumor cells showed a positive staining for MCP-1. *c*, a strong immunostaining was seen in lymphoreticular cells, especially macrophages infiltrated into tumor stroma. *d*, negative control.

MCP-1 Expression in Primary Breast Tumors. Immunohistochemical analysis in primary breast tumors showed that MCP-1 was expressed in both stromal cells and tumor cells (Fig. 1). Monocytic cells were major components of the stromal cells that expressed MCP-1, but some fibroblastic spindle-shaped cells were also positive for MCP-1 staining.

Survival Analysis. The prognostic significance of each molecule was assessed with the cutoff value set at the median value. Univariate analysis demonstrated that, in addition to node status ($P < 0.0001$) and tumor size ($P = 0.038$), MCP-1 and VEGF had significant prognostic values for relapse-free survival ($P = 0.028$ and $P = 0.034$, respectively; Table 5). All

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Table 5 Univariate analysis for survival in breast cancer patients

Markers	Log-rank (Mantel-Cox)	
	χ^2	*P*
Menopause (pre *vs.* post)	1.369	0.50
Tumor size (<3 cm *vs.* ≥3 cm)	4.287	0.038
Node (negative *vs.* positive)	22.59	<0.0001
ER	6.03	0.014
PgR	3.28	0.070
VEGF	4.49	0.034
TP	1.35	0.25
TNF-α	0.53	0.47
IL-1	0.42	0.52
IL-4	1.16	0.28
IL-6	2.59	0.11
IL-8	0.004	0.94
IL-10	1.20	0.27
IL-12	4.96	0.26
MCP-1	4.82	0.028

Table 6 Multivariate analysis for survival in breast cancer patients (n = 135 patients)

Marker	χ^2	*P*	SE	EXP
n (negative *vs.* positive)	10.998	0.0009	0.727	0.090
ER (negative *vs.* positive)	5.527	0.0187	0.604	0.513
VEGF/MCP-1[a] (−/−, +/−, −/+ *vs.* +/+)	4.417	0.0356	0.338	2.215
Tumor size (<3 cm *vs.* ≥3 cm)	1.220	0.2693	0.429	0.406

[a] VEGF/MCP-1 −/−, VEGF level below median and MCP-1 level under detection level; VEGF/MCP-1 +/−, VEGF level above median and MCP-1 level under detection level; VEGF/MCP-1 −/+, VEGF level below median and MCP-1 detectable level; VEGF/MCP-1 +/+, VEGF level above median and MCP-1 detectable level.

variables with $P < 0.2$ in the univariate analysis were included in the multivariate analysis. VEGF and MCP-1 were assessed as a combined phenotype because of a close interfactorial correlation. A combined VEGF and MCP-1 status had an independent prognostic significance after node status and ER status (P = 0.0356; Table 6; Fig. 2).

DISCUSSION

In the present study, high levels of MCP-1 were found to be expressed in primary breast cancers by ELISA and immunohistochemical analysis. Both tumor cells and stromal cells were observed to express MCP-1. MCP-1 is a potent chemoattractant for macrophages, and it was shown that monocyte recruitment was disordered in MCP-1-deficient mice (42). Our study confirmed that the expression of MCP-1 was correlated significantly with TAM accumulation in primary breast tumors. Interestingly, MCP-1 was closely correlated with positive endothelial growth regulators including VEGF, TP, and IL-8. The importance of these endothelial growth regulators in neovascularization and tumor progression has been demonstrated unequivocally in a variety of tumors (20, 32–36, 43). VEGF has been reported to induce MCP-1 in endothelial cells (44). Although MCP-1 has been suggested to be angiogenic in experimental studies (21), endothelial cells have no receptors for MCP-1 (45). Rabbit corneal assays demonstrated that MCP-1 induced neovascularization with accumulation of macrophages, suggesting the important role of macrophages in MCP-1-induced angiogenesis (21). Because TAMs are known to be an important source of angiogenic factors, our data indicate that MCP-1 may induce angiogenesis through production of angiogenic factors by macrophages.

A number of studies have revealed the relationship between angiogenesis and the immune system. Several cytokines including TNF-α, IL-1, and IFN-γ are known to be inducers of TP (46, 47). In fact the close correlation between TP and TNF-α, and between TP and IL-1, was confirmed in this study. MCP-1 is known to be a regulator of T-cell differentiation. In particular, MCP-1 induces Th2 cytokine IL-4, whereas it inhibits generation of Th1-type cells, suggesting that MCP-1 might induce Th2 dominance in tumor surroundings (15, 16). Our results failed to demonstrate a direct correlation between MCP-1 and Th2 cytokines but showed that MCP-1 levels tended to be associated with IL-6 and IL-10. It was also observed that Th2-type cytokines, including IL-4 and IL-10, were correlated with each other in breast tumors. The lack of correlation between MCP-1 and IL-4 might be attributable to the fact that IL-4 is derived not only from lymphocytes but also from other types of cells such as mast cells (22, 23).

IL-12 and IFN-γ, both of which are Th1 cytokines, have been shown to inhibit angiogenesis (30, 31, 48–51). In this study, VEGF correlated with the Th2 cytokine IL-6, and TP had an inverse correlation with the Th1 cytokine IL-12, suggesting that angiogenic factors might be regulated cooperatively with Th2 cytokines in tumor surroundings. Indeed, it has been reported that VEGF has an inhibitory effect on the differentiation of dendritic cells (36, 52). In lung cancer, it has also been noted that the Th2-type immune stage is dominant rather than the Th1-type stage, which implies that evasion of Th1-dominant status might be crucial for tumor cells to grow (53).

The prognostic analysis showed that MCP-1, as well as VEGF, was a significant prognostic indicator. In the multivariate analysis, a combined MCP-1 and VEGF status was an independent prognostic indicator. The immune-regulating function and angiogenic function of MCP-1 might contribute to the poor prognosis of breast cancer patients with high MCP-1 levels. Because the prognostic significance of VEGF has been confirmed in various tumors, further assessment of the prognostic value of MCP-1 is warranted.

In conclusion, this study showed that MCP-1 expression was associated with macrophage accumulation and correlated with the concentration of various angiogenic regulators, and that MCP-1/VEGF was an independent prognostic indicator in breast cancer. MCP-1 might be a candidate for regulation of the relationship between angiogenesis and the immune balance in human breast carcinoma. MCP-1 could be a novel target in cancer treatment once its role in immune regulation and angiogenesis is better understood.

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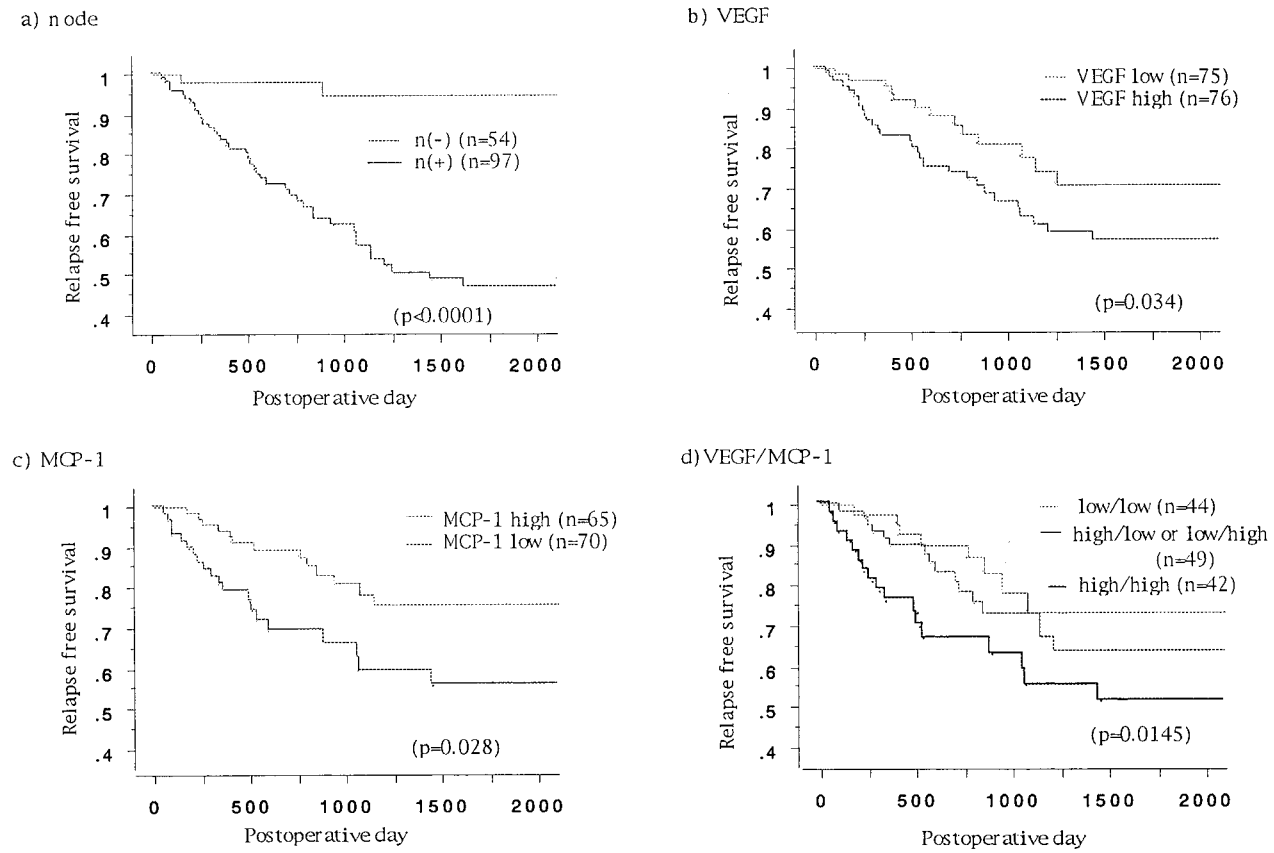

Fig. 2 Relapse-free survival curves stratified by nodal status, VEGF status, and MCP-1 status. *a*, node-positive patients showed a worse prognosis as compared with node-negative patients. *b*, VEGF-high patients showed a significantly worse prognosis as compared with VEGF-low patients (log-rank test, $P = 0.034$). *c*, MCP-1-high patients also showed a significantly worse prognosis as compared with MCP-1-low patients (log-rank test, $P = 0.028$). *d*, in a combined analysis, the patients with VEGF-high and MCP-1-high phenotype showed a significantly poorer prognosis as compared with those with other phenotypes.

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