Antibody Response against the c-erbB-2 Oncoprotein in Breast Carcinoma Patients

Serenella M. Pupa, Sylvie Ménard, Salvatore Andreola, and Maria I. Colnaghi

Divisions of Experimental Oncology E and Anatomical Pathology and Cytology; Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezia 1, 20133 Milan, Italy

Abstract

Indirect immunofluorescence analysis of sera from breast carcinoma patients whose tumors were characterized for overexpression of the c-erbB-2 oncogene (p185HER2) and for lympho-plasma cell infiltration, revealed no circulating antibodies specifically directed against the p185HER2 molecule in the 20 samples tested, whereas supernatants of B-cell clones, derived from Epstein-Barr virus-transformed peripheral blood lymphocytes from 10 of these patients, contained such antibodies in 6 of the 7 c-erbB-2- and lympho-plasma cell infiltration-positive cases. The antibodies contained in two of the positive supernatants immunoprecipitated a Mr 185,000 molecule from oncoprotein-positive cell extracts that was identified as the oncoprotein in sequential immunoprecipitation experiments with anti-p185HER2 monoclonal antibodies. No cells producing antibodies with a similar reactivity were obtained from Epstein-Barr virus-transformed peripheral blood lymphocytes from breast carcinoma patients with p185HER2-negative tumors or from healthy donors. These data prove the existence of an antibody response specifically directed against the p185HER2 oncoprotein in breast carcinoma patients that may represent an important effector mechanism in the control of c-erbB-2 overexpressing tumors.

Introduction

Abundant evidence obtained in vitro indicates the presence of an antitumor immune response in patients, including breast carcinoma patients (1), although the existence of effective in vivo antitumor protection and the identification of the antigens involved are still a matter of debate (2).

In human breast cancer, one of the most frequently found genomic alterations is amplified expression of the c-erbB-2 oncogene (3, 4). A statistically significant association has been found between overexpression of this oncogene and a marked LPI3 in the tumor, composed predominantly of macrophages and B-cells (5, 6). Moreover, the finding that LPI is indicative of a good prognosis only in the c-erbB-2-positive subgroup (6) suggests the possible involvement of the oncoprotein in triggering the patients' antitumor immune response.

In our investigation of the antibody response directed against the p185HER2 oncoprotein, we detect no antibodies directed against the oncoprotein in sera of patients with breast carcinomas characterized for p185HER2 overexpression and tumor LPI; however, we do find antibodies specifically reactive with c-erbB-2-positive target cells and able to immunoprecipitate the oncoprotein in culture supernatants of EBV-transformed patients’ PBL.

Materials and Methods

Patients and Histopathology. Sera from 20 patients with ductal breast carcinomas were collected before surgical removal of the primary tumors. Tumor specimens were obtained from all patients and evaluated for oncoprotein overexpression. LPI was evaluated by morphological examination. PBL were collected 48 h after surgery.

Cell Lines. Human p185HER2-positive cell lines included breast carcinoma SK-BR-3, ovary carcinoma SK-OV-3, and lung adenocarcinoma Calu-3; human p185HER2-negative cell lines included breast carcinomas MCF-7 and MDA-MB-468, colon carcinoma HT-29, vulvar epidermoid carcinoma A431; and small cell lung carcinomas N592, NCI-H69, NCI-H128, NCI-H345, and NCI-H209 (all from the American Type Culture Collection, Rockville, MD). Human p185HER2-negative ovary carcinoma cell lines IGROV1 and OVCA432 and the melanoma cell line MeWo were kindly provided by Dr. J. Bénérd (Institut G. Roussy, Villejuif, France), Dr. R. Knapp (Dana Farber Institute, Boston, MA), and the late Dr. J. Fogg (Memorial Sloan Kettering Cancer Center, New York, NY), respectively. Bone marrow cells were obtained from iliac crest aspirates. All cell lines were maintained in RPMI 1640 (Microbiological Associates, Walkersville, MD) supplemented with 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml).

EBV Infection. Human PBL from 7 breast carcinoma patients with tumors positive for LPI and c-erbB-2 overexpression, from 2 patients with LPI- and c-erbB-2-negative tumors, and from 4 healthy donors were isolated by density gradient separation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) from 20 to 30 ml of heparinized peripheral blood. Lymphocytes were incubated for 2 h with a supernatant of the EBV-producing marmoset cell line B95-8. After 3 washes, cells were seeded in 96-well microplates (1 X 10^3/well) and cultured at 37°C, adding in the culture medium 2.5 μg/ml of cyclosporin A (Sandoz) for the first 2 weeks.

Immunohistochemistry. IF on live cell suspensions was carried out as described (7), using the patients’ sera or supernatants from EBV-transformed PBL. Briefly, after a 30-min incubation with the test sample, cells were washed and incubated for a further 30 min at 0°C with 0.1 ml fluorescein-conjugated goat anti-human IgA + IgG + IgM (heavy and light chains) (Kpl, Inc., Gaithersburg, MD), diluted 1:30. To determine antibody isotype, biotinylated goat anti-human heavy chain (Amersham, United Kingdom) and fluorescein-conjugated avidin-biotin peroxidase complex kit (Vector, Burlingham, CA). Tumors were considered positive when a strong membrane labeling was observed.

Radiolabeling and Solubilization of Cells. Calu-3 and SK-BR-3 live cells in suspension (10 x 10^6) were membrane-labeled by lactoperoxidase-catalyzed radioliodination and solubilized as described (9). Cellular lysates were cleared by centrifugation (15 min at 10,000 x g) and the soluble extracts were immunoprecipitated.

Sequential Immunoprecipitation. Immunoprecipitation experiments were carried out as described (9). For immunodepletion, an aliquot (500 μl) of precleared radiolabeled SK-BR-3 cell extract containing 15 x 10^6 cpm was immunoprecipitated with Sepharose coated with 3 different anti-c-erbB-2 oncoprotein mAbs (MGR2 + MGR3 + MGR4) (10). The immunodepleted supernatant was again immunoprecipitated with these Sepharose-coupled mAbs and with Sepharoses coupled with human antibodies contained in two supernatants from infection 7, respectively, p185HER2 positive and negative by IF. The precipitated molecules were extracted from each Sepharose using sample buffer and heated for 10 min at 90°C. Samples were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions and subjected to autoradiography at ~80°C.

Results and Discussion

Based on our previous data (6) identifying c-erbB-2 as a prognostic factor in breast carcinoma and demonstrating the association of c-
erbB-2 overexpression with the presence of plasma cells as a component of the lymphoid infiltration of tumors (Fig. 1), we analyzed the sera of patients with tumors characterized for both p185HER2 oncoprotein overexpression and LPI for the presence of anti-c-erbB-2 antibodies. Fifteen sera obtained from patients with LPI-positive tumors, 9 of which were also p185HER2-positive, and 5 sera from patients with breast carcinomas negative for both LPI and c-erbB-2 overexpression, were tested by IF on live cells of two oncoprotein-positive and two negative cell lines in order to detect only antibodies reactive with the extracellular domain of the oncoprotein, i.e., the antibodies that might exert a protective effect against the tumor. However, no antibodies specifically reactive with the p185HER2-positive cell lines were detected in any serum sample. It is possible that such circulating antibodies are present at levels undetectable by IF or that they are absorbed by p185HER2-overexpressing tumor cells or by the circulating soluble p185HER2 molecule released by the tumor (11).

To address this issue, we investigated the in vitro production of antibodies by patients' lymphocytes in a tumor-free system. PBL from 7 patients with breast carcinomas positive for both LPI and p185HER2 overexpression were infected with EBV. Supernatants from a total of 774 wells containing growing cells were tested by IF on two p185HER2-positive and two negative cell lines (Table 1). The first screening of supernatants derived from PBL of 6 of the 7 patients revealed antibodies specifically reactive with the oncoprotein-positive cells in 42 cultures. At 2–3 months after EBV infection, only 6 of the 42 positive cultures still produced the specific antibodies. Indeed, 4 supernatants reacted with 2 c-erbB-2-positive targets and 2 supernatants with 3 such target cells, whereas all supernatants were negative on the 14 p185HER2-negative cells which included 11 carcinomas, 1 melanoma, and 2 suspensions of normal bone marrow cells. The antibodies were all of IgM isotype, as determined by IF using biotinylated goat antibody directed against human immunoglobulin heavy chains.

To further analyze the target molecule recognized by these human antibodies, cell extracts from two p185HER2-positive cell lines and one negative cell line (Calu-3, SK-BR-3, and A431), solubilized after membrane radiiodination, were immunoprecipitated using two different specific supernatants obtained from infections 1 and 7, one nonspecific supernatant from infection 1 and one negative supernatant from infection 7. Two major bands with molecular weights of 69,000 and 85,000 were detected after immunoprecipitation with the nonspecific supernatant, which reacted on both oncoprotein-positive and -negative target cells (Fig. 2, Lane 1), whereas specific supernatant from infection 1 immunoprecipitated a Mr 185,000 molecule together with other lower molecular weight proteins (Fig. 2, Lane 2) as did specific supernatant from infection 7 (Fig. 3A, Lane 2). No Mr 185,000 molecule was immunoprecipitated by the human antibodies contained in specific supernatant from infection 7 from cell lysate immunodepleted with the anti-p185HER2 mixture of mAbs (Fig. 3B, Lane 2). Also the other lower molecular weight proteins were removed by immunodepletion indicating that they are likely degradation products of the oncoprotein arising during the solubilization process. With the negative supernatant, no molecules were detected either before or after sequential immunoprecipitation (Fig. 3, A and B, Lanes 3). Fig. 3A, Lane 1, shows the molecules precipitated by anti-c-erbB-2

![Fig. 1. Plasma cellular infiltrate around a ductal breast carcinoma. × 480.](image)

| Table 1 Reactivity of EBV-transformed PBL from patients with breast carcinoma positive or negative for both LPI and c-erbB-2 overexpression |
|------------------|------------------|------------------|
| Infection        | Infected PBL (×10^6) | No. of supernatants tested | No. of supernatants c-erbB-2 specifically positive |
| c-erbB-2-positive tumor |
| 1                | 15               | 86              | 2               | 1               |
| 2                | 21               | 180             | 8               | 1               |
| 3                | 29               | 205             | 7               | 2               |
| 4                | 6                | 65              | 1               | 0               |
| 5                | 25               | 58              | 8               | 1               |
| 6                | 16               | 76              | 0               | 0               |
| 7                | 10               | 104             | 16              | 1               |
| Σ = 7            | Σ = 774          | Σ = 42          | Σ = 6           |
| c-erbB-2-negative tumor |
| Σ = 3            | Σ = 358          | 0               |
| Healthy donors   |
| Σ = 4            | Σ = 160          | 0               |

*Performed on 2 oncoprotein-positive and 2 oncoprotein-negative target cells.

*Performed on 3 oncoprotein-positive and 14 oncoprotein-negative target cells (see "Materials and Methods").
mAbs that are almost completely removed by immunodepletion (Fig. 3B, Lane 1). Moreover with the specific supernatants, no molecules were immunoprecipitated from the p185HER2-negative cell extract (data not shown). These data indicate that the human antibodies, selected for reactivity with the oncoprotein-positive cells, actually recognize the c-erbB-2 oncoprotein.

EBV infection of PBL obtained from 4 healthy donors and from 3 breast carcinoma patients with oncoprotein-negative tumors produced no cultures that contained antibodies specifically reactive with the p185HER2-positive target cells (Table 1). Comparison of this outcome (0 positive cultures in 7 experiments) with the findings using PBL from patients with p185HER2-positive tumors (6 positive cultures in 7 experiments) by χ² analysis indicates a significant (P < 0.01) relationship between the PBL source and the presence of specific antibodies suggesting that the induction of the anti-oncoprotein antibodies requires an oncoprotein-overexpressing tumor and is not the result of a natural immune response against cross-reactive epitopes.

It remains unclear why the oncoprotein, which is also expressed by normal cells, becomes immunogenic when presented by tumor cells. Recently, several studies have described circulating antibodies, directed against various nuclear oncoproteins involved in the control of growth and differentiation, such as c-myb, c-myc, and p53, in the sera of some cancer patients (12–15). Although mutations as well as exposure of immunocompetent cells to nuclear molecules consequent to tumor cell lysis might explain the immunogenicity of these oncoproteins, mutations in c-erbB-2 have been reported only in rats (16) and in a few human tumors of the central nervous system (17). In breast carcinomas only c-erbB-2 overexpression has been found (18). Instead, the humoral immunogenicity of p185HER2 might rest in its aberrant glycosylation in tumor cells or its overexpression to levels sufficient to trigger immune response or in a different presentation to the immune system. Also, a mechanism of intermolecular help, i.e., B- and T-cells recognizing distinct molecules present in the same complex, may be implicated in the break of split tolerance of anti-p185HER2 B-cells (19).

In agreement with recently reported data on the immunogenicity of polymorphic epithelial mucin (20), we present here that also the c-erbB-2 oncoprotein may be immunogenic for the cancer patients' humoral immune system. Our data together with the good prognosis found in patients with LP1- and c-erbB-2-positive tumors raise the possibility of exploiting the oncoprotein product as a vaccine to further stimulate the patients' immune response.

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References

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