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Measurement of an apoptotic product in the sera of breast cancer patients

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Abstract

During apoptosis, a number of intracellular proteins are cleaved by caspases. The intermediate filament protein cytokeratin 18 (CK18) is cleaved at Asp238 and Asp396. A monoclonal antibody, M30, specifically recognises a fragment of CK18 cleaved at Asp396 (M30-antigen). We used an enzyme-linked immunosorbent assay (ELISA) to measure M30-antigen levels in the sera of 82 healthy subjects and 201 patients with breast cancer. Patients with primary cancer had higher M30-antigen levels than healthy subjects ($P=0.0001$). Patients with recurrent cancer showed higher M30-antigen levels than healthy controls and patients with primary cancer ($P<0.0001$ and $P=0.008$, respectively). In patients with primary cancer, M30-antigen levels were higher in the oestrogen receptor (ER)-negative subgroup than the ER-positive subgroup. In patients with recurrent cancer, M30-antigen levels correlated with the number of involved organs and performance status ($P=0.041$ and $P=0.014$, respectively). There was no association between serum M30-antigen levels and patient prognosis. We conclude that the levels of circulating M30-antigen are increased in patients with breast cancer.

Keywords: Apoptosis; M30; Cytokeratin 18; ELISA; Serum; Breast cancer; Caspase

1. Introduction

Apoptosis is a form of cell death that is either developmentally regulated, caused by specific stimuli, or activated in response to various forms of cell stress. Structural changes of cells during apoptosis are mediated by proteases such as those of the caspase family, which cleave a number of intracellular substrates. One of these substrates is cytokeratin 18 (CK 18) which is a major component of intermediate filaments of simple epithelial cells and tumours derived from such cells. CK18 is cleaved into proteolytic fragments by caspases during apoptosis [1–3].

Recently, a monoclonal antibody which recognises a neo-epitope of CK18 exposed after caspase cleavage during apoptosis was described [4]. The antibody, M30, detects only the caspase cleaved fragment of CK18, but not native or intact CK18. The epitope was mapped to the C-terminal caspase cleavage site of CK18 (amino acids 387–396). M30 can be used to demonstrate apoptotic cells by immunohistochemistry, while viable and necrotic cells are not recognised. The antibody detects cells earlier during apoptosis than TdT-mediated dUTP nick-end labelling (TUNEL) [4] and M30 reactivity detected by immunohistochemistry has been shown to correlate with the apoptotic index detected by TUNEL [5].

In this study, we used an enzyme linked immunosorbent assay (ELISA) to detect the caspase cleaved fragment of CK18 (M30-antigen) and examined its correlation with clinical factors in patients with breast cancer.

2. Patients and methods

2.1. ELISA

M30-antigen levels were measured using an ELISA kit, M30-Apoptosense (Peviva AB, Sweden). This ELISA uses a monoclonal antibody recognising an epitope on the

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238–396 fragment of CK18 as catcher and horseradish peroxidase conjugated M30 as detector. M30-antigen levels are expressed as U/l [6]. One unit (U) corresponds to 1.24 pmol of a synthesised peptide containing the M30 recognition motif according to the manufacturer. The intra- and interassay coefficients of variation of the ELISA were 0.7–5.8% and 2.8–4.8%, respectively.

2.2. Cell culture

HBL100, an oestrogen receptor (ER)-positive human breast epithelial cell line, was grown in Dulbecco's Modified Eagle Medium (GibcoBRL, Paisley, Scotland, UK) supplemented with 5% fetal calf serum and 50 μg of streptomycin and 50 units of Penicillin/ml (GibcoBRL, Paisley, Scotland, UK). On the day before the addition of paclitaxel, cells were plated at 0.12×10^6 cells per well in 24 well plates (Falcon 3047) in 0.5 ml of medium. The next day, cells received fresh medium containing 0.2 μM paclitaxel (Calbiochem, La Jolla, CA, USA), 50 μM cisplatin (Bristol-Myers Squibb) and 40 μM z-VAD(Ome)FMK (ESP, Livermore, CA, USA) as indicated in the results. Heat treatment was conducted for 10 minutes at 55 °C. At the times indicated, medium was collected and centrifuged at 2000*g* for 5 min and the supernatant was used for the analyses.

2.3. Patients and serum collection

Sera were collected from 82 healthy people (49 females and 33 males) and 201 breast cancer patients who were treated in Tokyo Metropolitan Komagome Hospital from 1996 to 1999. Healthy people were unselected volunteers. The average age of the healthy people and the patients were 50.4 (range: 22–75) and 53.3 (range: 30–86) years old, respectively. The patients included 152 consecutive patients with primary cancer without distant metastases and 49 patients with first recurrence after surgical operations of the primary tumours. Sera from cancer patients were collected prior to the initial treatments. Patients with liver and renal dysfunction and other complications were excluded from this study. Informed consent was obtained from all healthy people and patients. The study was approved by the local institutional review board. Performance status was determined according to the Eastern Cooperative Oncology Group (ECOG) criteria. For all patients, liver, lung and distant lymph-node metastases were diagnosed using computed tomographic (CT) scan and bone metastases were diagnosed using X-ray or bone scintigraphy. For patients with stage II or more, brain metastasis was examined using a CT scan. None of the patients with primary cancer had detectable distant metastases.

2.4. Immunoprecipitation and western blotting

Three hundred microlitres of serum from 2 cancer patients with high M30-antigen values were diluted with 300 μl IP buffer (PBS, 20 mM ethylene diamine tetra acetic acid (EDTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF), 10 μM pepstatin A, 10 μM leupeptin, 25 μg/ml aprotinin, 1% (v/v) Elugent (Calbiochem, Darmstadt, Germany). The sera were incubated with 50 μl protein A sepharose (Pharmacia Biotech AB, Uppsala, Sweden) at 4 °C overnight to reduce non-specifically bound proteins. After centrifugation, the supernatant was incubated with 5 μg of the antibody used as catcher in the ELISA-assay at 4 °C for 2 h and further incubated for 2 h after addition of 20 μl protein A sepharose. The sepharose was washed with the IP buffer five times and bound proteins were dissolved in 10 μl 2×sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% glycerol, 2% (v/v) 2-ME, 0.01% (w/v) bromophenol blue) by boiling for 5 min. Samples were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunodetection was performed with horseradish peroxidase conjugated M30. Peroxidase activity was developed by SuperSignal West Pico (Pierce, IL, USA) according to the manufacturer's protocol.

2.5. Hormone receptor assay

The amounts of ER and progesterone receptor (PgR) in the cytoplasmic fractions of the tumour extracts were measured by ligand binding assay (dextran coated charcoal assay) using the E2R Otsuka assay kit (Otsuka Assay Institute, Tokushima, Japan) and by ABBOTT PgR enzyme immuno assay kit (Dynabott, Tokyo, Japan), respectively. The cut-off value for ER was 5 fmol/mg protein, and that for PgR was 10 fmol/mg protein.

2.6. Statistical analysis

Patients' data are presented as medians (25th–75th percentile) and graphically displayed by box plots. The Mann–Whitney U test was performed to test for differences in the M30-antigen levels between two groups. Kruskal–Wallis analysis of variance by ranks was used to compare M30-antigen levels among patients with different recurrent sites. Spearman's correlation coefficient by rank was used to evaluate the correlation between M30-antigen levels and tumour size, number of involved nodes in patients with primary breast cancer and between M30-antigen levels and performance status, number of involved organs in patients with recurrent breast cancer. The survival curves were drawn by the Kaplan–Meier method and the difference in prognosis was analysed by the log-rank test. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Detection of M30-antigen in cultured cell medium after induction of apoptosis

Human HBL100 breast epithelial cells were treated with paclitaxel to induce apoptosis and the culture media were assayed for M30-antigen. Fig. 1a shows the average level ± standard deviation (S.D.) of M30-antigen in three experiments. An increased activity was observed after the treatment ($P=0.049$ between control and treated cells at 48 h). This increase was blocked by the addition of the caspase inhibitor z-VAD-fmk, indicating that the increase is dependent on caspases. In a second set of experiments, heat treatment (55 °C, 10 min) was used to induce necrosis. Heat treatment of HBL100 cells induced a complete loss of viability, but did not increase M30-antigen levels (Fig. 1b). Cisplatin was used as an apoptosis inducer in parallel and was found to induce M30-antigen.

3.2. Detection of the M30-antigen in sera from cancer patients

Sera from 2 cancer patients were immunoprecipitated with the antibody used as catcher in the ELISA-assay, and bound proteins were analysed by western blotting with the M30 antibody. The western blotting showed two bands at sizes of approximately 40 and 24 kDa (Fig. 2). The serum from the other patient showed the same pattern (data not shown). This is in accordance with the report by Leers and colleagues which demonstrated that MR65 cells treated with etoposide gave two M30-reactive protein bands of approximately 40 and 24 kDa [4].

3.3. M30-antigen levels in sera

The median (25th–75th percentile) serum M30-antigen levels were 156.2 (136.9–188.8) U/l in the 82 healthy controls, 165.7 (136.9–192.8) U/l in the 152 patients with primary breast cancer and 180.5 (140.5–227.4) U/l in the 49 patients with recurrence. In one patient with recurrence, four samples were taken on different days over 1 month before a treatment. The average±standard deviation was 184.4±2.8 U/l (range 182.6–188.5 U/l), suggesting little variation in the M30-antigen values from day to day.

Fig. 3 shows that patients with primary cancer had higher M30-antigen levels than healthy controls ($P=0.0001$) and that patients with recurrent cancer had higher M30-antigen levels than healthy controls and patients with primary cancer ($P<0.0001$ and $P=0.008$, respectively). The correlation of M30-antigen levels with clinicopathological parameters in 152 patients with primary breast cancer is shown in Table 1. ER-negative patients showed a higher level of M30-antigen than ER-positive patients ($P=0.040$). Survival analyses were

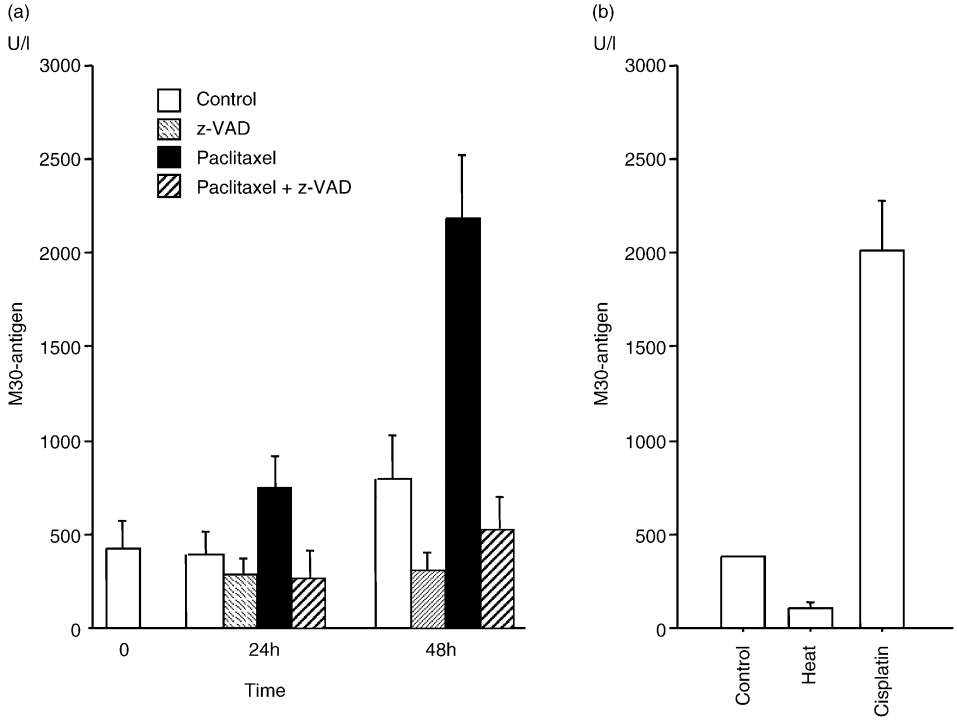

Fig. 1. M30-antigen levels in breast carcinoma cell cultures undergoing apoptosis or necrosis. (a) M30-antigen in culture media of HBL100 treated with paclitaxel. The average ± standard deviation (S.D.) in three experiments is shown. (b) M30-antigen in culture media of HBL100 heated at 55 °C or treated with cisplatin. The average ± S.D. in three experiments is shown.

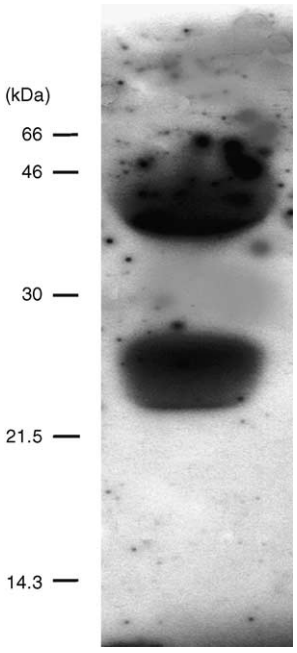

Fig. 2. M30-antigen in serum detected by Western blotting. Serum from a patient with breast cancer was immunoprecipitated with the antibody used as catcher in the ELISA, and bound proteins were analysed by western blotting with the M30 antibody.

Table 1
Association between M30-antigen levels and clinicopathological parameters in patients with primary breast cancer ($n = 152$)

Clinical parameter	Sample number	Median (25th–75th percentile) (U/l)	*P* value
Tumour size			
<2 cm	41	170.1 (137.4–190.8)	
2–5 cm	94	162.3 (136.4–189.7)	NS[a]
>5 cm	17	181.4 (141.3–217.3)	
Number of involved nodes			
0	87	161.3 (136.0–183.5)	
1–3	31	175.2 (152.1–203.7)	NS[a]
>4	28	144.8 (135.0–196.6)	
Unknown	6	193.8 (167.0–206.7)	
Menopausal status			
Pre	66	161.6 (135.8–185.0)	NS[a]
Post	86	171.7 (136.9–196.4)	
Oestrogen receptor			
+	69	142.7 (135.8–180.9)	
–	55	175.2 (136.6–205.2)	0.040
Unknown	28	178.6 (149.9–194.2)	
Progesterone receptor			
+	67	159.7 (135.8–183.4)	
–	57	162.8 (136.6–199.4)	NS
Unknown	28	178.6 (149.2–194.2)	
Histological type			
NIDC[b]	13	161.3 (137.7–188.1)	NS
Invasive ductal Carcinoma	139	166.5 (136.9–194.3)	

[a] NS, non significant.
[b] NIDC, non-invasive ductal carcinoma.

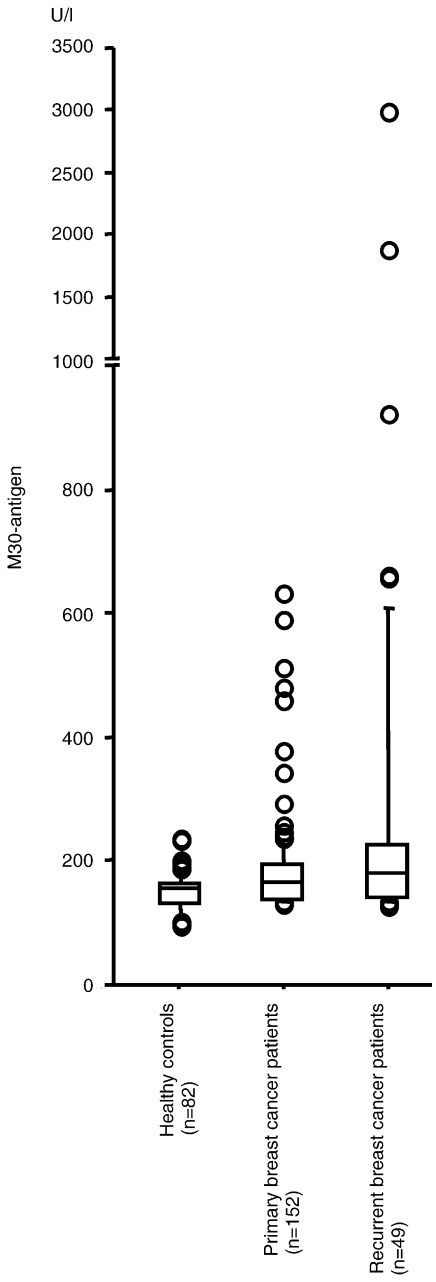

Fig. 3. M30-antigen levels in healthy controls and breast cancer patients. M30-antigen levels in 82 healthy individuals, 152 patients with primary cancer and 49 patients with first recurrence are illustrated by box plots. A box shows the 25th percentile and the 75th percentile in each group. The median is shown as a line across the box. Samples were collected before the initial treatments.

performed with the cut-off value set at the median (165.7 U/l), 180, 200, 250 and 300 U/l. None showed a significant value of serum M30-antigen level as a prognostic factor. Fig. 4 shows the survival curves with the cut-off value at 200 U/l (Fig. 4a, relapse-free survival, $P = 0.68$; Fig. 4b, overall survival, $P = 0.30$).

The levels of M30-antigen in 49 patients with recurrent cancer are shown in Table 2. A difference in M30-

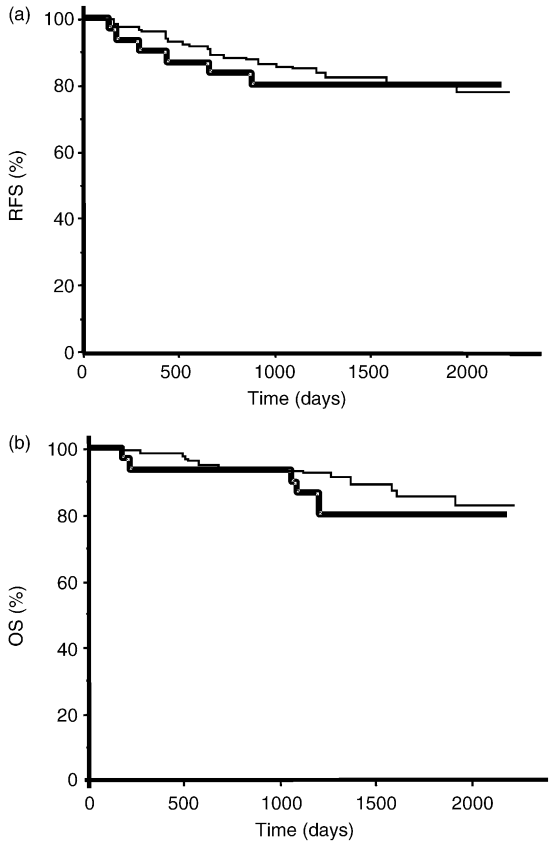

Fig. 4. Survival curves for patients with primary breast cancer: (a) relapse-free survival (RFS); (b) overall survival (OS). Patients were categorised into two groups according to serum M30-antigen level. Bold lines show curves of patients with M30-antigen >200 U/l ($n=122$) and thin lines show curves of patients with M30-antigen ≤200 U/l ($n=30$).

Table 2
Association between M30-antigen levels and clinical parameters in patients with recurrent breast cancer ($n=49$)

Clinical parameter	Sample number	Median (25th–75th percentile) (U/l)	*P* value
Major recurrent site			
Liver	9	176.8 (142.3–267.9)	
Lung	6	156.2 (137.3–531.6)	
Bone	9	199.3 (191.5–355.4)	0.020
Pleura	2	144.8 (140.3–149.4)	
Skin	5	235.0 (224.8–363.6)	
Local	3	136.4 (135.2–139.4)	
Lymph node	15	180.4 (147.3–202.9)	
Number of involved organs			
1	22	178.2 (140.5–202.2)	
2	21	195.9 (140.1–240.0)	0.041
3	6	524.9 (180.5–661.2)	
Performance status			
0	31	176.8 (140.3–199.0)	
1	11	205.5 (145.8–354.1)	0.014
2	6	228.5 (205.2–255.0)	
3	1	656.6	

antigen levels was observed among patients with different major recurrent sites ($P=0.020$). Patients with skin metastasis had higher M30-antigen levels than those without skin metastasis ($P=0.016$) and patients with local recurrence showed lower M30-antigen levels compared with those with other recurrent sites ($P=0.024$). M30-antigen levels were significantly correlated with the number of involved organs and the performance status ($P=0.041$ and 0.014, respectively). Patients with a larger number of involved organs had higher M30-antigen levels. Interestingly, patients with impaired performance status showed a higher concentration of M30-antigen levels in their sera.

4. Discussion

The possibility that tumour apoptosis may generate circulating products, detectable in serum, has an important diagnostic potential. It is well established that serum levels of nucleic acids are increased in patients with various cancers [7–9] and it has recently been reported that the serum levels of nucleosomes have been increased in cancer patients [10,11]. The origins of these materials are unknown but may, at least in part, be derived from apoptotic cells. To measure a product derived from apoptotic cells in serum, we used an ELISA based on the M30 monoclonal antibody [6]. This antibody specifically recognises a neo-epitope of CK18 formed by caspase cleavage. Previous reports showed that the M30 antibody detects only apoptotic cells, but not viable or necrotic cells by immunohistochemistry and that reactivity of the M30 antibody is associated with the apoptosis index by TUNEL [4,5]. Our results from *in vitro* experiments showed that M30-antigen levels increased during apoptosis and that this increase was blocked by a pan-caspase inhibitor. In addition, necrosis induced by heating did not lead to increases in the M30-antigen levels, suggesting that M30-antigen detected by the ELISA is specific to caspase-dependent apoptosis.

In this study, we demonstrated increased serum levels of M30-antigen both in patients with primary and recurrent breast cancer. To our knowledge, this is the first demonstration of a circulating apoptosis-specific product in the sera of cancer patients. In addition to the observations of increased serum M30-antigens in breast cancer, we have observed significantly increased levels also in the sera of liver and lung cancer patients (our unpublished observations). It is likely, therefore, that M30-antigen levels are elevated also in other carcinomas expressing CK18.

Our results showed correlations between serum M30-antigen levels and clinico-pathological factors. Elevated serum M30-antigen levels were associated with an ER-negative status of tumour tissues. It is known that ER-negative breast tumours have higher apoptotic indices than ER-positive tumours [12], consistent with the results in this report. In patients with recurrent breast cancer, serum M30-antigen levels correlated with the number of involved organs and performance status, suggesting that the increase in serum M30-antigen level is associated with cancer progression. Although we did not find any correlation between the M30-antigen level and prognosis of patients with primary breast cancer, some reports have shown correlations between tumour apoptosis and the prognosis of breast cancer patients [12,13]. It will be important to conduct larger prospective studies with longer observation periods to examine the correlation between M30-antigen levels and prognosis.

A number of chemotherapeutical agents and other anti-cancer treatments induce apoptosis [14–16]. It will be interesting to see whether changes in M30-antigen occur in patients' sera after the onset of therapy and whether such changes can be used to monitor the treatment effects. Studies to address this question are currently being pursued in our laboratory and others.

In conclusion, we showed that an apoptosis product, M30-antigen, was detected in sera from patients with breast cancer and that serum M30-antigen levels were increased in patients with breast cancer compared with healthy controls. In addition, serum M30-antigen levels were correlated with some of the clinical factors including ER-status and performance status. Further studies including therapeutic monitoring will clarify the clinical usefulness of the serum level of the apoptotic product, M30-antigen.

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