Table 3 Clinical response after FEC and after docetaxel following FEC treatment (n = 194)

Clinical response, N (%)	Overall	
	Responder	Non-responder
FEC		
Responder	106 (90%)	13 (10%)
Non-responder	38 (51%)	37 (49%)

cCR + cPR responder, *cSD + cPD* non-responder, *FEC* fluorouracil, epirubicin, cyclophosphamide, *CI* confidence interval

Discussion

We have presented results from the largest study to date that enrolled Japanese women undergoing preoperative chemotherapy for early stage breast cancer. Our findings demonstrated that four cycles of preoperative FEC followed by four cycles of docetaxel conferred a high rate of BCS, even among patients with primary tumors larger than 3 cm. We found a significant improvement in DFS when QpCR could be achieved, compared to the absence of QpCR. HER2 overexpression, response to FEC and response to docetaxel were significant predictors of QpCR with this regimen.

Regarding toxicity, there were no fatal events and no significant differences in the types and severity of toxicity as compared to other recent studies using similar regimens outside of Japan [6, 8, 9, 16–18]. Compared with overseas studies that also did not allow rh G-CSF the incidence of fever was the same in this study [8, 19]. In another studies which showed lower incidence of febrile neutropenia (13.5%) all patients were treated with rh G-CSF [16].

One of the merits of neoadjuvant chemotherapy for operable breast cancer is to decrease the size of the primary tumor in order to allow for BCS. The study protocol did not provide guidelines for breast conservation; therefore, the BCS rate that we observed reflected the biases that may occur in real-life clinical practice in Japan. Nevertheless, the BCS rate of 80% that we observed was favorable compared with other neoadjuvant studies performed overseas [3, 16].

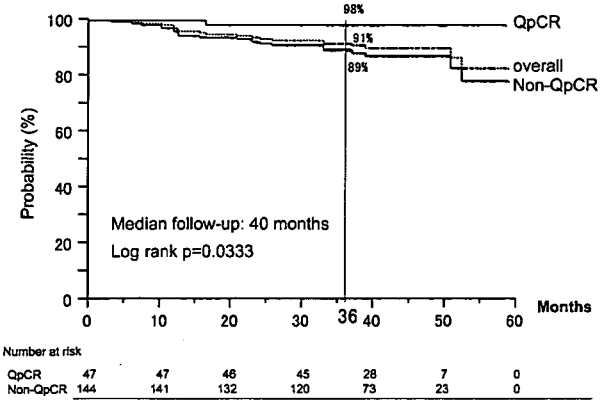

Fig. 1 Relationship of QpCR and non-QpCR to disease free survival

The PACS 01 trial which compared six cycles of adjuvant FEC with a sequential regimen of three cycles of FEC followed by three cycles of docetaxel 100 mg/m^2 (FEC-D) demonstrated an 18% risk reduction in DFS and 27% risk reduction in OS with FEC-D (adjusted P = 0.017). This study supports the conclusions that sequential adjuvant chemotherapy with FEC followed by docetaxel significantly improves DFS and OS in node-positive breast cancer patients [9]. In the current study the dose of docetaxel 75 mg/m^2 was selected based on the recommended doses for docetaxel in Japan, and we showed that the actual 3-year DFS rate of 91% was better than expected based on the results of overseas studies [7, 9, 20]. This confirms that the approved doses of 75 mg/m^2 is an appropriate does in Japanese women.

Furthermore a new definition of QpCR was defined for pathological effect in this study. When stratified between QpCR and non-QpCR, patients with QpCR had significantly favorable DFS. Indeed by adding docetaxel to FEC patients with QpCR resulted in improved survival similar to previous studies.

Even without anti-HER2 targeting therapy, a QpCR rate >60% was achievable in ER negative and HER2 positive tumors. A multivariate analysis has indicated the significant value of HER2 overexpression, which seems to suggest the importance of HER2 in the prediction of QpCR with this regimen. In this study both an anthracycline and docetaxel were used, so it is not clear which treatment was more strongly associated with HER2 as a predictive value of QpCR. Data in the metastatic and adjuvants setting suggest that docetaxel regimens may be more active than non docetaxel regimens in HER2 positive tumors [8, 21]. The value of HER2 status as a predictor of response to anthracycline-based chemotherapy is still a matter debate. On the other hand, there are several implicative data showing the predictive value of topoisomerase (Topo)-II for anthracyclines because Topo-II is a molecular target of anthracyclines [22–25]. There is evidence that HER2 amplification and Topo-II amplification usually occur in parallel and it is rare to have Topo-II amplification without HER2 amplification [23, 26]. In this study QpCR rate might clarify the difference between HER2 positive tumors and HER2 negative tumors. No patient has received trastuzumab in the adjuvant setting. Future translational studies will be necessary to explore the significance of Topo-II amplifications as well as HER2 gene amplifications in the prediction of the pathological response of this regimen. This result will be included the information in the future if

Table 4 Predictive variables for QpCR

Variables	Before treatment OR 95% CI (*P*)	After FEC treatment OR 95% CI (*P*)	After docetaxel following FEC treatment OR 95% CI (*P*)
Menopausal status	1.43	1.38	1.37
Pre (versus post)	0.94–2.15 (NS)	0.89–2.14 (NS)	0.87–2.12 (NS)
Tumor size	0.89	0.93	0.87
>3 cm (vs ≤3 cm)	0.61–1.3 (NS)	0.63–1.37 (NS)	0.59–1.28 (NS)
ER	1.4	1.44	1.35
Negative (versus Positive)	0.87–2.27 (NS)	0.88–2.36 (NS)	0.81–2.23 (NS)
PgR	1.61	1.49	1.65
Negative (versus Positive)	0.97–2.67 (NS)	0.89–2.51 (NS)	0.98–2.79 (NS)
HER2	2.02	2.24	2.11
3+ (vs <3+)	1.31–3.11 (0.0014)	1.42–3.53 (0.0005)	1.36–3.3 (0.0009)
Clinical response to FEC treatment	–	1.78	–
Response (versus non-response)	–	1.15–2.76 (0.0096)	–
Clinical response to docetaxel following FEC treatment	–	–	1.99
Response (versus non-response)	–	–	1.14–3.47 (0.0154)

QpCR quasi pathological complete response, *FEC* fluorouracil, epirubicin, cyclophosphamide, *OR* odds ratio, *ER* estrogen receptor, *PgR* progesterone receptor, *CI* confidence interval, *NS* not significant

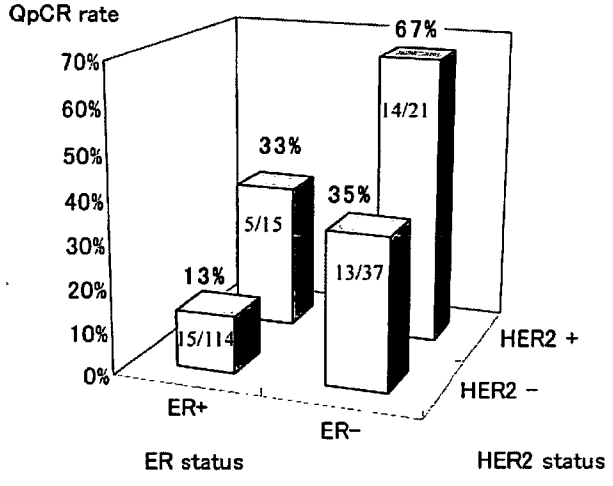

Fig. 2 Relationship between QpCR and HER2/ER status (n=187)

we use anthracycline and trastuzumab for all HER2 positive patients.

In the present study, though a multivariate analysis hasn't indicated the significant value of the status of hormone receptor, QpCR rate was higher in ER negative tumors than ER positive tumors, and QpCR rate in ER negative and HER2 positive tumors was remarkably high compared with ER positive and HER2 negative tumors. This model suggests that ER status is a dependent predictor, for QpCR possibly because it is related to HER2 expression. The sample size was perhaps too small to effectively determine the true impact of ER negative status as a predictor of QpCR. As most patients who are HER2 positive are also ER negative, it is likely that ER status will have some predictive value. However, larger studies are needed to determine this. These results are important for considering individual preoperative systemic therapy. This trend was similar to previous studies using AC followed by paclitaxel regimens, though the therapeutic situations are different [10, 12, 27, 28]. According to recent meta-analyses of post-operative adjuvant therapy, chemotherapy including cyclophosphamide/methotrexate/5FU (CMF)-type regimens, anthracycline-containing regimens and anthracycline followed by paclitaxel are more effective for hormone receptor negative tumors than for hormone receptor positive tumors [10–12, 27–32]. However, while hormone receptor negative tumors may be more responsive to preoperative regimens, a survival benefit can be observed regardless of receptor status [2]. In this study a multivariate analysis hasn't indicated the significant value of the status of hormone receptor. This may be affected by addition of docetaxel. Dose response with anthracycline is also different between hormone receptor positive tumors and hormone receptor negative tumors. For ER negative tumors, higher anthracycline doses may be required for improved prognosis, however, for ER positive tumors it might not be necessary [29].

In this study, most tumors responded to docetaxel even if they did not respond to FEC. However, some tumors showed a response to the initial therapy but a lesser response to the second therapy. This underscores the need to include non-cross resistant treatments in the

management of early stage breast cancer [33]. Various non-cross resistance molecules may be involved in this clinical phenomenon. Recent investigations indicate that initial chemotherapy may change the phenotype of the tumor by inducing pro-survival molecules in tumor cells or stroma [2, 3, 5, 7, 16]. In particular, key mediators such as nuclear factor-kappa B, cyclooxygenase-2 and thymidine phosphorylase are known to be induced by chemotherapy frequently, which may change those tumors relatively anti-apoptotic to the second chemotherapy [34–36]. From the clinical point of view, it would be useful to modify the treatment schedule based on initial response to treatment. Since the types of pro-tumor molecules and the magnitude of induction are different between agents, it might be reasonable to consider a different sequence (taxane followed by anthracycline), if information on the tumor phenotype could be obtained before starting treatment. Various treatment scenarios for non-responders to FEC could be considered. According to recent study results, surgery might be an option for non-responders to initial anthracyclines [37]. In order to enhance the effect of docetaxel, the combination with fluoropyrimidines such as capecitabine may be an option. Obviously for HER2 overexpressing tumors, anti-HER2 containing therapy should be considered. For the ER positive and HER2 negative phenotype, hormone therapy might be an option if tumors are relatively well differentiated. Individual treatment based on ER/HER2 status and the clinical response to the initial anthracyclines may be integrated as future direction [37].

In conclusion, 8-cycle preoperative chemotherapy with non-cross resistant regimens, FEC followed by docetaxel, is safe, feasible, and effective as primary systemic therapy for women with early stage breast cancer. In particular, the regimen allows a majority of Japanese patients to avoid the need for mastectomy. Patients with QpCR demonstrated significantly superior survival results. HER2 over-expression, response to FEC and response to docetaxel were significant predictors for QpCR. Based on our results, preoperative FEC followed by docetaxel should be considered a standard option for the treatment of Japanese women with operable breast cancer.

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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

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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

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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^{-12}
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

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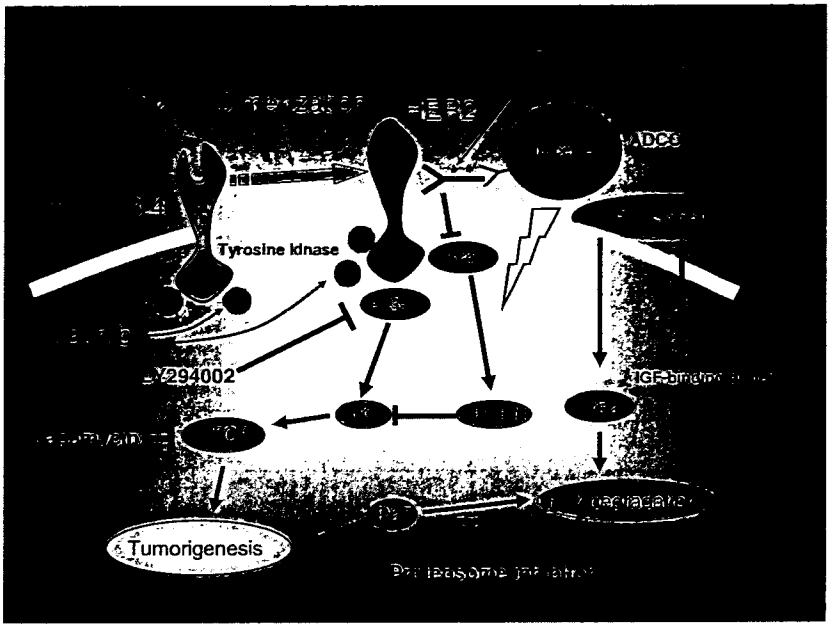

Fig. 1. The mechanisms of action of trastuzumab. Direct activity (induction of apoptosis: phosphatidylinositol-3-kinase [PI3K]–Akt pathway) and antigen-dependent cellular cytotoxicity (ADCC). The mechanisms of resistance: Downregulation of p27, loss of PTEN activity and activation of insulin-like growth 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

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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.

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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.

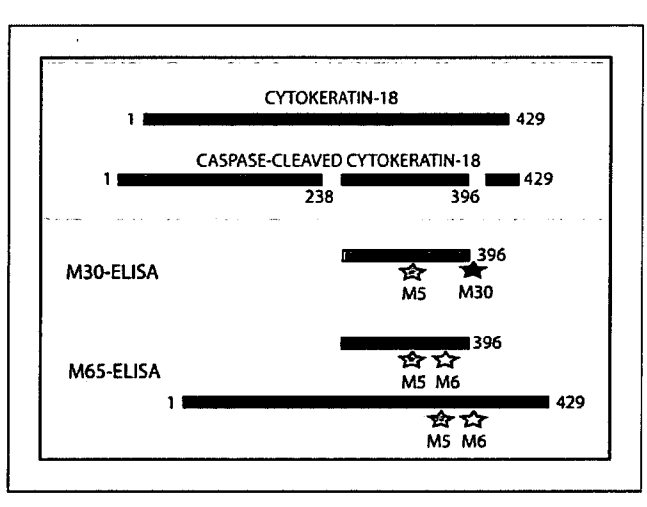

Fig. 1. CK18 is cleaved at Asp^{238} and Asp^{396} by caspases during apoptosis. The M30-Apoptosense ELISA assay uses antibody M30, which detects a neo-epitope of CK18 formed after caspase cleavage at Asp^{396} (12, 17). The M65-ELISA assay will detect all CK18 fragments that contain epitopes in the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

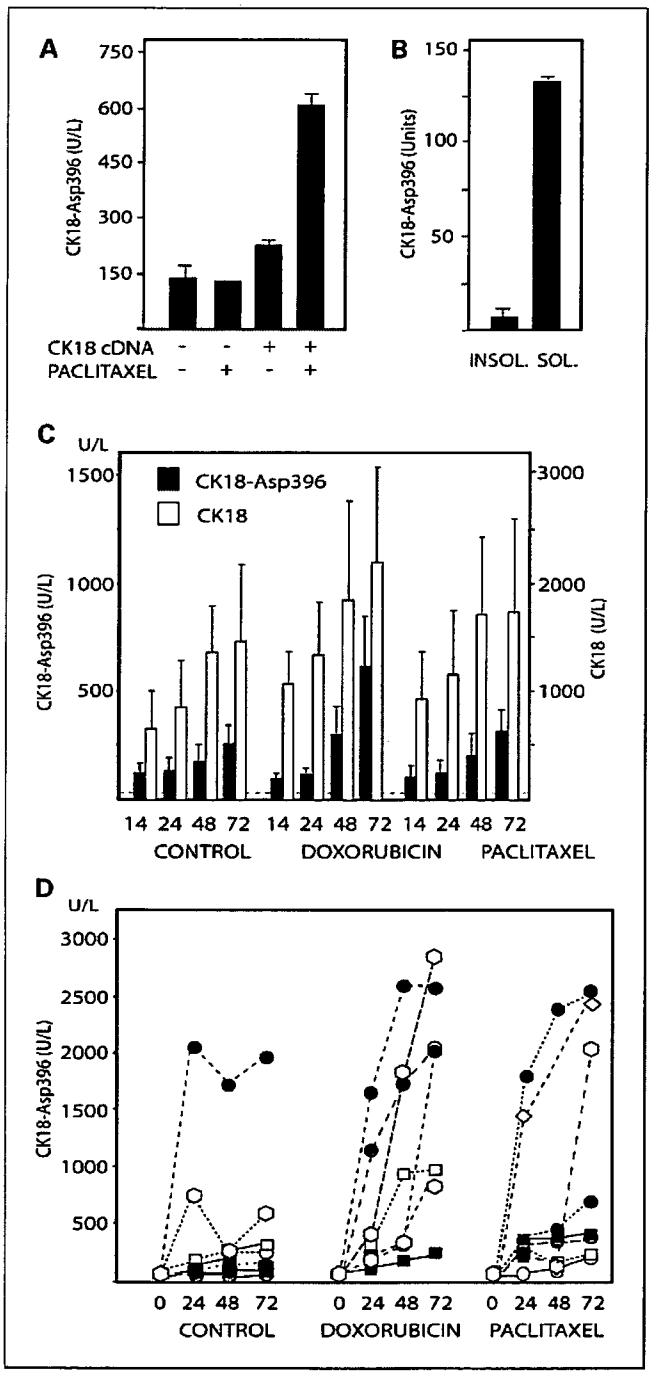

Fig. 2. Release of CK18-Asp^{396} from carcinoma cells. *A*, specificity of the M30-ELISA for caspase-cleaved CK18. Mouse embryo fibroblasts were transfected with a cDNA expression plasmid and treated with paclitaxel as indicated. Note that paclitaxel only induces increases in CK18-Asp^{396} in transfected 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

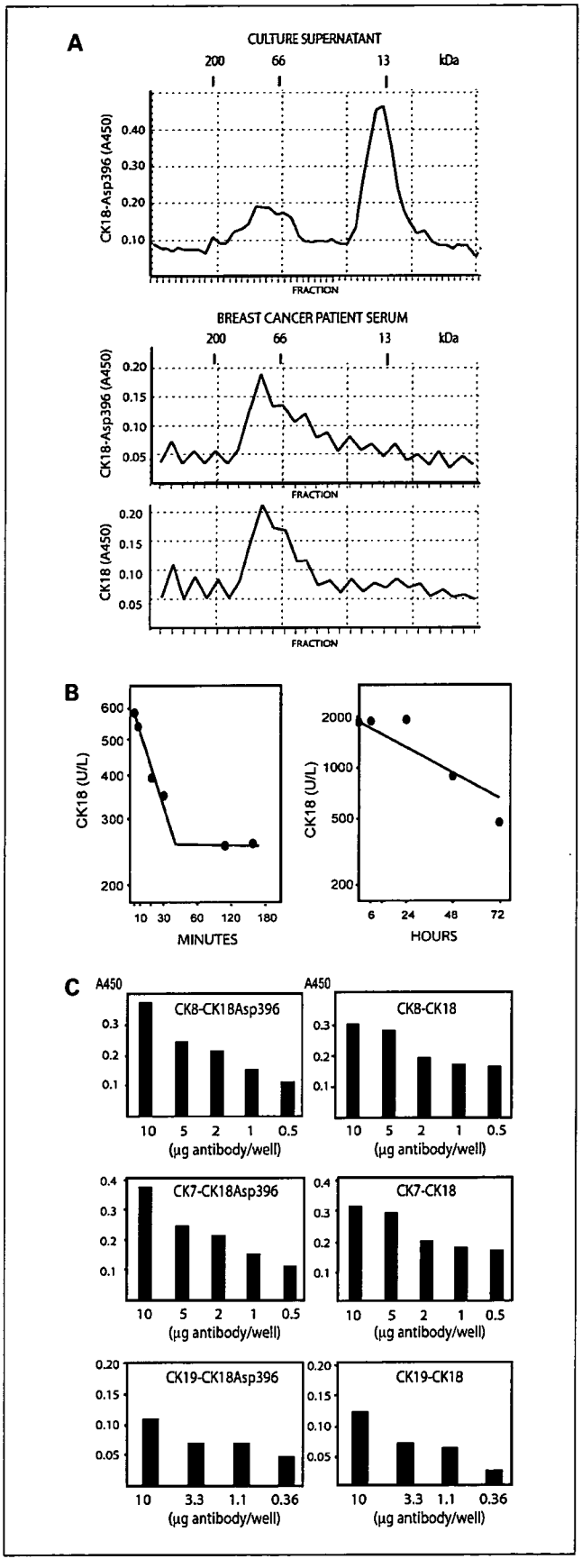

Fig. 3. CK18 complexes released from cells into circulation. *A*, Superose G200 gel filtration of tissue culture medium from apoptotic cells or patient serum (*top*) $CK18\text{-}Asp^{396}$ is present in the 10 to 20 kDa and 50 to 100 kDa range in tissue culture medium from apoptotic MDA-MB-231 cells; $CK18\text{-}Asp^{396}$ (*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

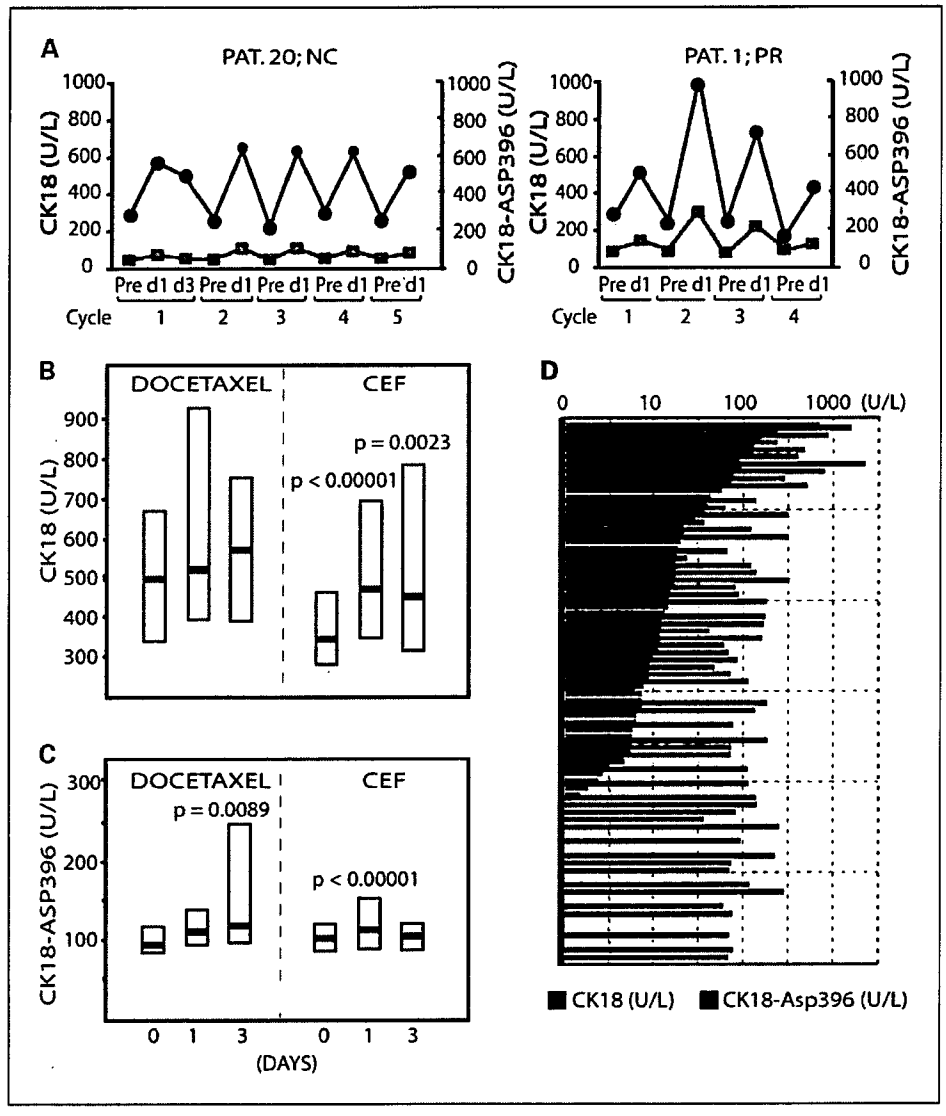

Fig. 4. Patterns of increase of CK18 during treatment of breast cancer patients. *A*, increases of CK18 (*blue points*; M65-ELISA) and CK18-Asp^{396} (*green points*; M30-ELISA) during CEF therapy of two breast cancer patients. The patients were followed for five or four cycles and showed stable disease (*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.

patients showed increases in both markers, whereas others only showed increases in total CK18. This result suggests that CEF therapy induces different death modes in different tumors.

Increases in CK18 are associated with clinical response. We examined the association between clinical response and serum CK18 increases in 43 patients receiving neoadjuvant CEF therapy. Patients normally leave the hospital after drug infusion—leading to difficulties to collect blood samples—but a limited number of paired samples is sufficient to achieve high statistical power using matched-pair statistics. Patients with partial clinical response showed significant increases in total CK18 at day 1 after treatment ($P < 0.0001$, Wilcoxon matched pair test; Fig. 5A). In contrast, nonresponding patients did not show significant changes in total CK18 levels ($P = 0.19$; Fig. 5A). Similar results were obtained using matched pair t test.

Patients who received neoadjuvant therapy were stratified according to the ratios of posttreatment to pretreatment values of CK18 and overall survival plots were constructed (Fig. 5B). A cutoff value of 18% increase in CK18 gave the best prognostic significance for survival ($P = 0.035$ by the log-rank test). The Cox proportional hazards model gave a hazard ratio of 7.28 (95% confidence interval, 0.84-62.9).

Discussion

Previous studies have shown that different anticancer agents induce increases in the levels of caspase-cleaved and total serum CK18 in prostate cancer patients and that serum CK18 is derived from tumor cells (11, 13). These results were promising with regard to the use of serum CK18 as a pharmacodynamic biomarker for tumor cell death. Previous studies have not established whether increases in serum CK18 occurring during treatment are associated with clinical responses because response monitoring is inaccurate in patients with hormone refractory prostate cancer (26). We here studied breast cancer patients receiving neoadjuvant treatment for local disease and from which accurate clinical data were available. The results show that increases in serum CK18 levels are associated with clinical response to CEF therapy. Interestingly, CK18 increases were not exclusively observed in patients showing clinical response but also in some patients showing stable disease during treatment (Fig. 4A), suggesting that serum CK18 is a sensitive response biomarker. In patients with stable disease, therapy-induced cell death may be balanced by tumor cell regrowth between treatment cycles (27). Both the sensitivity of the assays and the favorable performance characteristics in terms of antigen stability during storage (14) and during freeze-thawing (Table 3) suggest that CK18 biomarkers will be useful for monitoring treatment effects.

Table 4. Increased levels of CK18 in patient serum during treatment using different agents

Treatment	Increased levels of CK18 during therapy (%)	
	Caspase cleaved*	Total*
Docetaxel (breast)†	19.8 ($P = 0.0089$)	16.5 (NS)
Docetaxel (prostate)‡	18.7 ($P < 0.0001$)	21.4 ($P < 0.0002$)
Vinorelbine (prostate)‡	7.2 ($P < 0.001$)	6.7 ($P < 0.011$)
Estramustine (prostate)‡	-1 (NS)	8.2 ($P < 0.0001$)
CEF (breast)§	12.9 ($P < 0.00001$)	32.7 ($P < 0.00001$)

*Increased median levels of CK18-Asp^{396} and total CK18 (measured by the M30-Apoptosense and M65 ELISA assays).
†Increase over pretherapy levels at 72 h.
‡Increase over pretherapy levels 48 h (prostate data are from ref. 13).
§Increase over pretherapy levels at 24 h.

Apoptosis has received considerable attention as a major cellular outcome of chemotherapy, including DNA-damaging agents (2). Recent studies have implied that necrosis may also be a possible consequence of treatment (28). Doxorubicin has been shown to induce both apoptosis (29) and necrosis *in vitro* (30, 31). Our studies of tumor organ cultures from seven clinical cases of breast carcinoma showed induction of caspase-cleaved CK18 by doxorubicin in all seven cultures, showing apoptosis. Apoptotic responses were also observed using CEF therapy (cyclophosphamide is converted by the liver into active metabolites; acrolein was used for these studies).[6] In contrast, the *in vivo* CK18 response to CEF therapy was heterogeneous, characterized by increases in caspase-cleaved CK18 in the serum of some, but not all, patients with increases in CK18. This heterogeneous response could be due to defects in apoptosis signaling in some tumors. Furthermore, differences in factors such as tumor hypoxia, nutrition, or variations in the drug concentrations reached in different tumors may also be determinants of cell death mode. It has been reported that DNA-alkylating agents induce a rapid necrotic response due to activation of poly(ADP)ribose polymerase, leading to poly(ADP)ribose polymerase–mediated depletion of β-NAD$^+$ (7). Tumor cells, which are dependent on glycolysis for ATP production, undergo rapid ATP depletion and necrotic death. This response by DNA-damaging agents could be speculated to be more pronounced in hypoxic and poorly nourished tumors. That anthracycline-based therapy may induce a necrotic response is supported by the finding that complete pathologic responses to doxorubicin/docetaxel are associated with the presence of tumor necrosis in tissue sections (32). Induction of a necrotic response could explain the efficiency of chemotherapy in tumors with defective apoptotic pathways (discussed in ref. 7), including efficacy in p53-defective breast cancers (33).

Taxanes induce mitotic catastrophe, characterized by the occurrence of aberrant mitosis followed by cell division. Mitotic catastrophe is not a cell death mode, but will trigger cell death, either by apoptosis or by nonapoptotic mechanisms (1, 34–36). The findings in the present and a previous study (13), demonstrating increases in caspase-cleaved CK18 molecules in serum during docetaxel treatment, shows that this agent induces apoptosis *in vivo* (Table 4). It is likely that the efficiency of microtubule-interacting agents does not rely on the presence of an apoptotic machinery in the target cells;

[6] Our unpublished data.

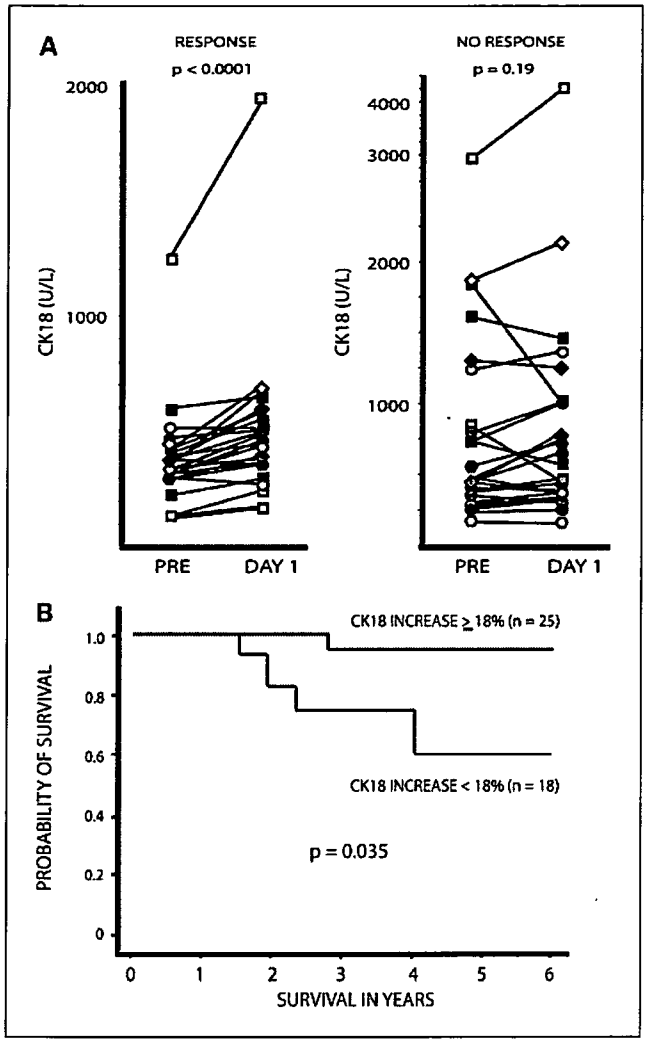

Fig. 5. Association to response. *A*, increases of total CK18 at day 1 of the first CEF treatment cycle are associated with therapy response. Pretherapy and day 1 CK18 levels are shown of patients showing response or no response; *P* values are from Wilcoxon matched pair test. *B*, improved overall survival curves of patients showing >18% increases in CK18 at the first cycle of CEF treatment. Kaplan-Meier plots, *P* value calculated by the log-rank test.

if the machinery is not present, cells are likely to die from other mechanisms. It should be noted that we have observed patients where docetaxel induces increases predominantly in total serum CK18 (11). However, there is a quantitative difference in the response to mitotic inhibitors and CEF, whereas mitotic inhibitors induce similar median increases in caspase-cleaved CK18 and total CK18, CEF induces larger increases in total CK18 reflecting a relatively larger proportion of tumors where necrotic cell death occurs.

Our data show that only a minor fraction of caspase-cleaved CK18 will remain in the insoluble, cytoskeletal fraction. Soluble caspase-cleaved CK18 consisted of molecular weight fragments in the 10 to 20 kDa range, which we presume to be monomeric caspase digestion products, and also of higher molecular weight material (50-100 kDa). Only the higher molecular weight material was present in serum from cancer patients, and we suggest that the smaller fragments are being filtered in the kidney glomerulus. The caspase-cleaved CK18 material present in serum reacted with CK7, CK8, or CK19 antibodies, showing that they at least partly are present in complexes. Although the precise nature of these interactions are unknown or if there are any alterations among the cytokeratin complexes under normal or abnormal physiologic conditions, our data showed that the serum CK18 levels from repeated blood draws of healthy donors were fairly stable without any trending (data not shown). The variations from multiple time points in normal donors are consistent with the data reported from cancer patients (15). The presence of caspase-cleaved CK18 in protein complexes is likely to explain the stability of the cleavage products in the circulation and in blood samples, explaining the excellent performance of serum CK18 with regard to stability and yields after repeated freeze-thaw cycles (refs. 14, 15; Table 3). It is likely that many other caspase-cleaved fragments released from apoptotic cells will not show a similar stability. The adequate assay performance adds to the advantages of serum CK18 as a biomarker for rapid monitoring of clinical response to cancer therapy.

We conclude that serum CK18 measurements may be useful for assessing treatment effects. The data suggesting that the initial cell death response determined by CK18 biomarkers is an important determinant of treatment outcome. The method is robust and samples can be frozen and stored before analysis, making the method suitable for multicenter clinical trials of novel anticancer drugs.

Acknowledgments

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A Nonfucosylated Anti-HER2 Antibody Augments Antibody-Dependent Cellular Cytotoxicity in Breast Cancer Patients

Eiji Suzuki,[1] Rinpei Niwa,[2] Shigehira Saji,[1] Mariko Muta,[1] Makiko Hirose,[1] Shigeru Iida,[2] Yukimasa Shiotsu,[2] Mitsuo Satoh,[2] Kenya Shitara,[2] Masahide Kondo,[3] and Masakazu Toi[1]

Abstract

Purpose: Removal of fucose residues from the oligosaccharides of human antibody is a powerful approach to enhance antibody-dependent cellular cytotoxicity (ADCC), a potential important antitumor mechanism of therapeutic antibodies. To provide clinically relevant evidence of this mechanism, we investigated ADCC of a fucose-negative version of trastuzumab [anti–human epidermal growth factor receptor 2 (HER2) humanized antibody] using peripheral blood mononuclear cells (PBMC) from breast cancer patients as effector cells.

Experimental Design: Thirty volunteers, including 20 breast cancer patients and 10 normal healthy control donors, were recruited randomly, and aliquots of peripheral blood were collected. ADCC of commercial trastuzumab (fucosylated) and its fucose-negative version were measured using PBMCs drawn from the volunteers as effector cells and two breast cancer cell lines with different HER2 expression levels as target cells. Relationships between cytotoxicity and characteristics of the patients, such as content of natural killer cells in PBMCs, type of therapy, *FCGR3A* genotypes, etc. were also analyzed.

Results: ADCC was significantly enhanced with the fucose-negative antibody compared with the fucose-positive antibody using PBMCs from either normal donors or breast cancer patients. Enhancement of ADCC was observed irrespective of the various clinical backgrounds of the patients, even in the chemotherapy cohort that presented with a reduced number of natural killer cells and weaker ADCC.

Conclusions: This preliminary study suggests that the use of fucose-negative antibodies may improve the therapeutic effects of anti-HER2 therapy for patients independent of clinical backgrounds.

Human epidermal growth factor receptor 2 (HER2) is a key contributor to normal cell growth and differentiation (1). However, when overexpressed, it is associated with neoplastic transformation of cells. Approximately 15% to 20% of breast cancers show HER2 overexpression and/or *HER2* gene amplification. Clinical benefit has been shown either as monotherapy with trastuzumab (Herceptin), a humanized anti-HER2 monoclonal antibody of human IgG1/κ isotype, or in combination with chemotherapy (2–8). Trastuzumab is known to show efficacy only in breast cancer patients with tumors strongly overexpressing HER2, graded 3+ by immunohistochemistry, or graded 2+ with *HER2* gene amplification as determined by fluorescence *in situ* hybridization (9); however, even in those HER2 overexpressors, some of the patients do not achieve clinical benefits with trastuzumab. Therefore, search for the prediction marker, which can select patients who would have better clinical benefits by trastuzumab, is important. On the other hand, to achieve better clinical outcome on HER2 overexpressors but with less efficacy, improvement of potential efficacy of conventional trastuzumab could be one of the important options.

Trastuzumab has been shown to have multiple mechanisms of action based on *in vitro* studies: antibody-dependent cellular cytotoxicity (ADCC) and direct growth inhibition of tumor cells (10–15). ADCC, a lytic attack on antibody-targeted cells, is triggered following binding of the Fc region of an antibody to the Fcγ receptor IIIa expressed on natural killer (NK) cells. The clinical importance of ADCC was first shown with rituximab (Rituxan), an anti-CD20 chimeric antibody approved for non-Hodgkin's lymphoma treatment in 1998 (16–18). These studies have focused on the relationships between the clinical response and Fcγ receptor IIIa 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

Authors' Affiliations: [1]Breast Group, Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Diseases Center; [2]Pharmaceutical Research Center, Kyowa Hakko Kogyo Co. Ltd.; and [3]Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tokyo, Japan

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Requests for reprints: Masakazu Toi, Breast Group, Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Diseases Center, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan. Phone: 81-3-3823-2101; Fax: 81-3-3824-1552; E-mail: maktoi77@wa2.so-net.ne.jp.

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FCGR3A-158V allele associated with strong IgG binding to the receptor and ADCC activation (19, 20). 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. (21) showed a significant correlation between clinical responses and ADCC-mediated killing by patients' peripheral blood mononuclear cells (PBMC). Furthermore, Arnould et al. (22) showed an increased infiltration of NK cells into tumor tissue of trastuzumab-responding patients. These reports support an *in vivo* role of ADCC in trastuzumab therapy and imply that ADCC enhancement could be a potential approach to improve its efficacy.

We and others reported that removal of fucose from antibody oligosaccharides attached to Asn^{297} of the heavy chain (defucosylation) significantly enhanced ADCC compared with that of conventional antibody (23-27). Thus, this modulation of antibody could be one of the most powerful approaches to improve efficacy in antibody therapy. One possible problem is that all these studies used PBMCs from normal healthy donors as effector cells. It is unclear whether this activity is also functioning for PBMCs from cancer patients whose immune system could be impaired either by the therapeutic agent used or by the immunosuppressing activity of tumor cells.

In this study, using PBMCs from breast cancer patients as effector cells, we evaluated ADCC of a defucosylated trastuzumab compared with commercial trastuzumab, which contained highly fucosylated oligosaccharides. In addition, relationships between ADCC and various clinical backgrounds of the patients were also analyzed.

Table 1. Patients' characteristics

Patients' characteristics	
Age (median), y	37-73 (55)
Total no. patients	20
HER2 status	
IHC 3+	4
IHC 0, 1+	14
Unknown	2
Hormone receptor status	
ER^+ and/or PgR^+	12
Disease progression status	
Recurrence	12
Primary	8
Metastatic site	
Lung	5
Liver	3
Bone	6
Lymph nodes	2
Brain	1
Soft tissue	2
Treatment status	
Chemotherapy	
EC, AC	2
FEC	2
Paclitaxel	5
Docetaxel	2
MMC + MTX	1
Capecitabine	2
Hormonal therapy	
Tamoxifen	3
Aromatase inhibitor	4
Medroxyprogesterone	1
Trastuzumab	2

Abbreviations: IHC, immunohistochemistry; ER, estrogen receptor; PgR, progesterone receptor; EC, epirubicin + cyclophosphamide; AC, doxorubicin + cyclophosphamide; FEC, 5-FU + epirubicin + cyclophosphamide; MMC, mitomycin C; MTX, methotrexate.

Materials and Methods

Blood samples. From April to October 2005, blood samples were randomly collected from both breast cancer patients (n = 20) who underwent various types of treatment, including surgery, chemotherapy, radiotherapy, hormonal therapy, and antibody treatment trastuzumab, and healthy volunteers (n = 10) registered at Breast Group, Komagome Hospital, Tokyo Metropolitan Cancer and Infectious Diseases Center (Tokyo, Japan). The numbers of patients composing each classification are shown in Table 1. The protocol of this study was approved by Institutional Review Board. All patients and healthy volunteers signed written informed consent statements before samples were taken and analyzed.

Cell lines. Human breast adenocarcinoma cell lines MCF-7 and SK-BR-3 were purchased from the American Type Culture Collection (Manassas, VA).

Anti-HER2 humanized antibodies. Trastuzumab (Herceptin) IgG1/κ-type anti-HER2 humanized antibody was purchased from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). For the generation of the nonfucosylated version of trastuzumab, the cDNA sequences of the VL and VH region were designed as the same with that of trastuzumab (2) and constructed by PCR-based method. The expression plasmid was constructed using humanized IgG1 expression plasmid pKANTEX93 (28) by joining the VL and VH cDNAs with human κ and γ1 constant region cDNAs, respectively. The expression vector was then introduced into α-1,6 fucosyltransferase gene knockout Chinese hamster ovary cells (29) via electroporation, and transfectant clones were selected in medium lacking hypoxanthine and thymidine as described previously (29). High-producing clones were selected by comparing IgG amounts in culture supernatants using an IgG-detecting ELISA method (28). Antibody was then purified from supernatant of confluent transfectant clone cultured in Excell 301 Medium (JRH Biosciences, Lenexa, KS) using protein A-Sepharose (Millipore, Billerica, MS).

Oligosaccharide analysis of anti-HER2 humanized antibodies. *N*-linked oligosaccharides were released by digestion of the antibodies with *N*-glycosidase F (Takara, Shiga, Japan). The released carbohydrates were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with positive ion mode as described previously (30).

Analysis of HER2 expression in breast tumor cell lines. Expression of cell surface HER2 molecules was determined by flow cytometry. Tumor cells (2×10^6) were stained with 10 μg/mL trastuzumab for 1 h on ice in the presence of 250 μg/mL human IgG (Welfide, Osaka, Japan) as blocking reagent. FITC-conjugated anti-human IgG (H+L; R&D Systems, Minneapolis, MN) was used as the secondary reagent. The stained cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter, Tokyo, Japan).

ADCC assay. Cytotoxicity was determined by the lactate dehydrogenase release assay as described previously (29), using human PBMCs as effector cells and either MCF-7 cells or SK-BR-3 cells as target cells at an E:T ratio of 15:1. Briefly, target cells (1×10^4) and effector cells (1.5×10^5) were distributed into 96-well U-bottomed plates and incubated with serial dilutions of antibodies for 4 h at 37°C. PBMCs were purified from peripheral blood using Lymphoprep (Axis-Shield, Dundee, United Kingdom). The supernatant lactate dehydrogenase activity was measured using a nonradioactive cytotoxicity assay kit (Promega, Madison, WI). Percentage cytotoxicity was calculated