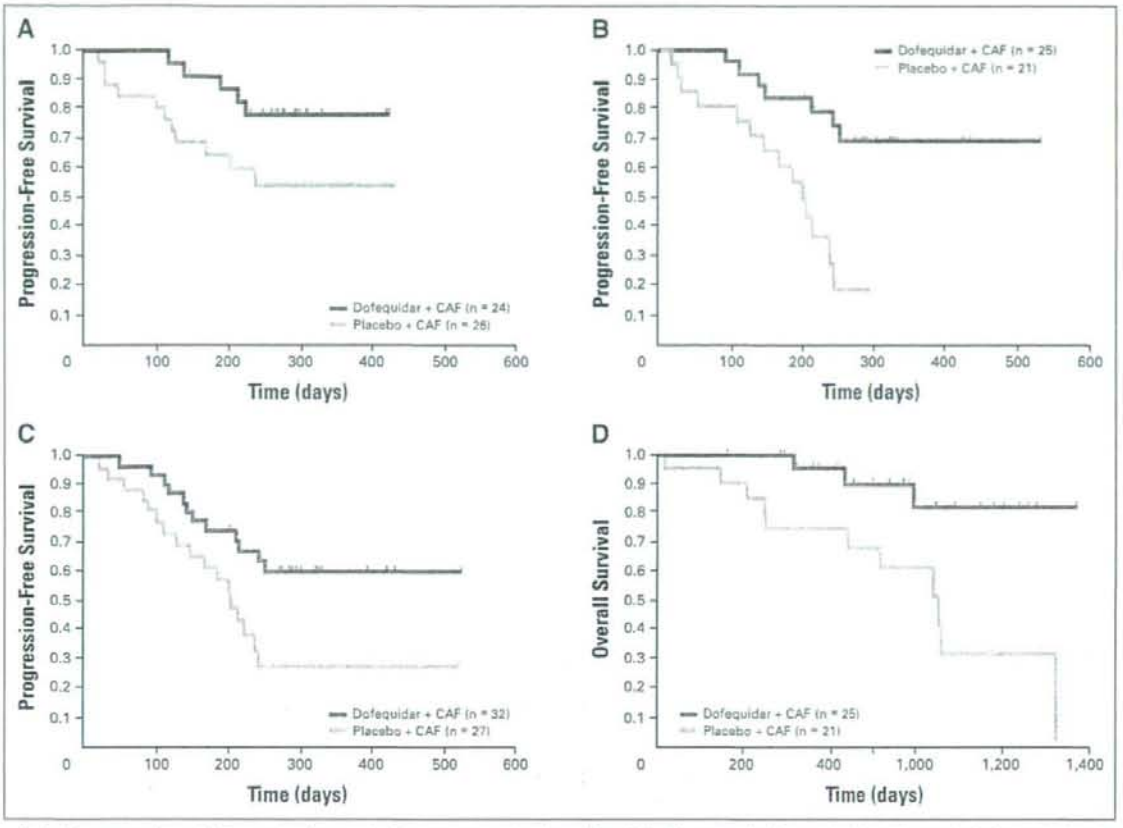

Fig 3. Subgroup analyses. (A) Progression-free survival in premenopausal patients ($P = .046$); (B) progression-free survival in patients who had no prior therapy ($P = .0007$); (C) progression-free survival in patients who were stage IV at diagnosis with an intact primary tumor ($P = .017$); and (D) overall survival in patients who had no prior therapy ($P = .0034$).

with advanced disease.[12] Significantly increased expression of P-gp and MRP-1 has also been reported in an immunohistochemical study of patients treated with preoperative chemotherapy, whereas pretreatment expression of MRP-1 was associated with significantly shorter PFS in patients.[26] In a more recent study, MRP-1 expression was shown to be an independent predictor for shorter relapse-free survival and overall survival, after adjuvant CMF treatment, in premenopausal, hormone receptor–positive patients.[27] However, MRP-1 expression did not affect patients' response to adjuvant tamoxifen plus goserelin treatment.[27]

These findings and our results support the view of Leonard et al,[3] who indicate that future patients will need to be carefully selected for the identification and development of effective drug-resistance modulators. Patient populations who may derive maximal benefit from MDR inhibition, for example, the no-prior-therapy, advanced-disease, or premenopausal patient group in the present study, could quite easily be overlooked or lost within a large, heterogeneous trial population.[3] Furthermore, by refining future clinical trials to incorporate specific disease and patient characteristics, a clearer picture of drug resistance in cancer will be obtained and the most effective MDR inhibitor/chemotherapeutic agent(s) selected.

Many MDR inhibitors have required high serum concentrations for MDR reversal, which resulted in unacceptable toxicity, thereby limiting their clinical impact.[7,28-32] Although more recent agents have shown improved tolerability profiles, this has been countered by unpredictable pharmacokinetic interactions with other transporter molecules (eg, cytochrome P450–mediated drug metabolism and excretion, necessitating dose reductions in chemotherapy agents and leading to inconsistent chemotherapy dosing among patients).[1,5] Similarly, the addition of the MDR-modulating agent valspodar (PSC 833) to chemotherapy agents did not improve treatment outcome.[33,34] Toxicity was increased in the valspodar-treated group compared with chemotherapy agents alone, despite the reduction of chemotherapy doses in the valspodar-containing regimen. In our study, dofequidar was well tolerated, with no indication of the unacceptable toxicity associated with early MDR inhibitors. Importantly, dofequidar did not affect the plasma concentrations of doxorubicin in patients during the study and displayed an acceptable pharmacokinetic profile.

In conclusion, this study suggests that treatment with dofequidar resulted in possible clinical benefit for patients who had not received prior therapy, who were premenopausal, or who were stage IV at diagnosis with an intact primary tumor. Dofequidar was also well

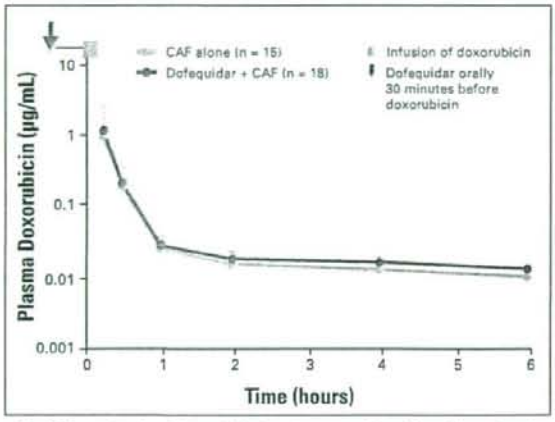

Fig 4. Plasma levels of doxorubicin in patients receiving dofequidar or placebo. CAF, cyclophosphamide, doxorubicin, and fluorouracil.

tolerated in the clinical setting and had no impact on doxorubicin pharmacokinetics. Further studies are merited to assess the effect of dofequidar in specific patient populations with breast cancer.

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment: N/A **Leadership:** N/A **Consultant:** Toshiaki Saeki, Nihon Schering; Takashi Tsuruo, Nihon Schering **Stock:** N/A **Honoraria:** N/A **Research Funds:** N/A **Testimony:** N/A **Other:** N/A

Conception and design: Toshiaki Saeki, Masakazu Toi, Yoshinori Ito, Shinzaburo Noguchi, Tadashi Kobayashi, Hironobu Minami, Tadashi Ikeda, Yasuo Ohashi, Wakao Sato, Takashi Tsuruo
Financial support: Wakao Sato
Administrative support: Toshiaki Saeki, Tadashi Ikeda
Provision of study materials or patients: Toshiaki Saeki, Tadashi Nomizu, Masakazu Toi, Yoshinori Ito, Shinzaburo Noguchi, Tadashi Kobayashi, Taro Asaga, Hironobu Minami, Naohito Yamamoto, Kenjiro Aogi, Tadashi Ikeda
Collection and assembly of data: Toshiaki Saeki, Tadashi Nomizu, Masakazu Toi, Yoshinori Ito, Shinzaburo Noguchi, Tadashi Kobayashi, Taro Asaga, Hironobu Minami, Naohito Yamamoto, Kenjiro Aogi
Data analysis and interpretation: Toshiaki Saeki, Masakazu Toi, Yoshinori Ito, Shinzaburo Noguchi, Tadashi Kobayashi, Hironobu Minami, Tadashi Ikeda, Yasuo Ohashi, Wakao Sato
Manuscript writing: Toshiaki Saeki, Wakao Sato
Final approval of manuscript: Toshiaki Saeki, Tadashi Nomizu, Masakazu Toi, Yoshinori Ito, Shinzaburo Noguchi, Tadashi Kobayashi, Taro Asaga, Hironobu Minami, Naohito Yamamoto, Kenjiro Aogi, Tadashi Ikeda, Yasuo Ohashi, Wakao Sato, Takashi Tsuruo

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Acknowledgment

We thank the investigators (physicians and staff) at the participating institutions; Shunzo Kobayashi, Tomoo Tajima, and Chikuma Hamada (Independent Monitoring Committee); Shigeto Miura, Morihiko Kimura, Hideo Inaji, Izo Kimijima, and Hirokazu Watanabe (Efficacy Evaluation Committeee); and Nihon Schering K.K. for their help.

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Overexpression of soluble vascular endothelial growth factor receptor 1 in colorectal cancer: Association with progression and prognosis

Tatsuro Yamaguchi,[1] Hiroko Bando,[2] Takeo Mori,[1] Keiichi Takahashi,[1] Hiroshi Matsumoto,[1] Michiya Yasutome,[1] Herbert A. Weich[3] and Masakazu Toi[1,4,5]

[1]Department of Surgery, Tokyo Metropolitan Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Disease Center, Tokyo 113-8677; [2]Department of Surgery, Tsukuba University Hospital, Tsukuba 305-8577, Japan; [3]Department of Gene Regulation and Differentiation, National Research Center for Biotechnology, D-38124 Braunschweig, Germany; [4]Department of Clinical Trials and Research, Tokyo Metropolitan Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Disease Center, Tokyo 113-8677, Japan

(Received October 3, 2006/Revised November 13, 2006/Accepted November 21, 2006/Online publication January 10, 2007)

We examined the expression of sVEGFR1 in colorectal cancer tissue and corresponding normal colorectal mucosa to assess the clinical significance of sVEGFR1 in colorectal cancer. We also assessed the relationship between sVEGFR1 levels and various clinicopathologic factors and prognoses. sVEGFR1 and VEGF levels were measured in fresh-frozen tumor extracts from 84 primary colorectal cancer tissues and 27 corresponding normal mucosa using ELISA. Mean of sVEGFR1 levels were 3.17 ng/mg protein. sVEGFR1 levels increased significantly in cancer tissue compared with normal mucosa. Although VEGF levels increased in cancer tissues, the ratio of sVEGFR1/VEGF in cancer tissue was significantly lower than that in normal tissue. No significant correlation between sVEGFR1 or VEGF levels and any clinicopathologic parameter was found. Overexpression of sVEGFR1 was significantly associated with a favorable prognosis. Based on sVEGFR1 levels in colorectal cancer without distant metastases, patients with higher sVEGFR1 levels (≥1.5 ng/mg protein) demonstrated significant longer recurrence-free survival than patients with lower sVEGFR1 levels (<1.5 ng/mg protein) ($P = 0.0017$). Multivariate analysis showed that the sVEGFR1 levels in cancer tissue were an independent prognostic indicator of disease progression. sVEGFR1 expression was significantly elevated in colorectal cancer tissue compared with normal mucosa and the intratumoral balance between sVEGFR1 and VEGF was significantly different between tumor tissue and normal controls. Furthermore, sVEGFR1 levels showed a significant prognostic value. Further studies concerning the biologic and therapeutic significance of sVEGFR1 in colorectal cancer are warranted. (*Cancer Sci* 2007; 98: 405–410)

Vascular endothelial growth factor, also known as VEGF-A, is one of the most important angiogenic factors.[1] It has been reported that overexpression of VEGF is an independent factor predicting poor prognosis in various types of malignant tumors.[2–7] VEGF binds to VEGFR1 and VEGFR2, which are tyrosine kinase receptors.[8,9] VEGFR1 mediates critical effects on physiologic and pathologic neovascularization; however, the function of VEGFR1 in the process of angiogenesis remains unclear. Some authors have reported that VEGFR1 functions as a positive regulator of angiogenesis,[10–12] whereas others have reported that it is a negative regulator of angiogenesis.[13,14] In contrast, VEGFR2 is widely accepted as an angiogenic receptor. VEGFR2 activates a phospholipase C gamma-protein kinase C-Raf-MAP kinase signaling pathway, which results in endothelial cell migration, proliferation, and vascular permeability.[15]

In addition to these receptors, a natural soluble form of the VEGF receptor (sVEGFR1) has been identified.[16,17] sVEGFR1, which was first cloned from the human umbilical vein endothelial cell cDNA library, is an alternative splicing variant of the VEGFR1 gene. It binds to VEGF with high affinity and inhibits its mitogenic response. Not only VEGF-A, but also other VEGF family ligands such as placenta growth factor, have the ability to bind to sVEGFR1.[18] sVEGFR1 is believed to be a modulator of or negative regulator of VEGF activity. Recently it has been reported that sVEGFR1 is expressed in breast cancer and astrocytic tumors.[19,20] In these tumors, sVEGFR1 correlated significantly with tumor growth and prognosis. In animal experimental models, transfer of *sVEGFR1* genes resulted in suppression of angiogenesis and regression of tumors.[21] Experimental data indicate strongly that sVEGFR1 is an important and intrinsic counterpart of VEGF and of angiogenesis, and the clinical importance of serum sVEGFR1 levels has been reported in some kind of tumors, including leukemia, lung cancer, and colorectal cancer.[22–24] However, the clinical significance of sVEGFR1 level in colorectal cancer tissue is largely unknown. We investigated the expression of sVEGFR1 in human colorectal cancer, as well as normal colorectal mucosa and explored the clinical significance of this receptor.

Materials and Methods

Patient population. We randomly selected tissue from 84 patients with operable colorectal cancer who underwent surgical resection at the Tokyo Metropolitan Komagome Hospital from January to December 2003. As controls, we obtained corresponding normal mucosa from 27 colorectal cancer patients. All patients signed an informed consent according to a protocol approved by the ethics committee of the hospital. Representative samples of tumor specimens and normal mucosa tissue were immediately frozen in liquid nitrogen after surgical resection and stored at −80°C until preparation for ELISA. Pathologic examinations were carried out on formalin-fixed, paraffin-embedded specimens.

Histopathologic analysis. Representative sections from all primary tumors were reviewed and analyzed by pathologists. Morphologic features examined included grade, lymph vessel/blood vessel involvement, and number of lymph nodes involved.

Sample preparation. Colorectal tumor tissue and normal mucosa samples were treated as previously reported.[22] Briefly, tissue samples were homogenized in a solution of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl2, 50 μM potassium phosphate, and a protease-inhibitor cocktail. The supernatants were then stored at −80°C until use. The

To whom correspondence should be addressed. E-mail: maktoi77@wa2.so-net.ne.jp

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PlGF, placental growth factor; sVEGFR1, soluble vascular endothelial growth factor receptor 1; VEGF, vascular endothelial growth factor.

doi: 10.1111/j.1349-7006.2007.00402.x

protein concentration of the supernatant extracted from tumor tissues was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA. Total VEGF protein concentrations in the tumor cytosols were measured using VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA). Measurements were made according to the methods recommended by the manufacturer. The minimal detection limit for total VEGF was 31 pg/mL.

ELISA for sVEGFR1 was carried out as previously reported.[3,25] A human sVEGFR1 ELISA kit (Bender MedSystems, Vienna, Austria) was used according to the manufacturer's protocol. The minimum detection limit was 100 pg/mL.

All protein level measurements made by ELISA were carried out in duplicate.

Statistical analysis. The correlation between two factors was evaluated using the Spearman's correlation coefficient by rank. Differences between groups were evaluated using the Student's *t*-test for continuous variables and the Kruskal–Wallis test for categorical values. Fisher's exact test was used to evaluate the relationship between two discrete and dichotomous variables. The analysis of disease-free survival was intended for colorectal cancer patients without distant metastases at the time of their operation. Survival curves were drawn using the Kaplan–Meier method and the log-rank test. Multivariate analyses were performed using logistic regression analysis. Significance was defined as a $P < 0.05$. All statistical evaluations were carried out by StatView (Abacus Concepts, Inc., Berkley, CA, USA).

Results

Clinicopathologic characteristics. Clinical and pathologic characteristics of the patients are shown in Table 1. All patients underwent surgical resection of the primary tumor and the diagnosis of adenocarcinoma was made microscopically. Of the 84 patients, 10 had distant metastases, including six with liver metastases, four with distant lymph node metastases, one with peritoneal dissemination, and one with lung metastases. Two patients had more than one distant metastasis. Of the 74 patients without distant metastases, 21 received adjuvant chemotherapy. The median follow-up period for all patients was 29.6 months (range, 1.0–35.3 months).

sVEGFR1 and VEGF concentrations. sVEGFR1 levels were detectable in 82 of 84 colorectal cancer tissues and ranged from 0.00 to 7.11 ng/mg protein. Mean sVEGFR1 and VEGF concentrations in colorectal cancer tissue were 3.17 ng/mg protein and 1.26 ng/mg protein, respectively, compared with 0.93 ng/mg protein and 0.25 ng/mg protein, respectively, in normal mucosa ($P < 0.0001$ for both comparisons, Wilcoxon signed rank test; Fig. 1a,b). The median sVEGFR1/VEGF ratio was 3.6 in colorectal cancer tissue and 11.8 in normal mucosa with a significant difference between groups ($P = 0.018$, Wilcoxon signed rank test; Fig. 1c). There was a significant correlation between sVEGFR1 and VEGF levels in colorectal cancer tissue ($\rho = 0.52$, $P < 0.0001$, Spearman's rank correlation test), whereas no correlation was seen between sVEGFR1 and VEGF levels in normal mucosa ($\rho = 0.24$, $P = 0.23$, Spearman's rank correlation test; Fig. 2a,b).

Predictive factor for recurrence of colorectal cancer. Table 2 shows the results of univariate analyses of angiogenic factors in colorectal cancer without distant metastases. sVEGFR1 levels were significantly higher in tissue from patients with colorectal cancer who did not experience a recurrence, compared with tissue from patients with colorectal cancer who did experience a recurrence ($P = 0.038$). However, there was no significance in VEGF levels between tissue from patients with colorectal cancer who did or did not experience a recurrence ($P = 0.46$). Based on recurrence status, lymph node metastases and sVEGFR1 levels were significantly different in tissues from patients with colorectal cancer that recurred and tissue from patients with colorectal cancer that did not recur ($P = 0.011$ and $P = 0.038$, respectively; Table 3). However, there was no correlation between VEGF levels and recurrence status ($P = 0.46$).

Table 1. Clinicopathological characteristics

Characteristics	Frequencies	Percentage (%)
Age		
Mean (years)	64 (37–83)	
Gender		
Male	48	57.1
Female	36	42.9
Location		
Colon	41	48.8
Rectum	43	51.2
T factor		
T1	3	3.6
T2	11	13.1
T3	54	64.3
T4	16	19.0
Size		
Mean (cm)	4.8 (1.8–11.5)	
Lymph node status		
Negative	46	54.8
Positive	38	45.2
Differentiation		
well	38	45.2
non-well	46	54.8
Dukes stage		
A	12	14.3
B	33	39.3
C	29	34.5
D	10	11.9
Chemotherapy		
Negative	53	63.1
Positive	31	36.9
Recurrence		
Negative	64	86.5
Positive	10	13.5

To assess the predictive value of sVEGFR1 status, we determined a cut-off level according to a step-wise method that provided the optimal separation between a low and high risk of recurrence. Table 4 shows the relationship between the increasing cut-off level and the statistical prognostic value by logrank test. The cut-off value of sVEGFR1 was determined as 1.5 ng/mg protein. When we compared recurrence-free survival based on sVEGFR1 levels in colorectal cancer, patients with higher sVEGFR1 levels (≥1.5 ng/mg protein) demonstrated significant longer recurrence-free survival than patients with lower sVEGFR1 levels (<1.5 ng/mg protein) ($P = 0.0017$; Fig. 3).

In the multivariate analysis, lymph node status and sVEGFR1 levels were independent predictive factors of recurrence in colorectal cancer (Table 5).

We investigated the correlation between angiogenic factors and overall survival in colorectal cancer. The two-year survival was 94.2% in the patients with high sVEGFR1 levels and 80.0% in patients with low sVEGFR1 levels. There was no significant correlation between the two groups ($P = 0.25$).

Discussion

Vascular endothelial growth factor overexpression is known to be associated with the progression of cancer. In many types of

doi: 10.1111/j.1349-7006.2007.00402.x

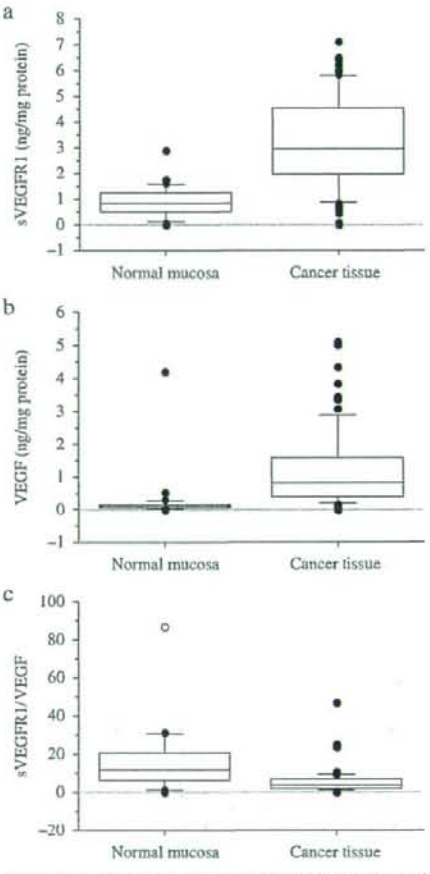

Fig. 1. Comparison of the concentration of soluble vascular endothelial growth factor receptor 1 (sVEGFR1) and vascular endothelial growth factor (VEGF) in colorectal cancer and normal mucosa. sVEGFR1 and VEGF levels were significant higher in colorectal cancer than in normal mucosa (Wilcoxon signed rank test; $P < 0.0001$; Fig. 1a,b). The sVEGFR1/VEGF ratio was significant lower in colorectal cancer than in normal mucosa (Wilcoxon signed rank test; $P = 0.018$; Fig. 1c).

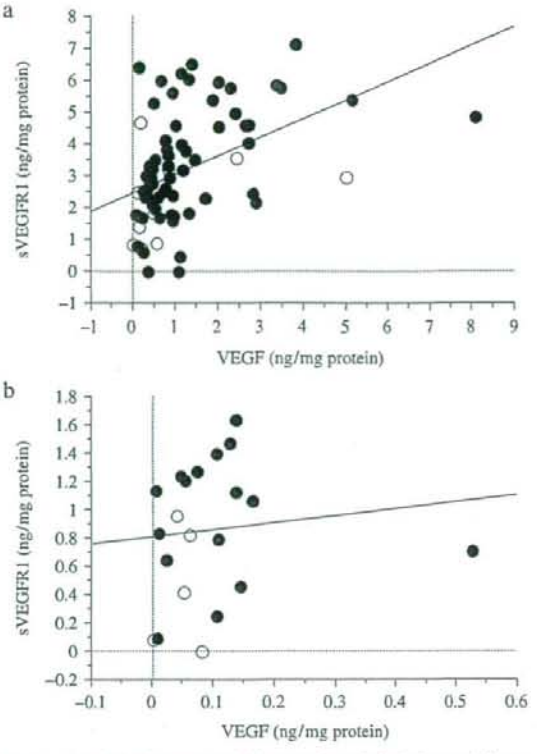

Fig. 2. Correlation between soluble vascular endothelial growth factor receptor 1 (sVEGFR1) and vascular endothelial growth factor (VEGF) concentrations in colorectal cancer (Fig. 2a) and in normal mucosa (Fig. 2b). There was a significant correlation between sVEGFR1 and VEGF levels in colorectal cancer (Spearman's rank correlation test; $\rho = 0.52$, $P < 0.0001$). There was no significant correlation between sVEGFR1 and VEGF levels in normal mucosa (Spearman's rank correlation test; $\rho = 0.24$, $P = 0.23$). ●, no recurrence; ○, recurrence.

human cancers, VEGF concentrations increase significantly in tumor tissues and correlate with prognosis. In this study, we found that sVEGFR1 levels were elevated in colorectal cancer tissue compared with corresponding normal mucosa. Previously, we reported that the means of VEGF and sVEGFR1 levels in breast cancer tissue were 0.53 ng/mg protein and 0.95 ng/mg protein, respectively.[25] Comparing those data with the current data measured by the same method, both VEGF and sVEGFR1 levels are significantly higher in colorectal cancer than breast cancer ($P < 0.001$ and $P < 0.001$, respectively). On the other hand, it has been reported that serum sVEGFR1 level was more often elevated in breast cancer than colorectal cancer.[24] The reasons for the difference between breast cancer and colorectal cancer and between tissue and serum levels are not clear, yet. Further studies are required to clarify the difference. In addition, there is a significant correlation between VEGF expression and sVEGFR1 expression in colorectal cancer tissues. The finding that VEGF and its intrinsic negative counterpart, sVEGFR1, tend to be coexpressed in a positive association was also observed in a study of primary breast cancer.[3,25] These similar findings indicate a possibility that a common regulatory mechanism exists between these molecules and that the mechanism is activated in the process of carcinogenesis or cancer progression. Interestingly, the ratio of sVEGFR1 and VEGF was significantly lower in cancer tissue compared with normal tissue, although little is known about the mechanism of this finding. According to accumulated experimental and clinical data, it is likely that the induction of sVEGFR1 in tumor tissue inhibits VEGF-induced tumor angiogenesis and retards tumor growth.[16,17,19,20] Thus, it is speculated that the shift in the tumor microenvironment from a sVEGFR1-dominant state to a relatively VEGF-dominant state helps tumors form new vessels, grow, and progress.

In terms of the up-regulation mechanism of sVEGFR1, Barleon *et al.* showed that media conditioned by various cancer cell lines grown under hypoxic conditions were able to up-regulate expression of VEGFR1 and sVEGFR1 but not of VEGFR2.[26] Theses effects were completely inhibited by VEGF-neutralizing extracellular VEGF receptor domains, indicating that expression of sVEGFR1 might be regulated by VEGF. Consequently, VEGF as well as hypoxia might play a significant role in the regulation of sVEGFR1 expression in the tumor microenvironment.

Table 2. The quantitation of soluble vascular endothelial growth factor receptor 1 (sVEGFR1) and vascular endothelial growth factor (VEGF) in colorectal cancer without distant metastases

Characteristics	sVEGFR1 (ng/mg protein)	*P*-value	VEGF (ng/mg protein)	*P*-value
Age				
≤60	3.48 ± 1.62	0.37	1.06 ± 1.08	0.34
>60	3.11 ± 1.79		1.38 ± 1.54	
Gender				
Male	3.46 ± 1.54	0.30	1.40 ± 1.65	0.36
Female	3.03 ± 1.91		1.10 ± 0.99	
Location				
Colon	3.12 ± 1.83	0.49	1.44 ± 1.63	0.24
Rectum	3.40 ± 1.64		1.07 ± 1.07	
T factor				
T1	2.90 ± 0.53	0.76	1.08 ± 0.66	0.77
T2	3.29 ± 2.31		1.33 ± 1.09	
T3	3.13 ± 1.56		1.12 ± 1.18	
T4	3.88 ± 2.05		1.82 ± 2.35	
Lymph node status				
Negative	3.44 ± 1.70	0.25	1.35 ± 1.48	0.47
Positive	2.97 ± 1.76		1.11 ± 1.22	
Differentiation				
well	3.38 ± 1.89	0.56	1.50 ± 1.67	0.15
non-well	3.15 ± 1.59		1.04 ± 1.04	
Dukes stage				
A	3.27 ± 2.01	0.44	1.27 ± 0.96	0.38
B	3.50 ± 1.61		1.38 ± 1.64	
C	2.97 ± 1.76		1.11 ± 1.22	
Adjuvant therapy				
Negative	3.37 ± 1.79	0.36	1.29 ± 0.36	0.77
Positive	2.97 ± 1.55		0.18 ± 0.47	
Recurrence				
Negative	3.42 ± 1.73	0.038	1.30 ± 1.35	0.46
Positive	2.21 ± 1.32		0.96 ± 1.59	

Intratumoral sVEGFR1 and VEGF levels were determined by enzyme-linked immunosorbent assay (ELISA). The results reflect the mean values and *P*-value. The correlations between each biological factor and clinicopathological parameters were analyzed using Student's *t*-test and Kruskal–Wallis test. The data shown are the mean values ± standard deviation.

Table 3. The differences of clinicopathological factors in colorectal cancer patients without distant metastases according to their recurrence status

Characteristics	Absence of recurrence	Presence of recurrence	*P*-value
Age			
≤60	25	4	>0.99
>60	39	6	
Gender			
Male	32	7	0.32
Female	32	3	
Location			
Colon	32	5	>0.99
Rectum	32	5	
Depth of invasion			
T1	3	0	0.26
T2	11	0	
T3	42	7	
T4	8	3	
Size			
Mean (cm)	4.5 + 2.0	5.5 + 2.1	0.19
Lymph node status			
Negative	43	2	0.011
Positive	21	8	
Differentiation			
well	33	2	0.091
non-well	31	8	
Dukes stage			
A	12	0	0.015
B	31	2	
C	21	8	
Adjuvant therapy			
Negative	45	19	0.71
Positive	8	2	
sVEGFR1			
Mean (ng/mg protein)	3.42 + 1.73	2.21 + 1.32	0.038
VEGF			
Mean (ng/mg protein)	1.30 + 1.35	0.96 + 1.59	0.46

The Fisher's exact test was used to evaluate the relationship between two discrete and dichotomy variables, and the Student's *t*-test was used to evaluate the differences between the two groups for continuous variables. sVEGFR1, soluble vascular endothelial growth factor receptor 1; VEGF, vascular endothelial growth factor.

Recently, circulating sVEGFR1 levels were discovered to increase remarkably in patients with preeclampsia.[27] It is thought that sVEGFR1 is made by the placenta and acts by neutralizing VEGF and PlGF. Higher concentration of sVEGFR1 and lower concentrations of PlGF and VEGF have been observed in the serum of patients with preeclampsia. Therefore, a reciprocal regulatory mechanism can be considered not only in the preeclampsia state, but also in malignant tumors.

There were two opposite reports regarding the serum sVEGFR1 levels in colorectal cancer patients. Kumar *et al.* reported that sVEGFR1 was detected in the serum of colorectal cancer patients, and after surgery, it was markedly decreased. On the other hand, Chin *et al.* showed that serum sVEGFR1 levels in colorectal cancer patients before surgery were significantly lower than those in normal controls, and after curative surgery, serum sVEGFR1 levels became equivalent to those in normal controls.[28] As in our study sVEGFR1 levels were significantly higher in colorectal cancer tissue than in normal mucosa, our data supported the former report.

To our knowledge, the current study is the first to show the clinical significance of sVEGFR1 expression in colorectal cancer tissue and normal mucosa. VEGF and sVEGFR1 levels had no correlation with any clinical or pathologic factors in colorectal cancers; however, overexpression of sVEGFR1 was significantly associated with absence of recurrence. Intratumoral VEGF concentrations showed no prognostic value in this study. Several reports have failed to demonstrate the prognostic value of tumor VEGF expression,[29,30] whereas many other studies have shown that VEGF has a significant value as a prognostic marker.[31–35] This discrepancy might be due to the difference in the measurement methodology of VEGF. We measured VEGF protein concentrations in fresh-frozen tumor materials by ELISA after confirming a significant relationship between VEGF protein levels measured by ELISA and those measured by Western-blot analysis.[25]

To characterize the prognostic value of sVEGFR1, we assessed a cut-off value and the ratio between sVEGFR1 and VEGF levels. A significant prognostic value was observed between 1.0 ng/mg protein and 2.0 ng/mg protein, and the highest value was seen at 1.5 ng/mg protein; thus, we used 1.5 ng/mg protein as the cut-off value for prognostic assessment. Patients with high sVEGFR1 levels (≥1.5 ng/mg protein) showed a significantly favorable prognosis compared with those with low sVEGFR1 levels (<1.5 ng/mg protein). In the analysis

doi: 10.1111/j.1349-7006.2007.00402.x

Table 4. Univariate prognostic value of soluble vascular endothelial growth factor receptor 1 (sVEGFR1)

Cut-off level (ng/mg protein)	*P*-value
1.0	0.0161
1.1	0.0161
1.2	0.0161
1.3	0.0161
1.4	0.0161
1.5	0.0017
1.6	0.0057
1.7	0.0087
1.8	0.0532
1.9	0.0110
2.0	0.0201
2.5	0.1242
3.0	0.0655
3.5	0.1531
4.0	0.1704
4.5	0.1974
5.0	N.D.

sVEGFR1 status showed a significant statistical prognostic value by univariate analysis. The cut-off value of sVEGFR1 was determined as 1.5 ng/mg protein. N.D., not determined.

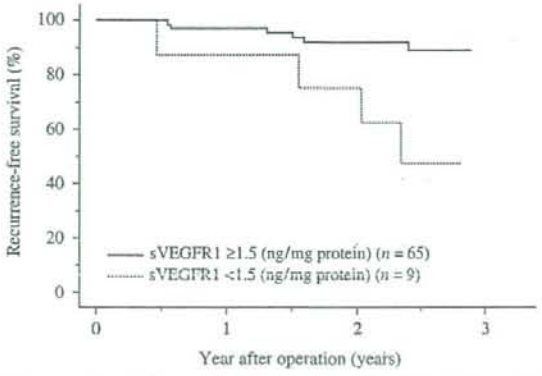

Fig. 3. Kaplan–Meier curve for recurrence-free survival in patients with colorectal cancer by soluble vascular endothelial growth factor receptor 1 (sVEGFR1) level. When we compared recurrence-free survival based on sVEGFR1 levels in colorectal cancer, patients with higher sVEGFR1 levels (≥1.5 ng/mg protein) demonstrated significant longer recurrence-free survival than patients with lower sVEGFR1 levels (<1.5 ng/mg protein) (log-rank test; $P = 0.0017$).

Table 5. Multivariate analysis for recurrence-free survival

		SE	χ^2	HR	*P*-value
Lymph node status	Negative : Positive	0.86	4.89	6.7	0.027
sVEGFR1 (ng/mg protein)	<1.5 : ≥1.5	0.85	4.13	0.17	0.042

HR, hazard ratio; SE, standard error; sVEGFR1, soluble vascular endothelial growth factor receptor 1.

of the sVEGFR1/VEGF ratio, we failed to demonstrate a significant prognostic value in the overall population. These results were different from results of our primary breast cancer study where we found a potent prognostic value of the sVEGFR1/VEGF ratio.[3,25] It is difficult to explain why the sVEGFR1/VEGF ratio was significant for breast cancer but not for colorectal cancer. However the difference in steroid hormone dependency between these two tumor types might contribute to these different findings, as recent studies have shown that estrogen is a significant regulator of VEGF and sVEGFR1 in human breast cancer cells.[36,37]

Recently, bevacizumab, which is a recombinant humanized monoclonal antibody of VEGF, has been demonstrated to improve the survival of metastatic colorectal cancer patients undergoing chemotherapy.[38] Bevacizumab blocks VEGF by inhibiting the VEGF signaling pathway, resulting in suppression of tumor angiogenesis and in retardation of tumor growth. Nevertheless, a recent report showed that VEGF levels had no significant correlation with the therapeutic effect of bevacizumab plus chemotherapy.[39] In a future study, it might be interesting to explore the value of sVEGFR1 and VEGF levels for predicting the therapeutic impact of bevacizumab-containing treatments.

Various types of antiangiogenic therapies are being tested clinically. In the future, more novel agents and new combinations will be examined in clinical trials. Combinations consisting of multiple antiangiogenic agents might be also investigated based on preclinical studies that demonstrate additional or synergistic effects. To create more effective antiangiogenic therapies and optimize treatment for patients, it is critical to study cancer biology further, with a particular emphasis on tumor angiogenesis. Because VEGF is regarded as the most important therapeutic target in an antiangiogenesis strategy, it will be important to pay an attention to sVEGFR1 expression in cancer tissues. It would also be interesting to study what regulates the balance between sVEGFR1 and VEGF levels in cancer tissues and in circulation. These investigations could help lead to an efficient individualized antiangiogenesis therapy for cancer.

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doi: 10.1111/j.1349-7006.2007.00402.x

Review Article

Improving the efficacy of trastuzumab in breast cancer

Eiji Suzuki[1] and Masakazu Toi[2,3]

[1]Department of Surgery, Tokyo Metropolitan Komagome Hospital, Tokyo 113-8677; [2]Department of Surgery, Graduate School of Medicine Kyoto University, Kyoto 606-8507, Japan

(Received January 2, 2007/Revised January 30, 2007/Accepted February 2, 2007/Online publication April 9, 2007)

Although overexpression of human epidermal growth factor receptor 2 (HER2) protein, amplification of the gene or both are associated with poor prognosis in breast cancer, trastuzumab has clearly provided clinical benefits in metastatic breast cancer, adjuvant treatment settings and primary systemic therapy. However, even in those HER2 overexpressors, the majority of patients who achieve an initial response generally acquire resistance within 1 year. Therefore, it is critical to elucidate the mechanism of resistance and to search for better combination treatments with chemotherapeutic agents or other novel modalities. Here, we discuss both clinical and preclinical data regarding these issues. (*Cancer Sci* 2007; 98: 767–771)

Overexpression of human epidermal growth factor receptor 2 (HER2) protein, amplification of the gene or both occurs in approximately 20–30% of primary breast cancers, and the beneficial effects of trastuzumab treatment are seen only in patients with HER2 overexpression. However, less than 35% of patients respond to trastuzumab as a single agent. Furthermore, the majority of the patients who achieve an initial response generally acquire resistance within 1 year.[1,2] To improve the efficacy of trastuzumab in breast cancer patients, it is critical to elucidate the mechanism of resistance of these tumors and develop better combination treatments with chemotherapeutic agents or other novel modalities.

Efficacy of trastuzumab in breast cancer patients

Metastatic breast cancer. Although 68% of the patients in a previous study were treated with anthracycline agents as adjuvant treatment after surgery, trastuzumab alone produced a response rate of only 26% in first-line treatment. To improve this, the investigators tested a combination of chemotherapeutic agents with trastuzumab and showed additive-to-synergistic effects with cisplatin, carboplatin, cyclophosphamide, docetaxel, paclitaxel, vinorelbine, doxorubicin and epirubicin, among others.[3–5] An attenuation of DNA repair activity was reported as the mechanism for synergy between trastuzumab and platinum salts (cisplatin and carboplatin).[6] Pegram *et al.* reported that the combination of docetaxel plus trastuzumab increased antitumor efficacy against MCF7/HER2-overexpressing xenografts compared with the combination of paclitaxel plus trastuzumab.[7] The mechanism behind the unique interaction between trastuzumab and docetaxel has yet to be defined, but at least five differences between paclitaxel and docetaxel might explain the observed interaction. First, docetaxel has more potent cytotoxic antitumor effects than paclitaxel on an equimolar basis.[8] Second, docetaxel achieves higher intracellular concentrations with less cellular efflux of the drug.[9] Third, docetaxel has a higher affinity for microtubules than paclitaxel does.[10,11] Fourth, coincubation of docetaxel with trastuzumab results in increased apoptosis in SK-BR-3 cells compared with that caused by equimolar concentrations of paclitaxel.[12] Fifth, docetaxel is associated with increased phosphorylation of Bcl-2, leading to increased apoptosis at lower concentrations of docetaxel than paclitaxel.[13] Given that the combination of trastuzumab plus the chemotherapeutic agents described above showed synergistic antitumor effects, many clinical trials have been conducted and have revealed an increase in response rate, up to 50–90%.[14–16]

Adjuvant treatment

HERA trial. In the third phase III trial (HERA) (Table 1), patients were randomized after adjuvant (or neoadjuvant) chemotherapy, with or without radiation, to receive trastuzumab every 3 weeks for 1 year or for 2 years, or to receive no trastuzumab therapy (control group).[17] An interim analysis was conducted after 475 events at a median follow-up period of 1 year. The analysis included 3387 patients in the 1-year trastuzumab arm plus the control group in whom a total of 347 events were reported (127 events in the trastuzumab group and 220 in the control group). Data from the 2-year trastuzumab arm were not included in the interim analysis. Disease-free survival rates 2 years after randomization were 86 and 77% for patients in the 1-year trastuzumab group and those in the control group, respectively (hazard ratio [HR] 0.54, $P < 0.0001$). The study included patients of any nodal status, and patients were required to be HER2-positive by immunohistochemistry (IHC) at the 3+ level and/or by fluorescence *in situ* hybridization (FISH).

NSABP-B31 (N9831). After a median follow-up period of 2 years, a joint interim analysis of data from 3351 patients in two cooperative group studies from the USA (NSABP-B31 and NCCTG N9831) showed significant improvements in the primary endpoint of disease-free survival and secondary endpoint of overall survival with paclitaxel plus trastuzumab compared with paclitazel alone (both following anthracycline plus cyclophosphamide).[18] Three years after randomization, disease-free survival was 87% among patients in the paclitaxel plus trastuzumab group compared with 75% in the paclitaxel group (HR 0.48, $P < 0.0001$). After 3 years, there was also a 33% relative reduction in the number of deaths with the addition of trastuzumab (62 vs 92 deaths; HR 0.67, $P = 0.015$).

BCIRG 006. Interim results of the fourth phase III trial (BCIRG 006) in 3222 patients with HER2-positive early stage breast cancer showed that, compared with a control adjuvant regimen of doxorubicin plus cyclophosphamide followed by docetaxel, there was a 51% reduction in the risk of disease recurrence with doxorubicin plus cyclophosphamide followed by docetaxel plus trastuzumab, and a 39% reduction when adjuvant therapy

[3]To whom correspondence should be addressed. E-mail: maktoi77@wa2.so-net.ne.jp

doi: 10.1111/j.1349-7006.2007.00455.x

Table 1. Summary of adjuvant trastuzumab trials

Study	Investigational treatment	Control treatment	n	HR for DFS (95% CI)	P-value
Romond *et al.*[18]	AC→PH	AC→P	3351	0.48 (0.39–0.59)	2×10^{-13}
Piccart-Gebhart *et al.*[17]	Chemotherapy→H	Chemotherapy	3387	0.54 (0.43–0.67)	<0.0001
Slamon *et al.*[19]	AC→TH	AC→T	3222	0.49 (0.37–0.65)	4.8×10^{-7}
	TCH			0.61 (0.47–0.79)	0.00015
Joensuu *et al.*[20]	TH→CEF	T→CEF	232	0.46 (0.21–0.83)	0.0078
	VH→CEF	V→CEF			

AC, doxorubicin and cyclophosphamide; CEF, cyclophosphamide, epirubicin and 5-fluorouracil; CI, confidence interval; DFS, disease-free survival; H, trastuzumab; HR, hazard ratio; P, paclitaxel; T, docetaxel; TCH, docetaxel, carboplatin, and trasuzumab; V, vinorelbine.

Table 2. Summary of neoadjuvant trastuzumab trials

Study	Regimen of primary systemic therapy	pCR rate	Clinical OR
Bines *et al.*[21]	Doc 36 mg/m² q1 week × 12 (over 14 week) + Tra q1 week × 14	13	72
Burstein *et al.*[22]	Pac 175 mg/m² q3 week × 4 + Tra q1 week × 12	18	75
Buzdar *et al.*[23]	Pac 225 mg/m² q3 week × 4 + Tra q1 week × 12 then FEC × 4 + Tra q1 week × 12	65	96
	Pac 225 mg/m² q3 week × 4 then FEC × 4	26	95
Coudert *et al.*[24]	Doc 100 mg/m² q3 week × 6 + Tra q1 week × 18	36	96
Harris *et al.*[25]	Vin 25 bg/m² q1 week + Tra q1 week × 12	21	92
Hurley *et al.*[26]	Doc 70 mg/m² + Cis 70 mg/m² q3 week × 4 + Tra q1 week × 12	21	
Van Pelt *et al.*[27]	Doc 100 mg/m² q3 week × 4 + Tra q1 week × 12		77
Kelly *et al.*[28]	AC q3 week × 4 then Tra + Pac q1 week × 12	19	86

AC, doxorubicine + cyclophosphamide; Cis, cisplatin; Doc, docetaxel; FEC, 5-fluorouracil + epirubicin + cyclophosphamide; OR, overall response; Pac, paclitaxel; pCR, pathological complete response; Vin, vinorelbine; qxwk, every x weeks.

comprised docetaxel, carboplatin and trastuzumab.[19] Results for both trastuzumab-containing treatment arms were statistically siginificant versus the control arm (HR 0.49, $P = 0.00000048$; HR 0.61, $P = 0.00015$). The second interim analysis, BCIRG 006, presented at the San Antonio Breast Cancer Symposium 2006 showed that compared with a control adjuvant regimen of doxorubicin plus cyclophosphamide followed by docetaxel, there was a 39% reduction in the risk of disease recurrence with doxorubicin plus cyclophosphamide followed by docetaxel plus trastuzumab, and a 33% reduction when adjuvant therapy comprised docetaxel, carboplatin and trastuzumab.[19] Results for both trastuzumab-containing treatment arms were again statistically significant versus the control arm (HR 0.61, $P = 0.000011$; HR 0.67, $P = 0.00028$). In the subset analysis, it was shown that coamplification of topoisomerase IIα may confer a therapeutic advantage to an anthracycline-based regimen.

FinHer Study. A smaller adjuvant therapy trial from Finland, FinHer, showed a significant advantage in the use of trastuzumab for only 9 weeks in the adjuvant therapy setting (in combination with docetaxel or vinorelbine).[20] The study involved 1010 patients randomized to docetaxel every 3 weeks for three doses versus 9 weeks of vinorelbine followed, in both groups, by three 3-week cycles of cyclophosphamide, epirubicin and 5-fluorouracil (CEF). The 232 patients found to have HER-2/neu-positive breast cancer by chromogenic *in situ* hybridization (CISH) were randomized to receive weekly trastuzumab for 9 weeks along with docetaxel and vinorelbine. At a median follow-up of 3 years, adjuvant trastuzumab was effective in preventing breast cancer recurrences (HR 0.46; $P = 0.0078$).

Primary systemic therapy. Several phase II trials have evaluated the use of trastuzumab in the neoadjuvant setting.[21–28] Although not always explicitly stated, pathological complete response was the primary endpoint in most of these studies. Various preoperative regimens that included trastuzumab patients with early stage HER2-positive breast cancer have shown promising results, as outlined in Table 2. The rates of pathological complete response ranged from 13 to 65% and those for clinical overall response ranged from 72 to 96%, with the majority being clinical complete responses.

One phase III trial with a planned sample size of 164 patients was halted early because an interim analysis showed a statistical advantage for trastuzumab plus chemotherapy versus chemotherapy alone in terms of the pathological complete response rate, which was the primary endpoint of this study.[23] Pathological complete response rates were 65 versus 26% ($P = 0.016$) among 42 randomized patients. Although clinical overall response rates were similar between the groups (96 vs 95%), clinical complete response rates were numerically higher with the trastuzumab-containing regimen (87 vs 47%). In addition, trastuzumab was generally well tolerated when used concurrently with the anthracycline-containing regimen in this study; however, as mentioned in a comment by Ahluwalia and Daw,[29] the addition of trastuzumab to anthracycline-based chemotherapy should not be used on a routine basis for the treatment of operable breast cancer. Further research is required, particularly to further establish the long-term cardiac safety of this regimen.

Mechanism of action of trastuzumab

Trastuzumab has been shown to have multiple mechanisms of action based on *in vitro* studies (Fig. 1). (1) The antibody binds to the extracellular domain of Her-2/neu and inhibits the downstream signaling cascade, resulting in growth inhibition of Her-2/neu-overexpressing tumor cells. This inhibitory capacity was found to be associated with internalization of the receptor–antibody complex and movement into endocytic vesicles.[30] (2) Treatment of HER2-overexpressing breast cancer cell lines with trastuzumab results in induction of p27KIP1 and the Rb-related protein p130, which in turn significantly reduces the number of cells undergoing the transition to S-phase (G_1 arrest).[31] (3) HER2 undergoes proteolytic cleavage that results in release of the extracellular domain and production of the

doi: 10.1111/j.1349-7006.2007.00455.x

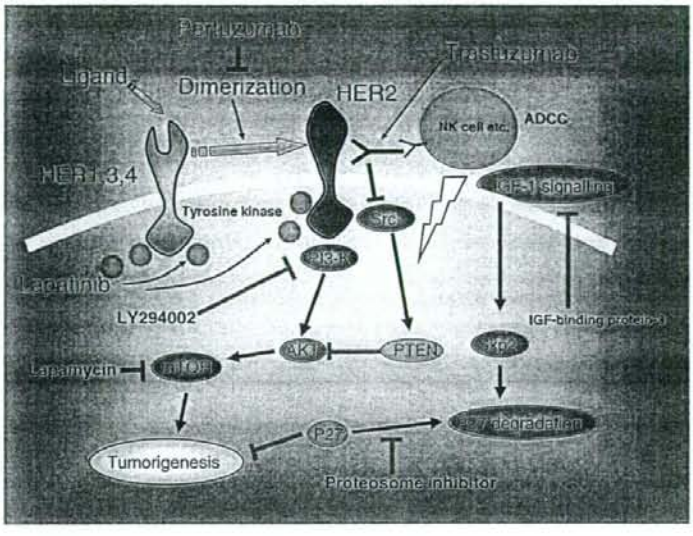

Fig. 1. The mechanisms of action of trastuzumab. Direct activity (induction of apoptosis: phosphatidylinositol-3-kinase [PI3K]–Akt pathway) and antigen-dependent cellular cytotoxicity (ADCC). The mechanisms of resistance: Downregulation of p27, loss of PTEN activity and activation of insulin-like growth factor (IGF)-I signaling. Lapamycin, IGF-binding protein-3, proteosome inhibitor or LY294002 might restore the resistance of trastuzumab. ADCC, antigen-dependent cellular cytotoxicity; HER, human epidermal growth factor receptor; IGF, insulin-like growth factor; NK, natural killer.

truncated membrane-bound fragment p95. This HER2 shedding is activated by 4-aminophenylmercuric acetate, a well-known matrix metallopratease activator, in HER2-overexpressing breast cancer cells. The HER2 p95 fragment is phosphorylated and has kinase activity. Trastuzumab inhibits basal and induced HER2 cleavage and, as a consequence, the generation of phosphorylated p95.[32] (4) Antigen-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells, is triggered following binding of the Fc region of an antibody to the Fcγ receptor IIIa (FcγRIIIa) expressed on natural killer (NK) cells. The clinical importance of ADCC was first demonstrated with rituximab (Rituxan), an anti-CD20 chimeric antibody approved for non-Hodgkin's lymphoma treatment in 1998.[33–35] These studies have focused on the relationships between the clinical response and FcγRIIIa gene (*FCGR3A*) functional polymorphism that generates either phenylalanine (F) or valine (V) at amino acid position 158, with significantly better clinical responses for patients having *FCGR3A*-158 V allele associated with strong IgG binding to the receptor and ADCC activation.[36,37] More recently, ADCC involvement in the clinical response was also suggested for trastuzumab therapy with methods seemingly more direct than *FCGR3A* genotyping. Gennari *et al.* showed a significant correlation between clinical responses and ADCC-mediated killing by patients' peripheral blood mononuclear cells (PBMC).[38] Furthermore, Arnould *et al.* showed an increased infiltration of NK cells into tumor tissue of trastuzumab-responding patients.[39] These reports support an *in vivo* role for ADCC in trastuzumab therapy. (5) Inhibition of angiogenesis has also been reported.[40]

Mechanism of resistance

Although trastuzumab provides important clinical benefits for a substantial proportion of HER2-positive breast cancer patients with well-defined HER2 overexpression or gene amplification, many patients do not respond to trastuzumab, thus underscoring the importance of determining the mechanisms of clinical sensitivity versus resistance. Currently, there is no clinically verified factor that can be used to predict trastuzumab resistance. However, possible mechanisms of resistance have been reported. (1) Nahta *et al.* created two trastuzumab-resistant (TR) pools from the SKBR3 HER2-overexpressing breast cancer cell line and demonstrated that the cyclin-dependent kinase inhibitor $p27^{kip1}$ was decreased in the TR cells and cyclin-dependent kinase activity was increased.[41] Exogenous addition of $p27^{kip1}$ increased trastuzumab sensitivity and the resistant cells displayed heightened sensitivity to proteasome inhibitor MG132, which induced $p27^{kip1}$ expression. Thus, it is suggested that trastuzumab resistance may be associated with decreased $p27^{kip1}$ levels and may be susceptible to treatments that induce $p27^{kip1}$ expression.[41] (2) PTEN (MMAC1/TEP) is a dual phosphatase that mainly dephosphorylates position D3 of membrane phosphatidylinositol-3,4,5 triphosphate (PI3,4,5P3), which is the site for recruiting the plecstrin-homology domain of Akt to the cell membrane. As phosphatidylinositol-3-kinase (PI3K) catalyzes the production of PI3,4,5P3, PTEN antagonizes this PI3K function and negatively regulates Akt activities. Trastuzumab treatment quickly increases PTEN membrane localization and phosphatase activity by recruiting PTEN tyrosine phosphorylation via Src inhibition. Reducing PTEN in breast cancer cells by antisense oligonucleotides confers trastuzumab resistance *in vitro* and *in vivo*. Patients with PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN. Interestingly, LY294002, PI3K inhibitors rescued PTEN loss-induced trastuzumab resistance, suggesting that PI3K-targeting therapies could overcome this resistance.[42] (3) Trastuzumab inhibited the growth of MCF-7/HER2-18 cells, which overexpress HER2/neu receptors and express insulin-like growth factor (IGF)-I receptors (IGFIR). In 1% fetal bovine serum (FBS), trastuzumab reduced cell proliferation by 42%; however, in 10% FBS or IGF-I, trastuzumab had no effect on proliferation. In SKBR3 cells, which overexpress HER2/neu receptor but express few IGFIR, trastuzumab reduced proliferation by 42% regardless of IGF-I concentration. When SKBR3 cells were genetically altered to overexpress IGFIR and were cultured with IGF-I, trastuzumab had no effect on proliferation. However, the addition of IGF-binding protein-3, which decreased IGFIR signaling, restored trastuzumab-induced growth inhibition. Thus, it is suggested that strategies that target IGFIR signaling may prevent or delay development of resistance to trastuzumab.[43]

Possibilities of improving the efficacy of trastuzumab therapy

Lapatinib. Lapatinib is an oral receptor tyrosine kinase inhibitor, targeting both epidermal growth factor receptor (EGFR) and HER2. Pre-clinical *in vitro* and *in vivo* models indicate that lapatinib is active as a monotherapy, synergistically in combination with trastuzumab, and in trastuzumab-resistant cell lines. Konecny *et al.* tested the therapeutic potential of lapatinib in a panel of 31 characterized human breast cancer cell lines, including trastuzumab-conditioned HER-2-positive cell lines, and reported that for the combination of lapatinib plus trastuzumab, synergistic drug interactions were observed in four different HER-2-positive cell lines. Moreover, lapatinib retained *in vitro* activity against cell lines selected for long-term outgrowth in trastuzumab-containing culture medium. Thus, these findings might provide a biological rationale to test lapatinib in combination with trastuzumab in HER-2-overexpressing breast cancer and in patients with clinical resistance to trastuzumab.[44] There has been one phase I trial of lapatinib plus trastuzumab in metastatic breast cancer[45] and two phase II trials of single-agent lapatinib in patients with refractory metastatic breast cancer.[46,47]

Pertuzumab. Pertuzumab, the recombinant humanized monoclonal antibody 2C4, binds to a different epitope on erbB2 than trastuzumab, and inhibits both homodimerization and heterodimerization with other erbB receptors and blocks ligand-activated signaling from HER-2/EGFR and HER-2/HER-3 heterodimers.[48] The combination of trastuzumab and pertuzumab synergistically inhibits the survival of BT474 breast cancer cell lines, in part because of increased apoptosis. Trastuzumab increases 2C4-mediated disruption of erbB2 dimerization with EGFR and erbB3. Combination drug treatment reduced levels of total and phosphorylated erbB2 protein and blocked receptor signaling through Akt, but did not affect MAPK. These results suggest that combining erbB2-targeting agents may be a more effective therapeutic strategy in breast cancer than treatment with a single erbB2 monoclonal antibody.[49] A phase II trial with trastuzumab and pertuzumab in patients with HER2-overexpressed locally advanced and metastatic breast cancer has been conducted.[50]

Mammalian target of rapamycin antagonist. Mammalian target of rapamycin antagonist (mTOR) is a serine-threonine kinase member of the cellular PI3K pathway that is involved in multiple functions such as transcriptional and translational control. Activation of mTOR as a consequence of nutrients and growth factors results in the phosphorylation and activation of the 40S ribosomal protein S6 kinase and the eukaryotic initiation factor 4E-binding protein-1. These proteins play a key role in ribosomal biogenesis and cap-dependent translation, which result in increased translation of mRNA that is important to the control and progression of the cell cycle. mTOR is a downstream mediator in the PI3K–Akt signaling pathway and plays a critical role in cell survival. In breast cancer the PI3K–Akt pathway can be activated by membrane receptors, including the HER family, the IGF receptor, and the estrogen receptor.[51] There is evidence suggesting that Akt promotes breast cancer cell survival and resistance to chemotherapy, trastuzumab and tamoxifen. This suggests that targeting the Akt–PI3K pathway with mTOR antagonists may increase the therapeutic efficacy of trastuzumab-resistant breast cancer.[52]

Fucose-negative trastuzumab. It was reported that removal of fucose from antibody oligosaccharides attached to Asn^{297} of the heavy chain (defucosylation) significantly enhanced ADCC compared to the conventional antibody.[53–56] Thus, this modulation of antibody could be one of the most powerful approaches to improve efficacy in cancer antibody therapy, and we evaluated the ADCC of commercial trastuzumab (fucosylated) and its fucose-negative version using PBMC drawn from the volunteers as effector cells and two breast cancer cell lines with different HER2 expression levels as target cells. ADCC was significantly enhanced with the fucose-negative antibody compared to the fucose-positive antibody. This preliminary study suggests that the use of fucose-negative antibodies may improve the therapeutic effects of anti-HER2 therapy in breast cancer.[57]

Future perspectives

Despite significant improvements in the analysis of mechanisms of action and resistance and clinical outcome with trastuzumab, it is still necessary to resolve the following questions. (1) Optimal timing for the induction of trastuzumab: The results of trastuzumab-based treatment in an adjuvant setting are more impressive than those in metastatic breast cancer. It might be better to start trastuzumab treatment earlier, such as in a primary systemic therapy setting, although neoadjuvant chemotherapy did not show clinical benefit when compared with an adjuvant setting. (2) Optimal duration: Final results from the HERA trial could reveal the optimal duration of trastuzumab treatment (1 vs 2 years). (3) Optimal combination treatment: In addition to chemotherapeutic agents or hormonal treatment, novel molecular targeting therapies, such as lapatinib or bevacizumab, could show clinical benefits. (4) Search for the prediction marker for responder: PTEN could be a promising marker for selecting responders to trastuzumab. Having a clinically useful prediction marker to select responders to trastuzumab is very important for improving health economics because of the high cost of trastuzumab treatment.

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Cytokeratin-18 Is a Useful Serum Biomarker for Early Determination of Response of Breast Carcinomas to Chemotherapy

Maria Hägg Olofsson,[1] Takayuki Ueno,[1,2] Yang Pan,[3] Ren Xu,[3] Feng Cai,[3] Heiko van der Kuip,[4] Thomas E. Muerdter,[4] Maike Sonnenberg,[4] Walter E. Aulitzky,[4] Stephan Schwarz,[1] Elina Andersson,[1] Maria C. Shoshan,[1] Aleksandra Mandic Havelka,[1] Masakazu Toi,[2,5] and Stig Linder[1]

Abstract

Purpose: With a widening arsenal of cancer therapies available, it is important to develop therapy-specific predictive markers and methods to rapidly assess treatment efficacy. We here evaluated the use of cytokeratin-18 (CK18) as a serum biomarker for monitoring chemotherapy-induced cell death in breast cancer.

Experimental Design: Different molecular forms of CK18 (caspase cleaved and total) were assessed by specific ELISA assays. Drug-induced release of CK18 was examined from breast carcinoma cells and tissue. CK18 protein composition was examined in serum. CK18 levels were determined in serum from 61 breast cancer patients during docetaxel or cyclophosphamide/epirubicin/5-fluorouracil (CEF) therapy.

Results: Caspase-cleaved CK18 molecules were released from monolayer cultures and tumor organ cultures to the extracellular compartment. CK18 was present in complexes with other cytokeratins in serum. Such CK18 protein complexes are remarkably stable, leading to favorable performance of CK18 biomarker assays for clinical investigations. Docetaxel induced increased levels of caspase-cleaved CK18 in serum from breast cancer patients, indicating apoptosis. CEF therapy led to increases predominantly in uncleaved CK18, indicating induction of necrotic cell death in many tumors. The increase in total CK18 at 24 h of the first treatment cycle correlated to the clinical response to CEF therapy ($P < 0.0001$).

Conclusions: Induction of necrotic cell death may explain the clinical efficacy of anthracycline-based therapy for breast carcinomas with defective apoptosis pathways. We suggest that CK18 biomarkers are useful for early prediction of the response to CEF therapy in breast cancer and may be useful biomarkers for clinical trials.

Chemotherapy induces multiple effects on tumor cells, including apoptosis, necrosis, autophagy, mitotic catastrophe, and senescence (1). The cellular outcome is dependent on several factors, including the type of drug used, the concentration of drug that will reach the tumor cells, and the properties of the tumor and its microenvironment. Apoptosis is a commonly described cellular outcome of treatment with many anticancer drugs (2, 3), and defects in the apoptotic machinery are believed to contribute to therapy resistance (4, 5). However, whether apoptosis is the primary antiproliferative mechanism of anticancer drugs in solid tumors is controversial (6). Other cell death modes than apoptosis are also possible. DNA-damaging agents have been reported to induce a necrotic response, due to hyperactivation of poly(ADP)ribose polymerase and depletion of cytosolic NAD (7). Photodynamic therapy has also been reported to induce necrosis (8). Various agents may also induce improper segregation of chromosomes during mitosis, leading to mitotic catastrophe (1, 9). Mitotic catastrophe is not a form of cell death per se, but rather a trigger for cell death by various mechanisms (9). Different classes of chemotherapeutic agents and ionizing radiation induce long-term growth arrest reminiscent of replicative senescence (10). An understanding of the mechanisms underlying these different outcomes is important to understand the antiproliferative activity of anticancer drugs and for understanding resistance to therapy.

Determining the mode of cell death is not trivial for cultured cells and is very difficult in tumor tissue. We have developed a method based on measurements of different molecular forms of CK18 that can be used to investigate cell death modes of epithelially derived cells *in vitro* and *in vivo* (11). This method is

Authors' Affiliations: [1]Cancer Center Karolinska, Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm, Sweden; [2]Kyoto University Hospital, Breast Surgery, Kyoto, Japan; [3]Department of Molecular Sciences, Amgen, Inc., Seattle, Washington; [4]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany; and [5]Department of Clinical Trials and Research, Metropolitan Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Disease Center, Tokyo, Japan

Received 1/3/07; revised 3/11/07; accepted 3/19/07.

Grant support: Cancerföreningen in Stockholm, the Swedish Cancer Society (Cancerfonden), the King Gustaf V Jubilee Foundation, EC FP6 (contract no. LSHC-CT-2004-505785), and Robert Bosch Foundation (project 02b), Stuttgart.

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Note: M.H. Olofsson and T. Ueno contributed equally to this work.

Requests for reprints: Stig Linder, Cancer Center Karolinska, R8:03, Karolinska Hospital, 17176 Stockholm, Sweden. Phone: 46-8-51772452; Fax: 46-8-339031; E-mail: Stig.Linder@cck.ki.se.

doi:10.1158/1078-0432.CCR-07-0009

Table 1. Characteristics of patients treated with neoadjuvant chemotherapy

Clinical variable	*n*	Total CK18		Cleaved CK18	
		Median (25th-75th), units/L	*P*	Median (25th-75th), units/L	*P*
Tumor size (cm)					
<2	6	338 (276-401)		114 (111-117)	
2-5	22	333 (309-383)	0.0044	110 (110-116)	0.005
>5	15	437 (356-1,115)		140 (115-249)	
Estrogen receptor					
+	27	343 (307-493)		114 (114-134)	
-	13	363 (306-391)	NS	112 (106-131)	NS
Unknown	3	437 (375-457)		118 (102-130)	
Progesterone receptor					
+	28	381 (308-397)		114 (111-122)	
-	12	346 (305-525)	NS	113 (104-138)	NS
Unknown	3	437 (375-457)		118 (102-130)	
Metastasis					
No. nodes					
0	21	322 (263-391)		115 (107-124)	
1-3	14	388 (339-401)	0.0046	113 (109-128)	NS
>4	8	436 (359-635)		118 (90-157)	
Bone metastasis					
+	3	1,871 (759-2641)		276 (138-295)	
-	40	351 (308-410)	0.028	114 (106-127)	NS

Abbreviation: NS, not significant.

based on the measurement of different molecular forms of cytokeratin 18 (CK18) released from dead cells, whereas apoptosis will result in the release of caspase-cleaved CK18 fragments; necrosis will result in release of uncleaved CK18 (11). These forms can be conveniently distinguished by the use of the monoclonal antibody M30, which recognizes a neo-epitope of CK18 generated during apoptosis (12). CK18 is therefore potentially both a quantitative and qualitative biomarker for cell death *in vivo*. Previous investigations have provided evidence that serum CK18 is derived from tumor cells (11, 13) and have been encouraging with regard to the usefulness of serum CK18 as a clinically useful biomarker (13–16). However, a number of issues with regard to the release of CK18 from cells into serum and the clinical utility of CK18 as a response marker remain to be answered. In this study, we examined treatment responses of breast carcinoma to paclitaxel and anthracycline-based therapy *in vitro* and *in vivo*. We provide evidence that increases of serum CK18 during cyclophosphamide/epirubicin/5-fluorouracil (CEF) therapy are associated with clinical responses to CEF therapy. Furthermore, we found that CEF therapy induces a heterogeneous response *in vivo* with regard to cell death mode.

Materials and Methods

Cell culture. MDA-MB-231 breast carcinoma cells were maintained in DMEM supplemented with 10% FCS, L-glutamate, penicillin, and streptomycin at 37°C in 5% CO_2. Tissue culture reagents were obtained from Life Technologies Cell Culture Products. Cells were treated with doxorubicin (Sigma Chemical Company), staurosporine (Sigma), or paclitaxel (Calbiochem) as indicated.

Tissue slice preparation and culture. Primary breast tumors >3 cm were obtained from patients at the Robert Bosch Hospital, Stuttgart, immediately after surgical resection and maintained in organ transportation medium (Euro-Collins) on ice until use. Tissue cores (5 mm in diameter) were prepared using a hand-held coring tool. From the cylinders, tissue slices with a thickness of 200 μm were prepared in cold PBS using a precision cutting tissue slicer (Krumdieck, Alabama Research and Development Corp.). Slices were then individually submerged in supplemented mammary epithelial growth medium as described (17). Incubation was done in 24-well plates at 37°C in a constant atmosphere of 5% CO_2 on a shaking platform. Treatment with drugs started after a recovery period of 24 h and was done for additional 72 h as described (17). In preliminary experiments, done in a panel of breast cancer samples, we found a higher mitochondrial tetramethylrhodamine methyl ester accumulation in the tumor cell compartment after a recovery period of 24 h.

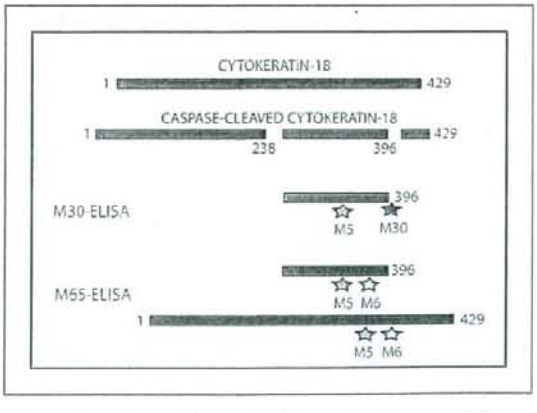

Fig. 1. CK18 is cleaved at Asp^{238} and Asp^{396} by caspases during apoptosis. The M30-Apoptosense ELISA assay uses antibody M30, which detects a neo-epitope of CK18 formed after caspase cleavage at Asp^{396} (12, 17). The M65-ELISA assay will detect all CK18 fragments that contain epitopes in the 300 to 390 amino acid region of the protein (11).

ELISA assays. Caspase-cleaved cytokeratin-18 (CK18-Asp^{396}) was determined by the M30-Apoptosense ELISA (ref. 18; PEVIVA AB). The M65-ELISA assay (PEVIVA AB) was used to measure total soluble CK18. ELISA tests for measuring cytokeratin complexes were done by coating different capture antibodies on 96-well Nunc Immuno Module plates overnight at 4°C in PBS at various concentrations. The capture antibodies used for the mixed ELISA assays were purchased from the following commercial sources: monoclonal antibodies for human CK7 (clone 4A39) and CK8 (clone 4A42) from US Biological, and monoclonal antibody for human CK19 (clone 17) from Abcam. The plates were washed thrice with PBST (PBS + 0.1% Tween 20) before incubation with human serum samples for 2 h at room temperature. Horseradish peroxidase–conjugated monoclonal antibody M30 or M5 from the M30 and M65 ELISA kits were used as detection antibody. 3,3′,5,5′-Tetramethyl-benzidine was used as the substrate. The intensity of the signal was determined by measuring the absorbance at 450 nm using the SpectroMax M5 microplate reader (Molecular Device).

Gel filtration. Serum from patients with breast cancer was fractionated on a Superose G200 column in PBS/10% horse serum. Similarly, medium from apoptotic MDA-MB-231 cells was collected and subjected to gel filtration. Fractions were collected and assayed for caspase-cleaved CK18 and CK18 by ELISA.

Patients. Sera were collected from 61 patients with primary breast cancer treated with chemotherapy (preoperative neoadjuvant chemotherapy for 43 patients and postoperative adjuvant chemotherapy for 18 patients) at the Tokyo Metropolitan Komagome Hospital from 1997 to 2003. The characteristics of the patients are shown in Table 1. Tumor sizes were determined by palpation. Patients were treated with CEF (600 mg, 60 mg, and 600 mg/m^2, respectively; average 3.8 cycles). Cyclophosphamide and 5-fluorouracil were given as 45 to 60 min infusions and epirubicin as a bolus injection. The chemotherapy was administered every 3rd week. Sera from cancer patients were collected before each cycle and at various times after each cycle of treatment. Patients with liver and renal dysfunction and other complications were excluded. For all patients, liver, lung, and distant lymph node metastases were diagnosed using computed tomographic scan, and bone metastases were diagnosed using X-ray or bone scintigraphy. For patients with stage II or more, brain metastasis was examined using computed tomographic scan. Of the 43 patients who received neoadjuvant chemotherapy, three patients had bone metastasis and none had other distant metastases. In 18 patients who received adjuvant chemotherapy, major metastatic sites were lung for seven patients, bone for three, liver for three, pleura for one, and lymph node for four. Clinical responses to treatments were evaluated according to the Union Internationale Contre le Cancer criteria. Informed consent was obtained from all patients, and the study was approved by the local institutional review board.

Statistical analysis. Patients' data are presented as median (25th-75th percentile) and graphically displayed by box plots. The Mann-Whitney *U* method was used to test for difference between two groups. The Wilcoxon matched pair signed ranks test was used to examine whether the members of pairs differ in size. The survival analysis was done by the log-rank test and the Cox proportional hazards model. All tests were done using a two-sided α level of 0.05.

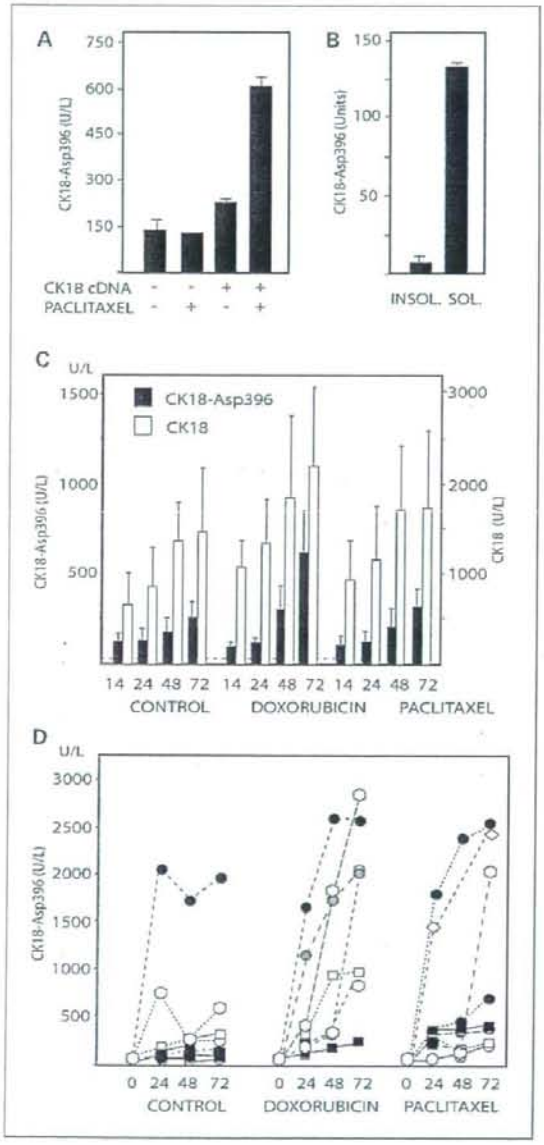

Fig. 2. Release of CK18-Asp^{396} from carcinoma cells. *A*, specificity of the M30-ELISA for caspase-cleaved CK18. Mouse embryo fibroblasts were transfected with a cDNA expression plasmid and treated with paclitaxel as indicated. Note that paclitaxel only induces increases in CK18-Asp^{396} in transfected cells. *B*, distribution of CK18-Asp^{396} epitopes between the insoluble (*INSOL.*) cytoskeleton and the medium after induction of apoptosis. MDA-MB-231 cells were treated with staurosporine (200 nmol/L) for 16 h and the medium was collected. The insoluble fraction was pelleted by centrifugation and washed thrice in PBS/0.5% NP40. The binding of the M30 antibody to the insoluble, nuclear/cytoskeletal fraction was determined by incubation with horseradish peroxidase–conjugated antibody followed by repeated washing and incubation with horseradish peroxidase substrate and expressed in total units (*U*). The total number of units released into the medium was determined by ELISA (U/L corrected with total volume). *C* and *D*, time-dependent increases in release of CK18-Asp^{396} from breast tumor organ cultures. Tumor tissue slices were treated with doxorubicin (1 μg/mL) or paclitaxel (6.8 μg/mL) and CK18-Asp^{396} was determined in the tissue culture medium. *C*, release of CK18 (*open columns*) and CK18-Asp^{396} (*filled columns*) from tumor tissue slices of MDA-MB-231 tumors from severe combined immunodeficient mice (mean values from three slides). Dotted line, baseline activity of tissue culture medium. *D*, release of CK18-Asp^{396} from tumor tissue slices from human breast carcinomas. Slices from seven different breast carcinomas were cultured in the presence or absence of drugs (concentrations as in *C*) *in vitro*, and medium was harvested at the indicated times. Points, mean from triplicate determinations. CK18-Asp^{396} median levels were 203, 544, and 2,056 units/L in control, paclitaxel-treated, and doxorubicin-treated cultures at 72 h.

Results

Release of caspase-cleaved CK18 molecules from drug-treated cells and tumor tissue. CK18 is a major component of the intermediate filament system of simple epithelial cells. During apoptosis, CK18 is cleaved by caspases at Asp^{238} and at Asp^{396} (refs. 12, 19, 20; Fig. 1). CK18 molecules cleaved by caspases at Asp^{396} ($CK18\text{-}Asp^{396}$) react with the M30 monoclonal antibody and soluble $CK18\text{-}Asp^{396}$ molecules are detected by the M30-Apoptosense ELISA (Fig. 1). Such caspase-cleaved CK18 fragments are convenient biomarkers for apoptosis of epithelially derived cells (11, 18, 21). To formally prove that detection of apoptosis by the M30 ELISA requires CK18 expression, we transfected mouse embryo fibroblasts with a CK18 expression plasmid. As expected, paclitaxel stimulated increases in $CK18\text{-}Asp^{396}$ in transfected, but not in untransfected, mouse embryo fibroblast (Fig. 2A). Induction of activity in the assay was blocked by a caspase inhibitor (data not shown; ref. 11).

CK18 is a constituent of the insoluble cytoskeleton and only a minor fraction is soluble (20). To examine whether most caspase cleavage events will generate soluble CK18 fragments, the fraction of $CK18\text{-}Asp^{396}$ epitopes was determined in the insoluble and soluble fractions after induction of apoptosis in a human breast carcinoma cell line. We found that >90% of the $CK18\text{-}Asp^{396}$ epitopes were present in the soluble fraction (Fig. 2B).

To further study the release of CK18 from tumor cells exposed to cytotoxic agents, we examined organ cultures of MDA-MB-231 breast tumors from severe combined immunodeficient mice. Tumor tissue slices were cultivated in the presence or absence of a taxane (paclitaxel) or an anthracycline (doxorubicin) for 3 days (17). Release of $CK18\text{-}Asp^{396}$ fragments to the culture medium was observed from untreated organ cultures, suggesting spontaneous apoptosis (Fig. 2C). Approximately 2-fold higher levels of $CK18\text{-}Asp^{396}$ were observed in doxorubicin-treated cultures at 72 h, whereas paclitaxel induced weaker increases (Fig. 2C). Similar patterns of release were observed when total CK18 was measured (using the M65-ELISA).

The release of $CK18\text{-}Asp^{396}$ fragments from organ cultures of different clinical cases of breast carcinoma treated with paclitaxel or doxorubicin was examined (Fig. 2D). The median level of $CK18\text{-}Asp^{396}$ was 2,056 units/L after 72 h of doxorubicin treatment, compared with 203 units/L in untreated control ($P < 0.05$, Wilcoxon two-sample test), demonstrating that doxorubicin induced apoptosis in the organ cultures. The median level of $CK18\text{-}Asp^{396}$ in paclitaxel-treated cultures was

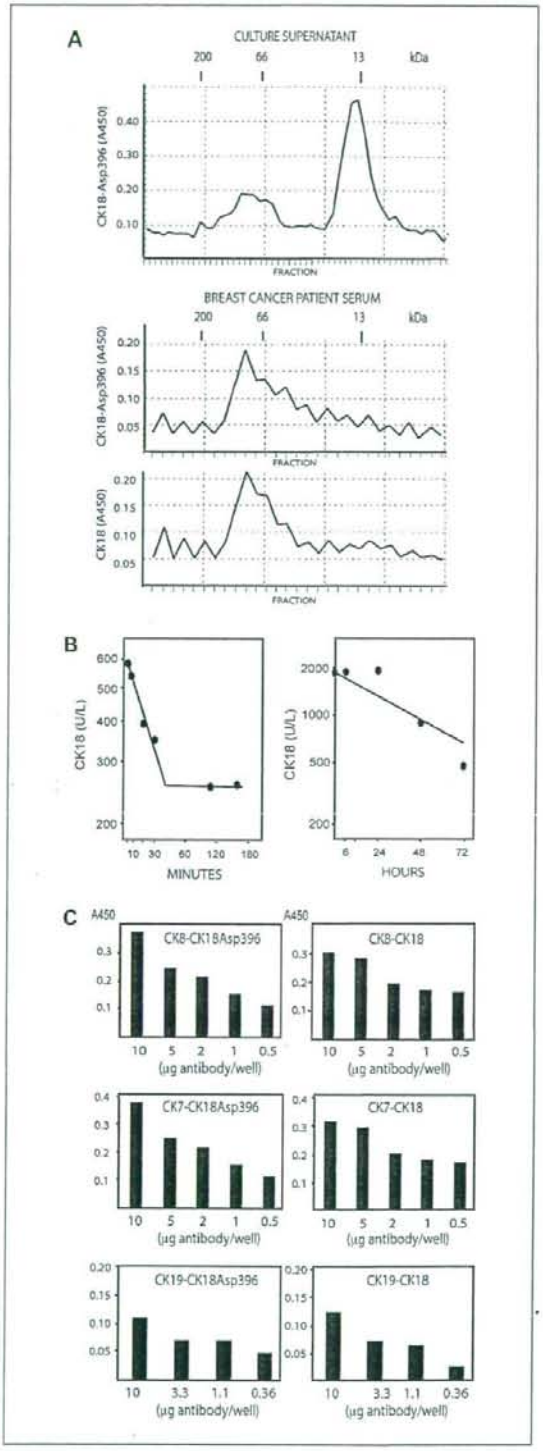

Fig. 3. CK18 complexes released from cells into circulation. *A*, Superose G200 gel filtration of tissue culture medium from apoptotic cells or patient serum (*top*) $CK18\text{-}Asp^{396}$ is present in the 10 to 20 kDa and 50 to 100 kDa range in tissue culture medium from apoptotic MDA-MB-231 cells; $CK18\text{-}Asp^{396}$ (*middle*) and CK18 (*bottom*) is present in the 50 to 100 kDa range in serum from a human breast cancer patient. *B* (*top*), plasma levels of the $CK18_{(284\text{-}396)}$ fragment after injection into mice. The fragment was synthesized in *Escherichia coli* and injected i.v. into mice. Bottom, plasma was collected after different times and assayed for CK18 (using the M65-ELISA assay): The $CK18_{(284\text{-}396)}$ fragment was incubated in mouse plasma at 37°C for the indicated times, and CK18 levels were examined using the M65-ELISA assay. *C*, CK18 is present in complexes with other cytokeratins in serum. Patient serum was analyzed by ELISA using plates coated with increasing amounts (in μg) of CK 8 (*top*), CK7 (*middle*), and CK19 (*bottom*) antibodies. Horseradish peroxidase–conjugated M30 or M5 antibodies were used to show $CK18\text{-}Asp^{396}$ or CK18 in the complexes.

Table 2. Assay precision for the M30 and M65 ELISA

M30 ELISA				M65 ELISA			
Serum sample	Average absorbance	CV%	No. assays	Serum sample	Average absorbance	CV%	No. assays
BRH84384	0.77	17.9	33	BRH84358	0.93	9.16	28
BRH80678	0.57	12.58	28	BRH84384	0.44	11.74	28
BRH84400	0.16	14.03	27	BRH84400	0.21	17.49	23
rCK18 Control	0.68	9.48	36	rCK18 Control	0.48	6.79	26

NOTE: Data were generated over a 8-mo period from two manufacturer's lots using various normal sera as well as the recombinant CK18 standard included in each assay kit.

not significantly different from control cultures at 72 h. Similar patterns of release were observed when total CK18 was measured (not shown).

We considered the possibility that soluble CK18-Asp^{396} fragments may be trapped in tumor tissue. Extensive digestion of tissue organ slices with collagenase did not, however, release significant amounts of activity detected by ELISA (data not shown).

Caspase-cleaved CK18 molecules are present as protein complexes in serum. The molecular composition of soluble proteins containing the M30 epitope was examined. Fractionation of medium from apoptotic cells on Superose G200 revealed one peak in the 10 to 20 kDa region and another in a higher molecular weight region (Fig. 3A, *top*). This pattern was distinct from that observed in sera of cancer patients where the M30 epitope was only found in the 50 to 100 kDa region (Fig. 3A, *middle*). Total CK18 (detected by the M65-ELISA) was found in the same fractions as CK18-Asp^{396} (Fig. 3A, *bottom*).

A 13-kDa CK18 form (CK18 residues 284-396) has been described in culture medium from apoptotic cells (22), consistent with the present findings. Injection of a recombinant 13 kDa fragment i.v. in mice showed a half-life of ~30 min (Fig. 3B, *top*). In contrast, incubation of the 13-kDa fragment in mouse plasma at 37°C *in vitro* showed a half-life of ~48 h (Fig. 3B, *bottom*). CK18 is a 45-kDa protein and the higher molecular weight material in serum were assumed to represent protein complexes. Using the same type of monoclonal CK18 antibodies both on solid phase and for detection in ELISA assays (M5-M5 or M30-M30), signals were detected using serum samples but not using recombinant CK18 (data not shown), consistent with CK18-CK18 complexes in serum. Signals were also observed in sera using mixed ELISA assays based on antibodies to different cytokeratin types (Fig. 3C). The results show that CK18 (and the CK18-Asp^{396} epitope) can be detected in complexes with CK7, CK8, and CK19 in serum, whereas CK18-CK14 complexes were not detected (not shown). A recombinant CK8 protein was tested in the CK8-CK18 ELISA but did not generate a signal (not shown). These findings suggest that small caspase-cleaved CK18 fragments are rapidly cleared from the circulation and that caspase-cleaved CK18 molecules are present as protein complexes in serum.

Caspase activity has been detected in circulation in patients with malignancies (23). A concern in the analysis of caspase-cleaved fragment in blood as a measure of cellular apoptosis is that cleavage of CK18 might occur in circulation. We addressed this issue using CK18-positive serum samples. Incubation with 1,000 units/mL recombinant caspase-3 for 4 h did not increase the levels of fragments containing the CK18-Asp^{396} epitope (data not shown). Control experiments showed that caspase-3 was active in serum under these conditions (data not shown).

Performance of CK18 assays. The clinical utility of the M30 and M65 ELISAs was investigated and qualified according to available bioassay validation guidelines established by a pharmaceutical industry consortium (24, 25). In brief, ELISA sensitivity, precision, specificity, assay range, reagent stability, sample stability, and variations from multiple blood draws of the same donor were investigated and determined. For example, assay precision profiles for the M30 and M65 ELISA

Table 3. Human serum sample freeze/thaw stability

Freeze thaw cycle	M30 (absorbance/% to control)			M65 (absorbance/% to control)		
	BRH84384	BRH80678	BRH84400	BRH84358	BRH84384	BRH84400
1	0.82/100%	0.58/118%	0.13/87%	0.84/105%	0.38/112%	0.14/108%
2	0.83/101%	0.57/116%	0.13/87%	0.86/108%	0.38/112%	0.14/108%
3	0.81/99%	0.56/114%	0.13/87%	0.87/109%	0.38/112%	0.14/108%
4	0.84/102%	0.59/120%	0.13/87%	0.83/104%	0.36/106%	0.13/100%
5	0.89/109%	0.61/125%	0.13/87%	0.80/100%	0.36/106%	0.13/100%
6	0.80/98%	0.56/114%	0.13/87%	0.79/99%	0.35/103%	0.12/92%
Control	0.82/100%	0.49/100%	0.15/100%	0.80/100%	0.34/100%	0.13/100%

NOTE: Serums samples for this study were generated by thawing at room temperature and refreezing for 24 h at -70°C for each cycle. Controls were the same samples taken directly from -70°C without any freeze-thaws.

assays are shown in Table 2. Both assays were run repeatedly using normal human serum controls of variable basal levels of intact and caspase-cleaved CK18 over a period of 8 months. The assay variability (CV%) ranged from 7% to 18% from two manufacturer's lots. Stability of native caspase-cleaved and intact CK18 proteins were also tested under repeated freeze-thaw conditions. Both forms of CK18 proteins were stable through six freeze-thaw cycles (Table 3). These data show an adequate performance of the assays for clinical studies. Because of the difference between the recombinant CK18 control (amino acids 284-396) and naturally existing serum CK18 analytes discussed above, the quantitation of CK18 should ideally be measured in absorbance instead of the unit based on the standard curve. In the following, CK18 unit is used to describe clinical data as nominal value to be consistent with conventional ELISA assay format so that the ELISA data here are comparable with data from other publications.

Serum CK18 levels during cancer treatment. The levels of total and cleaved CK18 were determined in blood during treatment of 61 patients with breast cancer (patient characteristics, see Table 1). The patients received either anthracycline-based therapy (CEF) or the semisynthetic taxane docetaxel. Blood samples were collected before each cycle of treatment and at 1 and 3 days after treatment. Examples of data from patients are shown in Fig. 4A. The levels of serum CK18-Asp^{396} and total CK18 increased between 24 and 72 h after initiation of treatment with docetaxel (Fig. 4B and C; Table 4). CK18-Asp^{396} levels at 72 h showed a larger spread than the values at 0 and 24 h showing a heterogeneous response between patients. CEF therapy induced more rapid increases in serum CK18-Asp^{396} and total CK18 levels compared with docetaxel. The median levels of total CK18 had increased with 114 units/L (32.7%) at 1 day, whereas CK18-Asp^{396} only increased with 13 units/L (12.9%).

The predominant increases in uncleaved CK18 during CEF therapy indicates a substantial component of caspase-independent cell death in some tumors. As shown in Fig. 4D, a heterogeneous response was observed with regard to the ratio of CK18-Asp^{396} to total CK18 in different patients; some

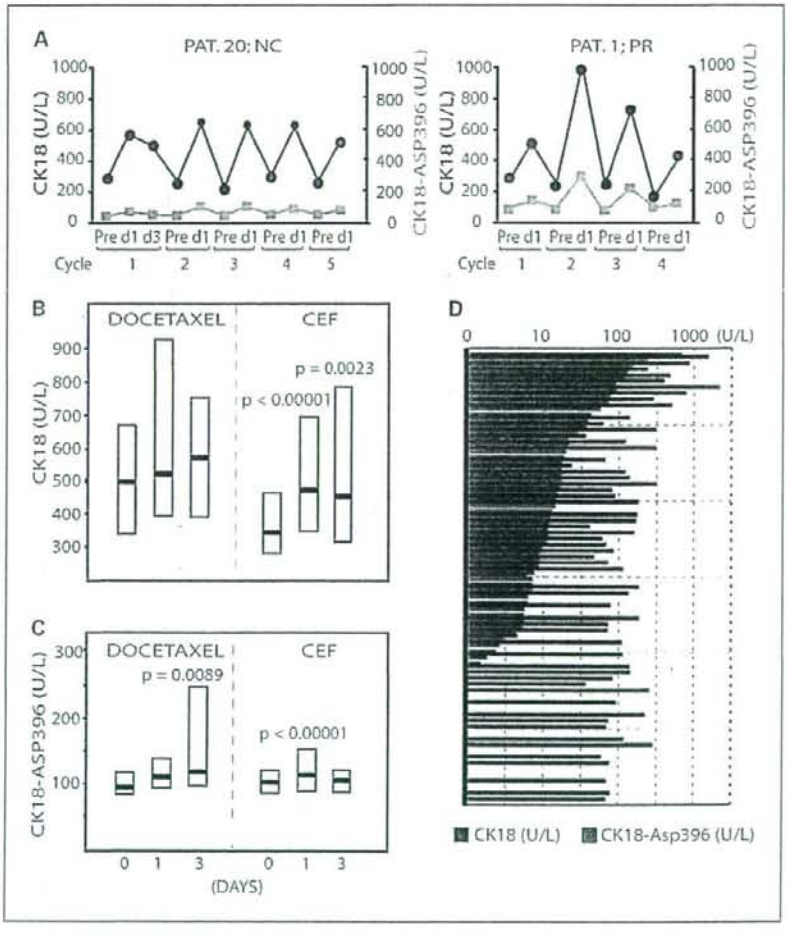

Fig. 4. Patterns of increase of CK18 during treatment of breast cancer patients. *A*, increases of CK18 (*blue points*; M65-ELISA) and CK18-Asp^{396} (*green points*; M30-ELISA) during CEF therapy of two breast cancer patients. The patients were followed for five or four cycles and showed stable disease (*NC*, no change) or partial response (*PR*). Note that patients received treatment every 3rd week (each cycle was 21 d in total). Samples were run in duplicates. *B*, median (25th-75th percentile) levels of CK18 during CEF or docetaxel therapy. Pretherapy levels and levels at days 1 and 3 are shown. Levels of statistical significance <0.01 (Wilcoxon two-sample test). *C*, median (25th-75th percentile) levels of CK18-Asp^{396} during CEF or docetaxel therapy at days 0, 1, and 3 (the same samples were used as in *B*). Levels of statistical significance <0.01. *D*, increases of levels of CK18 and CK18-Asp^{396} levels in individual patient sera from pretreatment to day 1; for each tumor, CK18 (*blue columns*) and CK18-Asp^{396} values (*red columns*) are shown pairwise. Occasional patients showed decreases in the cytokeratin levels between day 0 and day 1; these decreases are shown as 0.