Overexpression of group II phospholipase A₂ in human breast cancer tissues is closely associated with their malignant potency

S.-I. Yamashita, J.-I. Yamashita & M. Ogawa

Department of Surgery II, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto 860, Japan.

Summary Membrane-associated phospholipase A₂ (M-PLA₂) is an enzyme that hydrolyses the sn-2 fatty acyl ester bond of phosphoglycerides. We measured M-PLA₂ concentration in tissue extracts from 325 human breast cancers using a specific radioimmunoassay recently developed. Correlation analyses between the tissue concentration of M-PLA₂ and clinicopathological factors showed that the enzyme level was significantly higher in patients with distant metastasis than in those without. In addition, M-PLA₂ concentration was significantly higher in scarred carcinoma than in other histological types. No significant association was found between M-PLA₂ concentration and age, menstrual status, tumour size, histological grade, vessel involvement or oestrogen receptor (ER) and progesterone receptor (PR) status. The expression of M-PLA₂ mRNA was examined in a fibroadenoma, a stage IV breast cancer and its metastatic site of skin. Northern blot analysis showed a clear hybridisation band corresponding to M-PLA₂ mRNA in both primary breast cancer and its metastatic site, while the fibroadenoma expressed a faint band corresponding to M-PLA₂ mRNA. Breast cancer patients with high M-PLA₂ concentrations exhibited significantly shorter disease-free and overall survival than those with low M-PLA₂ concentration at the cut-off point of 5 ng 100 mg⁻¹ protein, which was determined in a separate study. In multivariate analysis, M-PLA₂ was found to be an independent prognostic factor for disease recurrence and death in human breast cancer. The possible significance of M-PLA₂ expression in human breast cancer tissue is discussed.

Materials and methods

Patients

The 325 breast cancer patients analysed in this study are those who underwent curative or non-curative mastectomy in the Department of Surgery II, Kumamoto University Hospital, during the 8 year period from 1982 to 1987. The median follow-up period for patients was 8.2 years (range 5.5–10.7 years). The clinicopathological parameters reviewed in this study were age, menstrual status, tumour size, number of positive nodes, presence or absence of distant metastasis, histological type, histological grade, vessel involvement, oestrogen receptor (ER) and progesterone receptor (PR). When histological typing was performed according to the World Health Organization (WHO) classification (1981), all tumours were classified into the same category, i.e. invasive ductal carcinoma. Therefore, each tumour was further analysed according to the classification of the Japanese Breast Cancer Society (1988), and was graded in parallel according to the criteria described by Bloom and Richardson (1957), except for 12 comedocarcinomas. ER and PR were determined by the dextran-coated charcoal method as described previously (McGuire et al., 1977). The results of ER and PR were summarised as negative (<10 fmol mg⁻¹ protein) or positive (≥10 fmol mg⁻¹ protein).

Assay for M-PLA₂

Tumour samples were drawn from a pool of frozen specimens (stored at −80°C) and each specimen was homogenised and extracted with 50 mM Tris–HCl buffer (pH 7.4) containing 0.25% Triton X-100 (Sigma, St Louis, MO, USA) as described previously (Yamashita et al., 1986). M-PLA₂ concentration was measured by a radioimmunoassay using anti-M-PLA₂ monoclonal antibody as described by Matsuda et al. (1991). There is no cross-reactivity of this antibody with human P-PLA₂, pancreatic trypsin, chymotrypsin, elastase 1 and pancreatic secretory trypsin inhibitor (Matsuda et al., 1991). The purified M-PLA₂ was iodinated with ²¹¹Iodine (New England Nuclear, Boston, MA, USA) by the chloramine-T method (Hunter & Greenwood, 1962), and the ²¹¹I-labelled M-PLA₂ was purified by gel filtration on a PD-10 column (Pharmacia Fine Chemicals, Sweden). Its specific activity was approximately 3.5 MBq µg⁻¹. The detection limit of M-PLA₂ was 7 ng 100 mg⁻¹ protein. The intra-assay coefficient of variation (CV) was obtained by testing one sample on the same kit ten times. Those for the high, middle and low sample levels were 3.8, 5.6 and 5.7% respectively. The inter-assay CV was calculated from assays using the same sample during a period of 1 month, and those for the three sample levels were 4.4, 4.5 and 3.2% respectively.
Northern blot analysis

Total RNA was extracted from a fibroadenoma, a primary breast cancer and its metastasis to skin by the guanidine thiocyanate–cesium chloride procedure (Sambrook et al., 1989). Total RNA (5 μg per lane) was separated by 1% agarose–formaldehyde gels, and transferred to nylon membrane (Hybond N+) by Northern blotting. The blots were hybridised with 32P-labelled specific probes. The 336 bp Neol–Scrl fragment of group II PLA2 cDNA (Seilhamer et al., 1989) was used for its mRNA detection. Filters were washed in 0.2 × SSC and 0.1% SDS at 65°C. As a control, filters were stripped and rehybridised with a radiolabelled G3PDH probe. The bands were quantitated by a BAS2000 image analyser.

Statistics

The Kruskal–Wallis test was used for the analysis of M-PLA2 concentration in relation to clinicopathological factors. The Cox proportional hazards model (Cox, 1972) was used in multivariate analysis to assess the independent prognostic significance.

Table I Correlation between M-PLA2 concentration and clinicopathological factors of human breast cancer

| Factor                      | M-PLA2 concentration* | P-value |
|-----------------------------|------------------------|---------|
| Age (years)                 |                        |         |
| < 50 (143)                  | 68 ± 21                |         |
| ≥ 50 (182)                  | 54 ± 9                 | NS      |
| Menstrual status            |                        |         |
| Pre/perimenopause (168)     | 72 ± 18                |         |
| Post-menopause (157)        | 47 ± 10                | NS      |
| Tumour size (cm)            |                        |         |
| > 2 (67)                    | 60 ± 10                |         |
| 2–5 (203)                   | 52 ± 14                | NS      |
| > 5 (55)                    | 89 ± 25                |         |
| Node involvement            |                        |         |
| 0 (184)                     | 49 ± 7                 |         |
| 1–3 (68)                    | 89 ± 21                | NS      |
| > 4 (73)                    | 61 ± 13                |         |
| Distant metastasis          |                        |         |
| Absent (290)                | 34 ± 10                |         |
| Present (35)                | 273 ± 45               | 0.025   |
| Histological type           |                        |         |
| Papillotubular (75)         | 21 ± 6                 |         |
| Solid tubular (120)         | 36 ± 12                |         |
| Scirrhous (114)             | 116 ± 24               | 0.016   |
| Others (16)                 | 24 ± 3                 |         |
| Histological grade*         |                        |         |
| Grade I (88)                | 46 ± 11                |         |
| Grade II (122)              | 68 ± 13                | NS      |
| Grade III (103)             | 69 ± 13                |         |
| Vessel involvement          |                        |         |
| Absent (207)                | 55 ± 22                |         |
| Present (118)               | 69 ± 15                | NS      |
| ER                          |                        |         |
| Positive (163)              | 62 ± 12                |         |
| Negative (128)              | 50 ± 17                | NS      |
| Unknown (34)                | 88 ± 27                |         |
| PR                          |                        |         |
| Positive (99)               | 71 ± 30                |         |
| Negative (184)              | 49 ± 15                | NS      |
| Unknown (42)                | 83 ± 26                |         |

*Mean ± s.e. Numbers in parentheses are the number of patients.

**Twelve comedocarcinomas were excluded from this analysis. NS, not significant.

Results

Relation between M-PLA2 concentration and clinicopathological factors

M-PLA2 was detected in tissue extracts from 311 of 325 specimens, the concentration ranging from 7.3 to 1,755 ng 100 mg−1 protein. The median value of M-PLA2 concentration was 52 ng 100 mg−1 protein. Table I shows the correlation between M-PLA2 concentration and the characteristics of the patients. When M-PLA2 concentration was compared in terms of age, menstrual status, tumour size, nodal status, histological grade, vessel involvement, ER and PR, no significant association was found between M-PLA2 concentration and any of these features. However, M-PLA2 concentration was significantly higher in scirrhous carcinoma than in other histological types (P = 0.016). Similarly, M-PLA2 concentration was significantly higher in distant metastasis-positive than in -negative patients (P = 0.025).

Relation between M-PLA2 concentration and survival

To evaluate the prognostic significance of M-PLA2, we analysed disease-free survival and overall survival in 290 breast cancer patients. Thirty-five patients with distant metastases at the time of primary therapy were excluded from this analysis. The cut-off point of 50 ng 100 mg−1 protein was used because our previous study of another group of patients demonstrated that this cut-off point could give a statistically significant separation for risk of relapse according to the method of Tandon et al. (1990). This cut-off point is close to the median value (52 ng 100 mg−1 protein) of the present series. As shown in Figure 1, patients with breast

Figure 1 Disease-free and overall survival curves in 290 breast cancer patients having no distant metastasis according to M-PLA2 concentration in tumour extracts. The cut-off point between high and low enzyme level was 50 ng 100 mg−1 protein. Number of patients in each group: high M-PLA2, 133; low M-PLA2, 157.
cancer tissue containing a high concentration of M-PLA₂ had a significantly shorter disease-free survival \((P = 0.008)\) and overall survival \((P = 0.011)\) time than those with a low content of the enzyme. In multivariate analysis including all variables, M-PLA₂ was found to be an independent prognostic factor for recurrence and for death of about the same import as lymph node involvement (Tables II and III).

**M-PLA₂ mRNA expression**

Northern blot analysis of total RNA from one fibroadenoma, one stage IV breast carcinoma and its skin metastasis is shown in Figure 2. Using a radiolabelled M-PLA₂ cDNA probe (336 bp Ncol–Scal fragment), M-PLA₂ mRNA was clearly demonstrated in total RNA preparations from breast carcinoma and its metastasis site, while fibroadenoma expressed only a faintly hybridising band of M-PLA₂ mRNA. Interestingly, the metastasis site expressed more M-PLA₂ mRNA than the primary tumour.

**Discussion**

We and other investigators have demonstrated that a transient increase in serum M-PLA₂ concentration is observed during surgery (Matsuda et al., 1991) and in various clinical conditions such as endotoxic shock (Vadas & Hay, 1983) and multiple injuries (Uhl et al., 1990), suggesting that this enzyme is one of the acute-phase reactants. We also showed that serum M-PLA₂ concentration was significantly elevated in patients with various malignant tumours (Matsuda et al., 1991). Since the incidence and magnitude of the elevation were greater in patients with advanced breast cancers than in those with early stages, we speculated that this enzyme might be produced by breast cancer cells themselves.

Immunohistochemical study showed that M-PLA₂ was preferentially stained in breast cancer cells rather than breast stromal cells (Yamashita et al., 1993), indicating that breast cancer cells produce a large amount of this enzyme.

In the present study, correlation analyses showed that tissue M-PLA₂ level was significantly higher in distant metastasis-positive than in -negative patients. Furthermore, of interest was the finding that the distant metastasis from a stage IV carcinoma showed even larger amounts of M-PLA₂ mRNA than the primary tumour. These results suggest that the expression of this enzyme may be related to the metastatic potency of human breast cancer.

Histologically scirrhous carcinoma, which is characterised by its prominent stromal cellularity, was also related to high M-PLA₂ concentrations in tissue extracts. In our previous report, the intensity of immunohistochemical staining was

**Figure 2** Northern blot analysis of total RNA from a fibroadenoma, a primary breast cancer and its metastasis to skin. Total RNA was hybridised with cDNA from human group II PLA₂ as probe. To assess equal load of mRNA per lane, the filter was subsequently hybridised to a G3PDH probe. 28S and 18S rRNA bands were used as molecular weight markers.

| Table II M-PLA₂ and clinicopathological parameters as prognostic factors for relapse in 290 stage I–III breast cancer patients |
|-----------------|-----------------|-----------------|-----------------|
| **Parameters** | **Univariate analyses** | **Multivariate analyses** |
|                 | **Relative risk** | **p-value** | **Relative risk** | **p-value** |
| **Independently associated with relapse** |
| Nodal status   |                  |              |                  |
| 0              | 1.0              | 0.9          | 1.0              | 0.9          |
| 1–3            | 2.2              | 0.001        | 1.7              | 0.003        |
| ≥4             | 4.1              |              | 2.9              |              |
| M-PLA₂ concentration |              |              |                  |
| <50            | 1.0              | 0.008        | 1.0              | 0.008        |
| ≥50            | 3.3              |              | 2.7              | 0.015        |
| **Associated with relapse only when evaluated alone** |
| Tumour size (cm) |                  |              |                  |
| <2             | 1.0              |              |                  |
| 2–5            | 1.5              |              |                  |
| >5             | 2.7              | 0.024        | 2.0              | 0.024        |
| Histological grade |                  |              |                  |
| Grade I        | 1.0              |              |                  |
| Grade II       | 1.7              |              |                  |
| Grade III      | 3.0              | 0.015        | 3.5              | 0.015        |
| Vessel involvement |                |              |                  |
| Absent         | 1.0              |              |                  |
| Present        | 2.0              | 0.050        | 2.0              | 0.050        |
| **Not associated with relapse** |
| Age            | NS               |              | NS               |              |
| Menstrual status | NS             |              | NS               |              |
| Histological type | NS            |              | NS               |              |
| ER             | NS               |              | NS               |              |
| PR             | NS               |              | NS               |              |

NS, not significant.
Table III  M-PLA₂ and clinicopathological parameters as prognostic factors for death in 290 stage I–III breast cancer patients

| Parameters                          | Univariate analyses | Multivariate analyses |
|-------------------------------------|---------------------|-----------------------|
|                                     | Relative risk | P-value | Relative risk | P-value |
| Independently associated with survival |                      |          |              |         |
| Nodal status                        |                      |          |              |         |
| 0                                   | 1.0                 | 0.001    | 1.0          | 0.001   |
| 1–3                                 | 2.1                 | 0.851    | 1.6          | 0.001   |
| ≥4                                  | 3.6                 | 0.780    | 2.5          | 0.022   |
| M-PLA₂ concentration                |                      |          |              |         |
| <50                                 | 1.0                 | 0.355    | 1.0          | 0.695   |
| ≥50                                 | 3.5                 | 0.007    | 2.5          | 0.110   |
| Associated with survival only when evaluated alone |                      |          |              |         |
| Tumour size (cm)                    |                      |          |              |         |
| <2                                  | 1.0                 | 0.579    | 1.0          | 0.475   |
| 2–5                                 | 1.1                 | 0.267    | 1.0          | 0.300   |
| >5                                  | 1.9                 | 0.034    | NS           |         |
| Histological grade                  |                      |          |              |         |
| Grade I                             | 1.0                 | 0.580    | 1.0          | 0.475   |
| Grade II                            | 1.2                 | 0.242    | 1.0          | 0.300   |
| Grade III                           | 2.9                 | 0.009    | NS           |         |
| Vessel involvement                  |                      |          |              |         |
| Absent                              | 1.0                 | 0.786    | 1.0          | 0.475   |
| Present                             | 1.5                 | 0.048    | NS           |         |
| PR                                  |                      |          |              |         |
| Positive                            | 1.0                 | 0.653    | 1.0          | 0.475   |
| Negative                            | 1.4                 | 0.044    | NS           |         |
| Not associated with survival         |                      |          |              |         |
| Age                                 | NS                  | NS       | NS           | NS      |
| Menstrual status                    | NS                  | NS       | NS           | NS      |
| Histological type                   | NS                  | NS       | NS           | NS      |
| ER                                  | NS                  | NS       | NS           | NS      |

NS, not significant.

greater in scirrhous carcinoma than in other histological types (S. Yamashita et al., 1993). Immunohistochemically M-PLA₂-positive cells were localised at the invading edge of the tumour where cancer cells were in contact with surrounding non-neoplastic tissues (S. Yamashita et al., 1993). Recently, we found that human M-PLA₂ itself has a mitogenic effect on fibroblasts (Kurizaki et al., 1992). In addition, M-PLA₂ augments the production of prostaglandin E₂ (PGE₂), which is known to stimulate mitogenesis in fibroblasts (Nolan et al., 1988; Hara et al., 1991). These findings suggest that M-PLA₂ may play an important role in stimulating the growth of stromal cells in breast cancer tissues in a paracrine fashion. Further, PGE₂ released at the tumour site inhibits the host immunological response and enhances tumour growth (Balch et al., 1984; Okada et al., 1990). The release of fatty acids is at least two orders of magnitude greater than eicosanoid production, and these fatty acids also have many direct biological effects on normal and malignant cells (Imagawa et al., 1989; Clerc et al., 1991).

Reliable predictors of survival or relapse in patients with breast cancer aid in determining the use of adjuvant chemotherapy or endocrine therapy. Established prognostic indicators, such as age, lymph node involvement, tumour grade and hormone receptor status, assist in predicting patient outcome or response to treatment, but are not entirely dependable. Several enzymes determined in the cytoplasm and organelles of tumour cells have been found to have prognostic value in human breast cancer. Most of these enzymes are proteinases, such as plasminogen activator (Janicke et al., 1989; Duffy et al., 1990; J. Yamashita et al., 1993) and cathepsin D (Spyratos et al., 1989; Tandon et al., 1990), which have been implicated in tumour infiltration and metastasis. The present study offers statistical evidence that M-PLA₂ concentration in tissue extracts is an independent prognostic factor that clearly identifies high- and low-risk patients using a cut-off point determined previously. To our knowledge, this is the first lipolytic enzyme which can be added to the list of second-generation prognostic factors in human breast cancer.

In conclusion, the present study provides evidence that M-PLA₂ expression is closely associated with the malignant potential of human breast cancer and that this new biological factor can be an independent prognostic factor.

This work was partially supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan to M. Ogawa. We are indebted to Mr M. Hara (NEC Co. Ltd, Tokyo, Japan) and Mr K. Akausa (IBM Co. Ltd, Tokyo, Japan) for assistance with statistical analyses.

References

BALCH, C.M., DOUGHERTY, P.A., CLOUD, G.A. & TILDEN, A.B. (1984). Prostaglandin E₂-mediated suppression of cellular immunity in colon cancer patients. Surgery, 95, 71–75.

BLOOM, H.J.G. & RICHARDSON, W.W. (1957). Histological grading and prognosis in breast cancer. A study of 1049 cases of which 359 have been followed for 15 years. Br. J. Cancer, 11, 359–377.

CLERC, P., Bensaadi, N., PRADEL, P., ESTIVAL, A., CLEMENTE, F. & VAYSSE, N. (1991). Lipid-dependent proliferation of pancreatic cancer cell lines. Cancer Res., 51, 3633–3638.

COX, D.R. (1972). Regression model and life tables. J. R. Stat. Soc. B, 34, 187–220.

DUFFY, M.J., REILLY, D., O'SULLIVAN, C., O'HIGGINS, N., FENNELLY, J.J. & ANDREASEN, P. (1990). Urokinase-plasminogen activator, a new and independent prognostic marker in breast cancer. Cancer Res., 50, 6827–6829.

HARA, S., KUDO, I. & INOUE, K. (1991). Augmentation of prostaglandin E₂ production by mammalian phospholipase A₂ added exogenously. J. Biochem., 110, 163–165.
Amino acid sequence of phospholipase A2 from the venom of Crotalus adamanteus. J. Biol. Chem., 252, 4913–4921.

Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature, 194, 495–496.

Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture. Proc. Natl. Acad. Sci. USA, 86, 4122–4126.

JAPAN MAMMARY CANCER SOCIETY (1988). Histological classification of breast tumors. In General Rule for Clinical and Pathological Record of Mammary Cancer, 9th edn, pp. 21–57. Kanehara: Tokyo.

A Laboratory Manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

The primary structure of a membrane-associated PLA2 from human spleen. Biochem. Biophys. Res. Commun., 163, 42–49.

Membrane-associated phospholipase A2 stimulates DNA synthesis in two murine fibroblasts. Res. Commun. Chem. Pathol. Pharmacol., 78, 39–45.

Evaluation of estrogen receptor assays in human breast cancer tissue. Cancer Res., 37, 637–639.

Development of a radioimmunoassay for human group-II phospholipase A2 and demonstration of post- operative elevation. Enzyme, 45, 200–208.

Role of arachidonic acid metabolism in the mitogenic response of BALB/c 3T3 fibroblasts to epidermal growth factor. Mol. Pharmacol., 33, 650–656.

Regression mechanisms of mouse fibrosarcoma cells after in vitro exposure to quercetin: diminution of tumorigenicity with a corresponding decrease in the production of prostaglandin E2. Cancer Immunol. Immunother., 31, 358–364.