Elevated expression of polymorphonuclear leukocyte elastase in breast cancer tissue is associated with tamoxifen failure in patients with advanced disease

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Besides a variety of other proteases, polymorphonuclear leukocyte elastase (PMN-E) is also suggested to play a role in the processes of tumour cell invasion and metastasis. Yet, there is only limited data available on the relation between the tumour level of PMN-E and prognosis in patients with primary breast cancer, and no published information exists on its relation with the efficacy of response to systemic therapy in patients with advanced breast cancer. In the present study, we have measured with enzyme-linked immunosorbent assay the levels of total PMN-E in cytosolic extracts of 463 primary breast tumours, and have correlated their levels with the rate and duration of response on first-line tamoxifen therapy (387 patients) or chemotherapy (76 patients) in patients with locally advanced and/or distant metastatic breast cancer. Furthermore, the probabilities of progression-free survival and postrelapse survival were studied in relation to the tumour levels of PMN-E. Our results show that in logistic regression analysis for response to tamoxifen treatment in patients with advanced disease, high PMN-E tumour levels were associated with a poor rate of response compared with those with low PMN-E levels (odds ratio: OR, 0.40; 95% CI, 0.22–0.73; P = 0.003). After correction for the contribution of the traditional predictive factors in multivariate analysis, the tumour PMN-E status was an independent predictor of response (P = 0.001). Furthermore, a high tumour PMN-E level was related with a poor progression-free survival (P < 0.001) and postrelapse survival (P = 0.022) in a time-dependent analysis. In contrast, the tumour level of PMN-E was not significantly related with the efficacy of response to first-line chemotherapy in patients with advanced breast cancer. Our present results suggest that PMN-E is an independent predictive marker for the efficacy of tamoxifen treatment in patients with advanced breast cancer.

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A complex cascade of proteinases, their receptors and inhibitors, are required for invasion of tumour cells through the extracellular matrix, for subsequent metastasis and angiogenesis (reviewed by Mignatti et al., 1986; Andreasen et al., 1997; Nelson et al., 2000). Tumour growth and angiogenesis are associated with a strong inflammatory response via the attraction of macrophages and polymorphonuclear (PMN) leucocytes by tumour cell-secreted chemoattractants (Jackson et al., 1997; Lee et al., 1997). In contrast to their attributed role in exterminating the tumour, recruited inflammatory cells have been reported to be associated with tumour progression as well (Schöll et al., 1994; Yamashita et al., 1994, 1996; Shamamian et al., 2000, 2001). The responsible protease is thought to be PMN-elastase (PMN-E), a serine protease that can degrade various components of the extracellular matrix directly (Janoff and Schere, 1968; Mainardi et al., 1980; McDonald and Kelley, 1980; Barrett, 1981), or indirectly through either the activation of other proteinases (Machovich and Owen, 1990; Shamamian et al., 2001) or the inactivation of their inhibitors (Levin and Santell, 1987; Gramse et al., 1984; Wu et al., 1995). Furthermore, it has been shown that PMN-E is not only produced by neutrophils but by human breast cancer cells as well (Kao and Stern, 1986; Yamashita et al., 1994), and moreover can promote the adhesion of tumour cells to vascular endothelial cells facilitating its role in tumour metastasis (Nozawa et al., 2000).

In many types of cancer, various tumour-associated proteases and their inhibitors have been shown to be independent prognostic factors for relapse-free and overall survival. The most extensively studied protease systems involve those of the urokinase-type plasminogen activator (uPA) (reviewed by Andreasen et al., 1997; Schmitt et al., 1997a; Look and Foekens, 1999) and a variety of matrix metalloproteinases (MMPs) (reviewed by Duffy et al., 2000; Nelson et al., 2000). Similarly, high tumour levels of PMN-E have been reported to be associated with a poor prognosis in patients with primary breast cancer (Yamashita et al., 1994, 1995a, b; Foekens et al., 2002) and nonsmall cell lung cancer (Yamashita et al., 1996). In addition to their prognostic relevance in primary disease, several proteases have been shown to be positive or negative predictive factors for the efficacy of adjuvant systemic endocrine therapy or chemotherapy in patients with breast cancer. These
MATERIALS AND METHODS

Patients and tissues

PMN-E levels were determined in cytosols of primary breast tumours and correlated with the type of response to first-line treatment with tamoxifen or chemotherapy in patients with advanced disease. In addition, we have studied the relation between the tumour level of PMN-E and the length of progression-free and postrelapse overall survival.

Table 1  Characteristics of patients, tumours, and treatment

| Characteristic                  | Tamoxifen | Chemotherapy |
|--------------------------------|-----------|--------------|
| Number of patients             | 387       | 76           |
| Menopausal statusa             |           |              |
| Premenopausal                  | 76        | 29           |
| Postmenopausal                 | 311       | 47           |
| M-statusb                      |           |              |
| M0                              | 360       | 93           |
| M1                              | 27        | 5            |
| Disease-free interval           |           |              |
| < 1 year                       | 106       | 31           |
| > 1 year                       | 281       | 45           |
| Dominant site of relapsec      |           |              |
| Soft                           | 57        | 19           |
| Bone                           | 169       | 17           |
| Viscera                        | 161       | 40           |
| ER statusd                     |           |              |
| Negative                       | 49        | 46           |
| Positive                       | 338       | 29           |
| Pgr statusd                    |           |              |
| Negative                       | 95        | 43           |
| Positive                       | 282       | 43           |
| Adjuvant treatment             |           |              |
| None                           | 309       | 62           |
| Hormonal therapy               | 0         | 2            |
| Chemotherapy                   | 78        | 12           |

*Owing to missing values, numbers do not always add up to 387 and 76, respectively.

In all patients were assessed by standard International Union Against Cancer criteria for complete and partial remission years. The characteristics of the patients who received the different types of systemic treatment with respect to menopausal status at the start of systemic treatment for advanced disease, the first dominating site, the number of patients with metastatic disease (M1 patients) at primary surgery, disease-free interval (DFI) between primary tumour removal and first recurrence, hormone receptor status, and the type and frequency of adjuvant therapy are listed in Table 1. The median follow-up of patients still alive after start of systemic therapy for advanced disease was 34 months (range 9–137 months) in the tamoxifen-treated patients (38 patients still alive), and 39 months (range 7–80 months) in those who received chemotherapy (four patients still alive). On first-line tamoxifen therapy, tumour progression occurred in 75 patients (99%) during follow-up. Of these patients, 42 were eventually treated with endocrine therapy (tamoxifen in 28 patients, progesterin in 14 patients).

All patients were assessed by standard International Union Against Cancer criteria for complete and partial remission.
(objective response). Patients with no change for >6 months (stable disease) have a postrelapse survival similar to patients with partial remission (Ravdin et al, 1992; Foekens et al, 1994a). Therefore, for overall response, objective response and stable disease were combined.

Assays of PMN-E, ER, PgR, and total protein

Tumour tissues were stored in liquid nitrogen and pulsed in the frozen state with a microdisemembrator as recommended by the European Organization for Research and Treatment of Cancer (EORTC) for processing of breast tumour tissue for cytosolic ER and PgR determinations (EORTC Breast Cancer Cooperative Group, 1980). The resulting tissue powder was suspended in EORTC receptor buffer (10 mM dipotassium chloride EDTA, 3 mM sodium azide, 10 mM monothioglycral, and 10% vv–1 glycerol, pH 7.4). The suspension was centrifuged for 30 min at 100000 × g to obtain the supernatant fraction (cytosol). ER and PgR levels were determined by ligand binding assay or with enzyme immunoassay as described previously (Foekens et al, 1989a). Cytosol protein was quantified with the Coomassie brilliant blue method (Bio–Rad Laboratories, CA, USA) with human serum albumin as a standard. Taking into account that most of the PMN-E in body fluids or tissue cytosols is complexed with its main antagonist, the α1-proteinase inhibitor, we used a commercial two-site enzyme-linked immunosorbsent assay for quantification of total PMN-E levels after adding a surplus of α1-proteinase inhibitor (100 μg ml–1) to each cytosol (Milena–PMN Elastase, Milena Biotec, Bad Nauheim, Germany).

Statistics

The strength of the associations of PMN-E with continuous variables was tested with Spearman rank correlation (rs). The strength of the association of PMN-E (used as a continuous variable) with other variables (used as grouping variable) was tested with the nonparametric Wilcoxon rank-sum test or Kruskal–Wallis test, followed by a Wilcoxon-type test for trend across ordered groups where appropriate. The length of progression-free survival was defined as the time from the start of treatment for advanced disease until the start of next treatment because of progressive disease or until the time of intercurrent death. Survival probabilities were calculated by the actuarial method of Kaplan and Meier (1958). The log-rank test was used to test for differences between survival curves. Both univariate and multivariate analyses were performed using the Cox proportional hazards model. The proportionality assumption was investigated using a test based on the Schoenfeld residuals (Grambsch and Therneau, 1994). The residuals were retrieved and a smooth function of time was fitted and then tested whether there was a relation. The likelihood ratio test in the Cox regression models was used to test for differences and interactions. The relation of predictive factors with response to therapy was examined with logistic regression analysis. In our search for the best categorization of PMN-E, we have used isotonic regression analysis (Barlow et al, 1972; Foekens et al, 1994b) using the rate of overall response to tamoxifen treatment as end point. All computations were done with the STATA statistical package, release 7.0 (STATA Corp., College Station, TX, USA). All P-values are two-sided.

RESULTS

PMN-E levels and patient and tumour characteristics

The levels of PMN-E in all the 463 primary breast tumours analysed ranged from 0.4 to 1667 ng mg–1 protein (median, 6.3 ng mg–1 protein). They were not related with age (rs = –0.04, P = 0.41), but were slightly higher in tumours of premenopausal patients (median, 6.7 ng mg–1 protein) compared with those of postmenopausal patients (median, 5.9 ng mg–1 protein) at time of primary surgery (P = 0.08). PMN-E levels were negatively related with ER (rs = –0.20, P < 0.001) and PgR (rs = –0.19, P < 0.001), but were not significantly related with tumour size (P = 0.74) or grade (P = 0.11), or with the lymph-node status of the patient (P = 0.48).

The median level of PMN-E in the tumour cytosols of the 387 patients, who were treated with first-line tamoxifen therapy, was 6.0 ng mg–1 protein (range 0.4–1667 ng mg–1 protein). This is significantly (P = 0.03) lower than the median level of 7.1 ng mg–1 protein (range 0.7–426 ng mg–1 protein) that was measured in tumours of the 76 patients who were treated with first-line chemotherapy. The data are consistent with the higher proportion of ER-negative tumours in the latter series of patients and the observed negative relation between the levels of PMN-E and ER.

Univariate analysis for response to treatment in advanced disease

Of the 387 patients who received tamoxifen as first-line treatment for advanced disease, 197 (51%) responded (11 complete remission, 48 partial remission, and 138 stable disease). The median duration of response in these responders was 15 months. Using logistic regression analysis, it was shown that older age and postmenopausal status were associated with a higher rate of response to tamoxifen treatment than younger age and premenopausal status (Table 2). In patients with a disease-free interval less than 1 year, 25% of the 221 patients with high PMN-E levels showed a worse rate of response on tamoxifen treatment than younger age and premenopausal status (Table 2). In patients with a disease-free interval greater than 1 year (57% response, OR = 2.47), adjuvant chemotherapy and first site of relapse were not related to the type of response on tamoxifen treatment. Patients with ER-positive or PgR-positive tumours had a more favourable response rate (OR = 3.73 and OR = 1.73, respectively) than patients with ER-negative or PgR-negative tumours (OR = 1). As expected, the traditional prognostic factors nodal status, and size and grade of the primary tumour were not significantly related with the type of response to tamoxifen in advanced disease, that is, they were no predictive factors. When analysing the relation of continuous PMN-E levels with response, it appeared that there was a trend towards a lower response rate with increasing levels of PMN-E (P = 0.07). Using isotonic regression analysis, 20.0 ng mg–1 protein was chosen as cutoff point to classify advanced breast cancer patients as PMN-E-high and PMN-E-low. Compared with the 331 patients with low PMN-E levels (54% response (16% complete and partial remission, 38% stable disease), OR = 1), the 56 patients with high PMN-E levels showed a worse rate of response (32% response (11% complete and partial remission, 21% stable disease), OR = 0.40; P = 0.003).

The median postrelapse survival time after the start of tamoxifen treatment was longer in the 331 patients with low tumour PMN-E levels compared with the 56 patients with high PMN-E levels (25 and 15 months, respectively). However, the median duration of response in the 179 responding patients with low tumour PMN-E levels was not different from that in the 18 responding patients with high PMN-E levels (median 15 and 17 months, respectively).

In Cox univariate regression analysis using continuous PMN-E levels, PMN-E was not significantly associated with progression-free survival (P = 0.51) or postrelapse survival (P = 0.06) when all failures during the total follow-up period were taken into account (385 and 349 events in the analyses of progression-free and postrelapse survival, respectively). However, both in the analysis for postrelapse and progression-free survival, the proportional hazards assumption was violated (P = 0.01 and P = 0.04, respectively). This was indicative for a time-dependent relation between the level of PMN-E and the survival analyses. Since the median progression-free survival time was 6.7 months and that of postrelapse survival was 23.5 months, we further explored the
short-term predictive value of PMN-E for the periods before 6 months in the analysis of progression-free survival (182 events of a total of 385) and before 20 months in the analysis of postrelapse survival (167 events of a total of 349). In these analyses, increasing levels of PMN-E were significantly related with a poor progression-free survival \((P = 0.01)\) and postrelapse survival \((P = 0.001)\). Furthermore, the proportional hazards assumption was no longer violated with its contribution was not statistically significant \((P = 0.31)\). The independent relation of PMN-E levels with the rate of response to tamoxifen treatment was studied using multivariate logistic regression analysis (Table 2). High levels of PMN-E were associated with a poor rate of response \((OR = 0.47, P = 0.01)\). In addition to PMN-E status, premenopausal status, a short DFI, and ER negativity were associated with a poor response rate in the multivariate analysis. In separate multivariate analyses in which PMN-E was added as a dichotomised variable to the model, the contributions of ER \((OR: 1.30; 95\% CI, 1.15–1.48; P = 0.001)\) and PMN-E \((OR, 0.47; 95\% CI, 0.25–0.89; P = 0.02)\) were statistically significant as well. However, when PMN-E was added as a continuous instead of a dichotomised variable to the multivariate model shown in Table 2, its contribution was not statistically significant \((P = 0.31)\). Furthermore, there was no statistically significant interaction between categorically added PMN-E and ER with respect to the rate of response to tamoxifen treatment.

### Response to tamoxifen treatment in ER subgroups of tumours

We explored the association of PMN-E with the response rate in the clinically important subgroups of ER-positive and ER-negative patients. The predictive value of a high PMN-E level for a lower response rate appeared to be confined to the subgroup of 338 patients who received adjuvant chemotherapy as first-line treatment for advanced disease. Of the 76 patients who received chemotherapy as first-line treatment for advanced disease, 33 (43%) responded (seven complete remission, 14 partial remission, 12 stable disease). In these patients, PMN-E levels, neither when analysed as a continuous nor as a dichotomised variable, were significantly related with the rate of response, duration of response, or the length of progression-free or postrelapse survival.

### Table 2 Univariate and multivariate analysis for response to first-line tamoxifen therapy in patients with advanced breast cancer

| Univariate analysis | Multivariate analysis* |
|---------------------|------------------------|
| **Menopausal status** |                         |
| Premenopausal       | 76                     | 34                  |
| Postmenopausal      | 311                    | 55                  |
| **Age (years)**     |                         |
| <40                 | 33                     | 42                  |
| 41–55               | 100                    | 43                  |
| 56–70               | 155                    | 53                  |
| >70                 | 99                     | 59                  |
| **First site of relapse** |                   |
| Soft tissue         | 57                     | 47                  |
| Bone                | 169                    | 54                  |
| Viscera             | 161                    | 49                  |
| **Disease-free interval** |                  |
| <1 year             | 106                    | 35                  |
| ≥1 year             | 281                    | 57                  |
| **Adjuvant chemotherapy** |               |
| No                  | 309                    | 51                  |
| Yes                 | 78                     | 50                  |
| **ER status** |                         |
| Negative            | 49                     | 24                  |
| Positive            | 338                    | 55                  |
| **PgR status** |                         |
| Negative            | 95                     | 41                  |
| Positive            | 282                    | 55                  |
| **PMN-E levels** |                         |
| Low                 | 331                    | 54                  |
| High                | 56                     | 32                  |

*The final multivariate model with all factors known included 387 patients. aOdds ratio (95% confidence interval). bAt the time of start of first-line tamoxifen treatment. \*Cutoff points: 10 fmol mg\(^{-1}\) protein. \^Low: \(\leq 20.0\) ng mg\(^{-1}\) protein, high: \(>20.0\) ng mg\(^{-1}\) protein. The increment in \(\chi^2\) after adding PMN-E to the model including traditional predictive factors was 6.1.
During the past 20 years, the presence of steroid receptors in the primary breast tumour has been the major guide to the physician to treat the patients with endocrine therapy. However, the steroid receptor status of the primary tumour does not fully predict which patient will benefit or fail from endocrine therapy, and many more potential cell biological predictive factors have been studied (reviewed by Klijn et al., 1999, 2002). The expression levels of several serine proteases, such as uPA, human kallikreins 3 and 10 have been reported to be associated with a poor relapse-free and/or overall survival in patients with primary breast cancer. Since the serine protease PMN-E is the only neutral protease that is able to degrade insoluble elastin (Janoff and Schere, 1968; Baugh and Travis, 1976), which is a structural component of breast tissues (Hornebeck et al., 1977), we considered it of interest to study the clinical relevance of PMN-E in breast cancer. Analogous to other serine proteases, the tumour level of PMN-E was also shown to be able to discriminate between primary breast cancer patients with high and low risk of recurrence (Yamashita et al., 1994, 1995b), irrespective of whether adjuvant tamoxifen treatment was given (Yamashita et al., 1995a). Yet, from the latter study, no conclusions can be drawn with respect to a possible relation between PMN-E and efficacy of tamoxifen therapy. Recently we showed that primary breast cancer patients with high tumour levels of uPA and its inhibitor PAI-1, being classified as high risk for recurrence, experienced an enhanced benefit from adjuvant chemotherapy (Harbeck et al., 2002a, b). For patients with advanced breast cancer, a very limited number of studies (all from our laboratory) addressing the relation of serine proteases with the efficacy of systemic treatment are available. In this respect, we showed that high tumour levels of uPA (Foekens et al., 1995), kallikrein 3 (Foekens et al., 1999), or kallikrein 10 (Luo et al., 2002) were significantly associated with a poor rate of response to tamoxifen therapy. In the present study, we investigated the predictive value of PMN-E for the efficacy of tamoxifen and chemotherapy in patients with advanced breast cancer. The direct measurable effect of the therapy on the size of relapse or the development of new relapses was considered the main end point of the study. This study is different from those performed in the adjuvant setting in which all intentionally cured primary breast cancer patients were evaluated with the development of a relapse as end point. In the case of nonrandomised patients, the occurrence of a relapse does not necessarily reflect an association of the marker studied with the efficacy of adjuvant treatment. Furthermore, response as in objective response and progressive disease cannot be studied because by definition there is no measurable tumour in the adjuvant setting. In the present study analysing the predictive value of PMN-E in the advanced setting, we corrected for traditional prognostic and predictive factors, including disease-free interval, in the multivariable logistic regression analysis for response to tamoxifen treatment. Thus, the possibility that the observed predictive value of PMN-E partly reflects its prognostic value is minimal excluded.

So far, the reason for the observed negative relations between the levels of PMN-E and ER and PgR, which we also showed in a larger series of 1143 primary breast cancer patients in which we studied the prognostic value of PMN-E (Foekens et al., 2003), is not clear. It may be explained by an inhibition in elastase activity by oestriadiol and progesterone that has been observed in breast cancer cells grown in vitro (Kao and Stern, 1986). On the other hand, the amount of immunoreactive PMN-E secreted by breast cancer cells into culture media was not affected by oestriadiol, which suggested that PMN-E synthesis is oestrogen independent (Yamashita et al., 1994). The only other laboratory that studied PMN-E expression in human breast cancer tissues reported a nonsignificant trend towards a negative correlation between PMN-E levels and those of ER and PgR in a small series of 62 tumours (Yamashita et al., 1994). In their subsequent study, Yamashita et al. (1995a) used Triton X-100 extracts, whereas Yamashita et al. (1995a) used cytosolic tissue extracts, whereas Yamashita et al. (1995a) used Triton X-100 extracts.

In the present study, high tumour levels of PMN-E were associated with a poor efficacy of tamoxifen treatment in patients with advanced disease, independent of the traditional predictive factors menopausal status, disease-free interval, and hormone
receptor status. From the exploratory subgroup analyses, it appeared that the association of PMN-E with the type of response to tamoxifen treatment was exclusively present in patients with ER-positive tumours. The association of PMN-E with the lengths of progression-free and postrelapse survival was only of statistical significance during short-term follow-up. These analyses were indicated since by taking all the failures during the total follow-up period into account, the proportional hazards assumption, on which the Cox regression analysis is based, was violated. Such a time-dependent relation between cell biological factors and (relapse-free) survival in primary breast cancer has recently been observed for PMN-E (Foekens et al, 2003), as well as for clinical and other biological factors like uPA (Yoshimoto et al, 1993; Schmitt et al, 1997b; Hilsenbeck et al, 1998). In the present study, no relation between the tumour PMN-E level and the efficacy of first-line chemotherapy in patients with advanced breast cancer was seen, suggesting that possible interfering effects of PMN-E are overruled by the chemotherapy regimens applied.

The underlying mechanisms explaining why the primary tumour level of PMN-E is associated with the efficacy of tamoxifen therapy in advanced disease is unclear. In addition to its described role in tumour cell invasion and metastasis, the serine protease PMN-E may, similar as has been suggested for uPA for human ovarian carcinoma cells (Fischer et al, 1998) and breast cancer cells (Foekens et al, 1995), provoke cell proliferation via enzymatic activation of several growth factor (receptor) pathways. In this respect, the ability of elastase to cleave or activate several growth factors or receptors, such as EGF and EGF receptor (Di Camillo et al, 2002), IL-2 and IL-6 receptor (Bank et al, 1999), stromal cell-derived factor-1 (Valenzuela-Fernandez et al, 2002), supports such a local elastase-directed growth interfering mechanism.

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PMN-elastase and tamoxifen therapy in breast cancer

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In summary, our study suggests that a high tumour level of PMN-E is associated with a poor rate of response to tamoxifen therapy, and a short progression-free and postrelapse survival. Thus, patients with low tumour levels of PMN-E may respond better to tamoxifen therapy. In contrast, since the levels of PMN-E do not affect the effectiveness of chemotherapy, patients with high tumour levels of PMN-E might benefit from chemotherapy rather than from endocrine therapy. The present pilot study is the first to report the relation between the tumour level of PMN-E and the efficacy of systemic therapy in patients with advanced breast cancer. However, confirmatory studies are needed before any conclusions can be drawn regarding suggestions to be made for the most promising systemic treatment based on the tumour level of PMN-E.

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