Jpn. J. Cancer Res. 81, 489–494, May 1990

In vitro and *in vivo* Release of Soluble *erbB*-2 Protein from Human Carcinoma Cells

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An enzyme-linked immunosorbent assay (ELISA) was developed to measure soluble *erbB*-2 protein in culture supernatants of various human cell lines and sera of patients suffering from recurrent breast carcinoma. Soluble *erbB*-2 protein was demonstrated in culture supernatants of cell lines that expressed high levels of *erbB*-2 protein as shown by western blot analysis of cell lysates. Increased levels of the protein, 40- to 190-fold higher than in healthy controls, were demonstrated in sera of 3 out of 12 patients with breast carcinomas. On immunohistological study of tumor tissues from 9 patients, high immune reaction with the anti-*erbB*-2 protein antibody was observed in 2 cases. These were two of the three patients who had elevated levels of *erbB*-2 protein in serum (a sample was not available from the third patient). These results raise the possibility that soluble *erbB*-2 protein level in serum can be used as an indicator for spread of carcinomas that overexpress *erbB*-2 protein.

Key words: Receptor — Oncogene — Carcinoma — Membrane protein — Breast

The human proto-oncogene c-*erbB*-2 encodes a 185 kilodalton (kDa) glycoprotein that is closely related to the epidermal growth factor receptor protein.[1-7] The c-*erbB*-2 gene product (*erbB*-2 protein) is likely to be a receptor for an as-yet unidentified growth factor. This protein is highly expressed in some human adenocarcinomas.[8-11] Breast carcinoma is one of those adenocarcinomas in which expression of the *erbB*-2 protein is increased: thirteen to thirty percent of breast carcinomas express high amounts of *erbB*-2 protein as demonstrated by immunohistological study of surgically resected specimens.[10,11] Several receptor-type transmembrane proteins, including the insulin receptor, epidermal growth factor receptor, CD8 protein and interleukin-2 receptor, have recently been shown to be released extracellularly.[12-14] As the predicted structure of the *erbB*-2 protein is that of a receptor-type transmembrane protein, there is a possibility that this protein is also released extracellularly. If this were the case, then the determination of the level of *erbB*-2 protein in sera or other body fluids might be useful as a diagnostic/monitoring tool for *erbB*-2-positive carcinomas. The present study was thus undertaken to determine if the *erbB*-2 protein is released extracellularly. Culture supernatants of cell lines that overexpress *erbB*-2 protein and sera of patients suffering from recurrent breast carcinomas were studied.

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MATERIALS AND METHODS

Cell lines Human breast carcinoma cell line YMB-1 and YMB-1-E were obtained from the Japanese Cancer Research Resources Bank (JCRB). Human breast carcinomas MDA-MB-453, ZR-7-5-1, MCF7 and human breast ductal carcinoma MDA-MB-175-VII were obtained from the American Type Culture Collection (ATCC). These five cell lines except MCF7 expressed the amplified c-*erbB*-2 mRNA.[15] MKN7, a gastric carcinoma cell line with amplified c-*erbB*-2 gene, NIH3T3, the murine fibroblast cell line with little expression of the *erbB*-2 protein and an additional cell line SV215 which overexpresses c-*erbB*-2 protein, were also studied. A transformed cell line EX-5, transfected with extracellular domain of c-*erbB*-2 DNA in NIH3T3 cells, was used for ELISA analysis.

Cell culture YMB-1 and YMB-1-E cells were cultured in minimum essential medium supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 4 m*M* HEPES, 2 m*M* L-glutamine and 0.18% $NaHCO_3$. MDA-MB-453-VII and MDA-MB-175 cells were cultured in L-15 medium with 10% FCS. ZR-7-5-1 cells and MCF7 were cultured in RPMI 1640 with 10% FCS. SV215 and NIH3T3 were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. These culture media were also supplemented with penicillin 100 U/ml and streptomycin 100 μg/ml. They were then maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

For ELISA analysis, the supernatants were collected from conditioned medium maintained for 24 h after the cells became confluent. A standard *erbB*-2 protein was purified by the use of an affinity column from the supernatant of serum-free DMEM with supplement B of Hymedium 606 (Kodjin Bio., Tokyo) and fibronectin 20 μg/ml in which EX-5 cells had been cultured.

Sera Sera taken from 12 patients suffering from recurrent breast carcinoma and under treatment at the Department of Clinical Oncology, Cancer Institute Hospital, were studied. Ten sera from healthy volunteers and 25 from patients with hematological diseases, obtained at the Department of Clinical Pathology, Institute of Medical Science Hospital, were also used as control sera.

Antibodies Three monoclonal antibodies that react with the extracellular domains of the *erbB*-2 protein, SV2-61γ and 6G10 (Nichirei, Tokyo), and 9G6 (kindly supplied by Dr. R. Nusse) were used for ELISA. The immunological specificities of SV2-61γ and 9G6 were reported previously.[16, 17] 6G10, recently obtained by immunizing mice with recombinant *erbB*-2 gene-transfected A4-15 cells,[16] reacts specifically with a 185 kDa protein on immunoprecipitation (unpublished). Rabbit polyclonal anti-*erbB*-2 protein antibody was used for immunohistological demonstration of the *erbB*-2 protein on paraffin-embedded tissue sections. The specificities of this antibody were described in the previous report.[4]

ELISA for *erbB*-2 protein Polystyrene microwell plates (NUNC, Roskilde, Denmark) were coated with 100 μl per well of 9G6 or 6G10 monoclonal antibodies (1 μg/ml in 0.15 *M* phosphate-buffered saline, PBS) and left overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20. Unbound sites were saturated by incubation with PBS containing 5% bovine serum albumin (BSA, Sigma) for 2 h at room temperature. The plates were washed again, 100 μl aliquots of sample were added and incubation was carried out overnight at 4°C. The plates were washed and incubated with 100 μl of biotinylated SV2-61γ monoclonal antibody (5 μg/ml) for 2 h at room temperature. The plates were then washed and incubated with 100 μl of peroxidase-conjugated streptavidin (Zymes Laboratories, Inc.) at a dilution of 1/4000 for 2 h at room temperature. After washing of the plate, 100 μl of 1 m*M* ABTS (Wako, Tokyo), 2.8 m*M* H_2O_2 in 0.1 *M* citrate buffer (pH 4.0) was added to each well. Absorbance was measured using a microplate reader (Dynatech, MR 650) at 410 nm. Units of *erbB*-2 protein were calculated from a standard curve constructed with the use of the partially purified serum-free supernatant from EX-5 cells. One ng of partially purified *erbB*-2 protein was taken as one unit.

Quantitation of the cell-bound *erbB*-2 protein To determine the amount of *erbB*-2 protein present in cultured cells, proteins were extracted from these cells and subjected to western blot analysis. The methods were described previously.[8, 18] After western blot analysis, the amount of *erbB*-2 protein was measured using a densitometer (LKB, 2400 Gelscan). The amount of MKN-7 lysate was regarded as a standard (100 U/ml).

Immunohistological study Formalin-fixed and paraffin-embedded pathological specimens were available from 9 out of 12 patients whose sera were studied by ELISA. Four micrometer sections of these tissues were used for immunohistological demonstration of *erbB*-2 protein. The avidin-biotin-peroxidase method[19] was employed, using a rabbit polyclonal anti-*erbB*-2 antibody as the first antibody. The sections were de-paraffinized through xylol and graded ethanol, washed with PBS, and immunostained thereafter following the original procedure of Hsu *et al.*[19] with slight modifications as described in a previous report.[8] The results were evaluated as ++, heavily stained; +, weakly stained; +/−, borderline and −, unstained.

RESULTS

Soluble *erbB*-2 protein levels in culture supernatants and sera In culture supernatants of 7 out of 9 cell lines, high levels of soluble *erbB*-2 protein, over 7.2 U/ml, were noted. Each of these seven cell lines also expressed large amounts of cell-associated *erbB*-2 protein, over 123 units, on western blot analysis of cell lysates. Meanwhile, NIH3T3 cells, which were found to express low levels of *erbB*-2 protein by western blot analysis, did not release detectable levels of the soluble *erbB*-2 protein (less than 4 U/ml). A soluble form was also not detected in culture supernatants of the MCF7 line, whereas the amount of the *erbB*-2 protein in lysates of these cells was at a moderate level (Table I). Sera from three patients with breast carcinomas showed high levels of soluble *erbB*-2 protein (9511, 3776 and 2046 U/ml with the combination of two antibodies 9G6 and SV2-61γ, and 9958, 3018 and 2674 with 6G10 and SV2-61γ, respectively). Two other breast carcinoma cases showed moderate increases in serum *erbB*-2 protein (less than 200 U/ml). The level of soluble *erbB*-2 protein in all the control sera, including 10 healthy volunteers and 25 sera from patients who were without solid tumor, was also under 100 U/ml (Table II).

Immunohistological study Heavy immunostaining was observed in 2 out of 9 specimens studied (Fig. 1, Table II). Two other cases showed borderline staining (+/−); and the remaining five cases were unstained. When these results were collated with the serum *erbB*-2 protein level, both of the heavily-stained tumor specimens were found to have been resected from patients with high levels of serum *erbB*-2 protein (Table II). A tissue specimen was

Table I. *erbB*-2 Protein Levels in Culture Supernatants and Cell Lysates of Various Cell Lines

Cells	*erbB*-2 protein in culture supernatants[a] (U/ml)		*erbB*-2 protein in cell lysates (U/ml)	c-*erbB*-2 mRNA and DNA[b]	
	9G6	6G10		mRNA	DNA
MKN7	15.4	12.0	100	≧20[c]	30[d]
NIH3T3	<4	<4	8	1[c]	1[d]
MCF7	<4	<4	72	1[c]	1[d]
MDA-MB-175-VII	10.1	11.7	123	nd	1[d]
ZR-7-5-1	10.9	13.8	132	3[c]	1[d]
MDA-MB-453	7.5	7.2	207	≧10[c]	2[d]
SV215	9.9	9.8	154	10[c]	10[c]
YMB-1	9.8	9.0	176	7[c]	1[c]
YMB-1-E	10.3	12.1	180	2[c]	1[c]

a) Two pairs of antibody combinations were used for determination of *erbB*-2 protein in culture supernatants: 9G6, SV2-61γ; 6G10, SV2-61γ.
b) The levels of c-*erbB*-2 mRNA and DNA in cell lysates of these cell lines are added in this table.
c) Data from Y. Yamada, *et al.* (unpublished).
d) Data from M. H. Kraus *et al.*[15]
nd: not done.

Table II. Soluble c-*erbB*-2 Gene Product (*erbB*-2 Protein) in Sera of Recurrent Breast Carcinoma Patients, Correlated with Immunopathological Reactivity of the Primary Tumor Cells against Anti-*erbB*-2 Protein Antibody, and Clinical Status

Patient	Serum *erbB*-2 protein (U/ml)		Immunostaining	Clinical status
	9G6	6G10		
1	2046	2674	++	Severe recurrence
2	<50	<50	−	Severe recurrence
3	<50	<50	−	Mild recurrence
4	9511	9958	++	Severe recurrence
5	<50	<50	−	Moderate recurrence
6	111	<50	−	Severe recurrence
7	55	<50	+/−	Severe recurrence
8	3776	3018	nd	Severe recurrence
9	<50	<50	nd	Severe recurrence
10	197	100	nd	Severe recurrence
11	<50	<50	+/−	Mild recurrence
12	<50	<50	−	Severe recurrence

Control sera	Serum *erbB*-2 protein[a] (U/ml)	Number of cases
Healthy controls (10)	<50	10
Hematological diseases (25)	<50	23
	<60	1
	<90	1

nd: not done;
a) The value with the two ELISA system, 9G6 and 6G10.

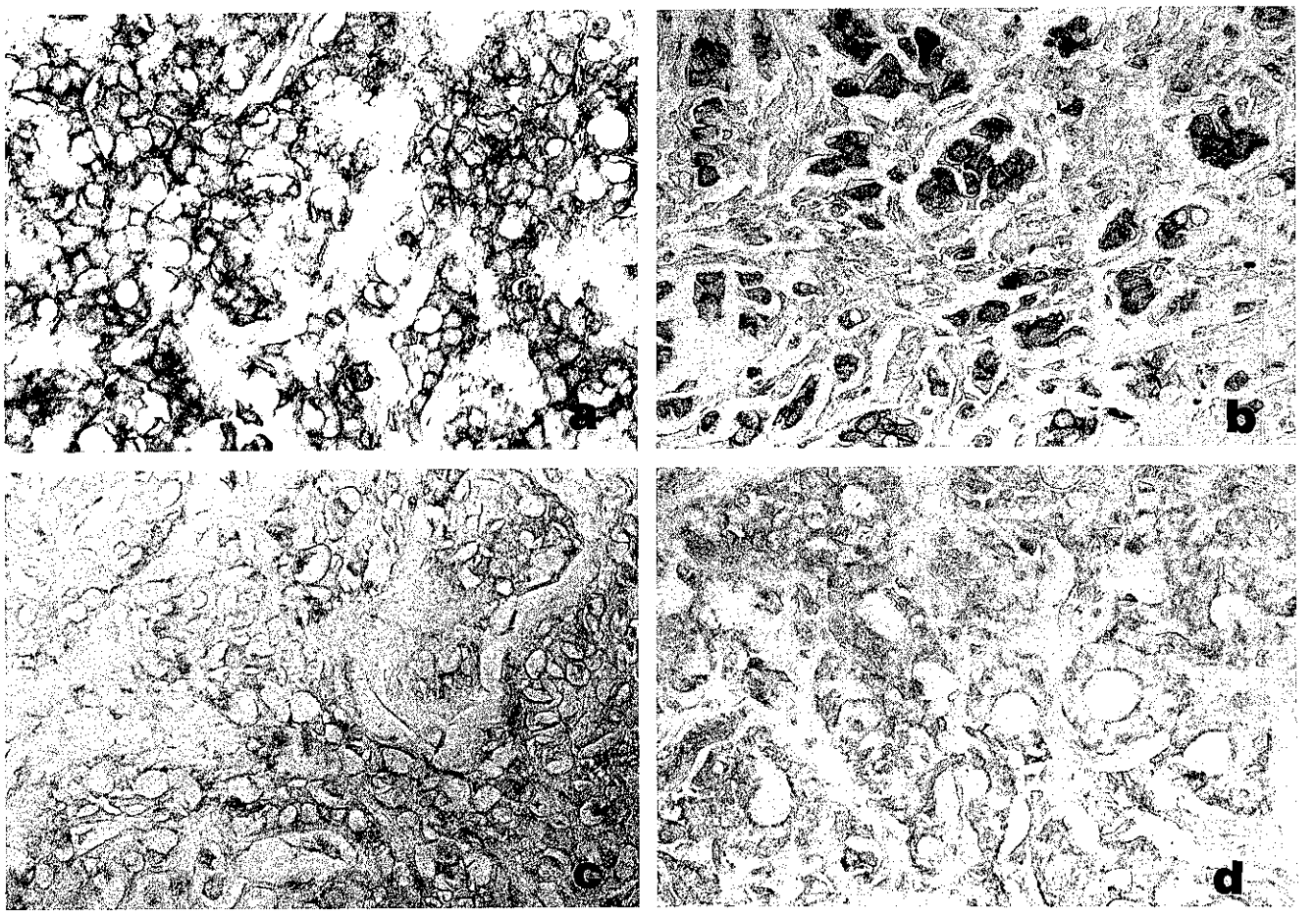

Fig. 1. Immunohistological findings of surgically resected primary breast carcinomas with rabbit polyclonal anti-*erbB*-2 protein antibody. The paraffin sections were stained by the avidin-biotin-peroxidase method. a: Case 4, heavily stained; b: Case 1, heavily stained, c: Case 7, borderline staining, d: Case 5, unstained.

not available from the third patient who had had an elevated level of serum *erbB*-2 protein.

DISCUSSION

Shedding of receptor molecules in soluble form has been shown with the receptors for insulin, epidermal growth factor, CD8 and interleukin-2. The present study clearly showed the presence of the soluble form of the *erbB*-2 protein in culture media and in sera of human carcinoma cases which overexpress the *erbB*-2 protein. The *erbB*-2 protein levels remained very low in sera of healthy volunteers and patients lacking solid tumors. This finding is substantiated by the previous immunohistological findings that adult human cells express the protein at very low levels.[18] Such low serum *erbB*-2 protein levels in healthy controls give an advantage in the determination of levels in the sera of patients who have tumors with highly expressed *erbB*-2 protein. Three patients with breast carcinoma showed over 40 to 140 times higher levels of serum *erbB*-2 protein by ELISA assay. The tumor cells of the two of the three patients were also shown to express a large amount of the *erbB*-2 protein on their cell surface by immunohistological study. In the remaining six patients whose carcinoma cells were unstained or very weakly stained, the serum *erbB*-2 protein levels remained at a low level. Thus, there is every reason to speculate that the third case having high serum *erbB*-2 protein was also an *erbB*-2-positive tumor, although her surgical specimen was not available for the present immunopathological study.

False-positive reaction in the present ELISA study is unlikely for the following three reasons: a) three monoclonal antibodies used for ELISA reacted specifically

with 185 kDa protein on western blot analysis of cancer cell lines overexpressing *erbB*-2 protein; b) three cell lines bearing increased *erbB*-2 protein, as seen in their cell lysates, showed the increase of *erbB*-2 protein in culture supernatants exclusively; c) cases with immunopathological *erbB*-2-positive tumor cells showed high serum *erbB*-2 protein while immunopathologically negative cases did not. However, many more cases have to be studied to justify fully the present ELISA system.

We have not shown how the *erbB*-2 protein is excreted into the sera or culture supernatant. It might be proteolytically cleaved on the cell membrane to release just the extracellular fragment of the molecule, or the entire receptor including extra- and intracellular domains might be excreted. Studies to determine the molecular size of the secreted *erbB*-2 protein are in progress.

The serum *erbB*-2 level is likely to be determined by the amount of *erbB*-2 protein on each tumor cell and the volume of tumor cells. Thus, before possible clinical use of this ELISA system as a diagnostic tool or for monitoring the extent of tumor spread and/or postoperative relapse, a much more extensive study will be necessary to determine the limits of detection or the conditions under which the level increases. However, the present study has clearly shown the possibility of its usefulness in a clinical setting.

Finally, the *erbB*-2 protein has been regarded as a kind of oncofetal protein,[18] as it is widely expressed in fetal epithelium at high level but is expressed at a very low level in adult tissues. This knowledge raises the possibility of immunotherapy in patients with *erbB*-2-expressing carcinomas by introducing antibody to the *erbB*-2 protein coupled with toxins. However, there may be some difficulties, because the present results suggest that the toxin bound to antibody to soluble *erbB*-2 protein would not be taken into the tumor cells and because serum *erbB*-2 protein would easily form immune complexes with the introduced antibody-toxin conjugates, which may produce further complications. Thus, some additional strategies might be needed to overcome these difficulties.

(Received January 12, 1990/Accepted March 1, 1990)

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