Neu Differentiation Factor (Heregulin) Induces Expression of Intercellular Adhesion Molecule 1: Implications for Mammary Tumors[1]

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ABSTRACT

Neu differentiation factor (NDF, also called heregulin) is a 44-kilodalton glycoprotein that stimulates tyrosine phosphorylation of the Neu/HER-2 receptor and induces phenotypic differentiation of certain mammary cancer cell lines to growth-arrested and milk-producing cells. To determine which molecules participate in the concomitant morphological alterations, we analyzed the expression of several cytoskeletal and surface molecules and found that NDF elevated the expression of the intercellular adhesion molecule 1 (ICAM-1) in cultured AU-565 human adenocarcinoma cells. The levels of both the protein and the mRNA of ICAM-1 were elevated after 3–5 days of treatment with NDF. Elevated expression of ICAM-1 was induced also by γ-interferon and by the tumor-promoting phorbol ester (PMA), albeit with different kinetics. Down-regulation of protein kinase C or its inhibition by calphostin C partially inhibited the effect of NDF, implying that the induction of ICAM-1 may be mediated by protein kinase C.

NDF transcripts were detectable in 3 of 9 human mammary tumors, suggesting that the in vitro effect of the factor may be relevant to breast cancer. By selecting Neu-positive human mammary tumors ($n = 39$), we found a significant correlation ($P < 0.001$) between the expression of ICAM-1 and histological features of invasive ductal carcinoma with a prominent carcinoma in situ component. When cultured in vitro the cells of these tumors grew in clusters and formed domelike structures reminiscent of comedo-type carcinoma in situ. In addition, the majority of patients with tumors that coexpressed ICAM-1 and Neu had no lymph node involvement, unlike most Neu-positive but ICAM-1-negative tumors, which metastasized to the lymphatic system. Taken together, our observations suggest that the induction of ICAM-1 by NDF may affect the morphology, differentiation state, and metastasis of Neu-expressing mammary tumor cells.

INTRODUCTION

The *neu* protooncogene (also called HER-2 and *erb*B-2) encodes a transmembrane tyrosine kinase that is structurally homologous to the receptor for EGF[3] (1–3). Ectopic overexpression of *neu* in mouse fibroblasts renders them tumorigenic (4, 5), and its overexpression in transgenic mice has led to a variety of malignancies, including adenocarcinomas (6). Although the mechanism by which elevated levels of the *neu*-encoded protein confers transformation is not understood, amplification and overexpression of the gene have been detected at high frequency in human adenocarcinomas from several tissues (7–10). Moreover, overexpression of *neu* appears to be associated with poor prognosis in breast cancer patients (7, 11, 12). These observations focused the attention on the Neu protein as a potential

target for antitumor strategies. Antibodies have been raised against the human protein and shown to inhibit the growth of tumor cells *in vitro* (13–15). Interestingly, anti-Neu monoclonal antibodies that could either stimulate tumor growth or significantly inhibit it have been generated (16), and the tumor-inhibitory action was correlated with an intrinsic capacity of the antibodies to induce cellular differentiation (17).

Despite extensive structural homology between Neu and the EGF receptor, no ligand of the latter can bind directly to Neu. However, a factor that can stimulate tyrosine phosphorylation of Neu was detected in the medium of *ras*-transformed rat fibroblasts (18, 19). Complete purification showed that the activity corresponded to a heat-stable M_r 44,000 glycoprotein that stimulated tyrosine phosphorylation of Neu in mammary cells and induced these cells to differentiate into milk-producing and growth-arrested mature cells (20). On the basis of this observation, the factor was named NDF. Molecular cloning of the NDF transcript showed that the precursor of the soluble factor is a membrane protein that contains an EGF-like domain (21). *In situ* hybridization analysis revealed that NDF is expressed in midgestation mouse embryos predominantly in neuronal tissues, suggesting that the factor has a neuronal function (22). Another unexpected finding was that the interaction of the factor with Neu is cell specific: Neu proteins of ovarian and fibroblastic cells are not stimulated by NDF, as if a still unknown membrane molecule participates in the interaction between the ligand and Neu (23). The human homolog of NDF, named heregulin, was isolated from human mammary tumor cells (24). Northern blot analysis and nucleotide sequencing revealed the existence of several distinct transcripts of NDF and heregulin that differ in the length of the cytoplasmic domain and in the EGF-like portion (21, 24). However, a recombinant form that includes only the latter region was found to be mitogenic for certain cultured adenocarcinoma cells (Ref. 24 and our unpublished observations).

The present study concentrated on the NDF-induced morphological alterations in mammary cells and identified the ICAM-1 as a surface component that undergoes up-regulation by NDF. ICAM-1 is a M_r 90,000 integral membrane glycoprotein, the extracellular region of which is divided into five immunoglobulin-like domains that are followed by a transmembrane region and a short cytoplasmic tail (25, 26). ICAM-1 plays an important role in inflammation (27), as well as in malignant diseases (28). Cytolytic conjugate formation between cytotoxic T-lymphocytes and target cells, such as endothelial and epithelial cells, occurs between the ICAM-1 molecule and the integrin lymphocyte function associated antigen (29). The release of certain cytokines (*e.g.*, interferon, interleukin 1, and tumor necrosis factor) at sites of inflammation and immune response causes cell activation and results in elevated expression of ICAM-1 on the cell surface (30). However, the molecule is not restricted to hematopoietic cells; it is expressed also on endothelial cells, dermal fibroblasts, melanomas, and certain kinds of carcinomas (31). Here we provide evidence that ICAM-1 is induced by NDF in mammary tumor cells and suggest that this process involves the action of protein kinase C. In an attempt to correlate the Neu-mediated induction of ICAM-1 with tumor biology, we found that Neu and ICAM-1 are coexpressed in a subset of inva-

sive human mammary tumors that are characterized by a prominent CIS component and low capacity to invade the surrounding stromal tissue and lymphatic system.

MATERIALS AND METHODS

Materials

PMA, cytochalasin B, mitomycin C, retinoic acid, tamoxifen, and calphostin C (all obtained from Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide and stored at −20°C. β-estradiol (Sigma Chemical Co.) was dissolved in ethanol and stored at −20°C. EGF (Bachem California, Inc., Torrance, CA) and prolactin (Sigma Chemical Co.) were dissolved in aqueous solutions and stored at −70°C and −20°C, respectively. NDF was purified to homogeneity from conditioned media derived from Rat1-EJ cells as described previously (20). IFN-γ was purchased from Collaborative Biomedical Products (Bedford, MA).

Antibodies

The following antibodies were used: N24 mAb to the human Neu (16); an anti-ICAM-1 mAb from Becton Dickinson (San Jose, CA); and a rabbit polyclonal antibody to α-lactalbumin from Accurate Chem. and Scientific Corp. (Westbury, NY). Biotinylated goat antibodies to mouse IgG or to rabbit immunoglobulins (for staining of Neu and for detection of α-lactalbumin, respectively) were from Jackson Labs (West Grove, PA), whereas biotinylated rabbit antibodies to mouse IgG$_{2b}$ were from Zymed Labs (San Francisco, CA).

Cells and Culture Conditions

The AU-565 cells, which were obtained from a pleural effusion of a breast carcinoma from the same patient as SKBR-3 cells (32), were received from the Cell Culture Laboratory, Navy Biosciences Laboratory (Navy Supply Center, Oakland, CA). MDA-MB453, SKBR-3, MCF-7, and SKOV-3 were purchased from the American Type Culture Collection (Rockville, MD). UACC-2116 cells were obtained from Dr. Beppino Giovanella of the Stehlin Foundation (Houston, TX). All of the cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified incubator with 8% CO$_2$ in air at 37°C. For the various experiments, exponentially growing cells were inoculated into Lab-Tek four-chamber slides (Nunc, Inc., Naperville, IL) at 1×10^4 cells/ml. Cell numbers were determined by hemocytometer chamber counting, and viability was monitored by trypan blue dye exclusion.

Primary Cultures of Breast Cancers

Fresh tissues were rinsed and minced finely in M15 medium (33) after removal of necrotic areas and blood clots. The cell-containing M15 medium was filtered through a Falcon 70-μm nylon mesh cell strainer and washed three times. The cells that passed through the filter were washed and centrifuged three times at 1000 rpm for 5 min. Trypan blue exclusion was used to determine cell number and viability. Cells were then plated in M41 medium (34) on collagen-coated Nunc chamber slides (as described in Ref. 35). NDF (10 ng/ml) was added directly to the chamber slides, and the cells were incubated at 37°C for 3 days. Neu and ICAM-1 staining were performed by a streptavidin alkaline phosphatase procedure (17).

Immunohistochemical Staining

ICAM-1. Cultured cells were fixed in 10% neutrally buffered formalin for 60 min after the chambers of the cultured slide (Nunc, Inc., Lisle, IL) were removed. After rinsing with Tris-buffered saline, they were treated with a blocking solution (10% rabbit serum, 0.1% bovine serum albumin, 0.5% Triton X-100 in phosphate-buffered saline). An ICAM-1-specific monoclonal antibody was then applied for 30 min. Biotinylated rabbit anti-mouse IgG$_{2b}$ was used as the linking antibody and applied for 20 min. The labeling reagent, alkaline phosphatase-conjugated streptavidin, was then applied for 15 min. The localized ICAM-1 was then visualized with CAS red chromogen (Cell Analysis Systems, Elmhurst, IL). DNA counterstaining was accomplished with the CAS DNA stain kit. All incubations took place at 37°C, but DNA counterstaining was performed at room temperature. Frozen tissue sections (5 μm) were

mounted onto polylysine-coated slides and immediately fixed for 60 min in 10% neutrally buffered formalin. After fixation, the frozen sections were stained in a manner similar to that used for the cell lines.

Neu/HER-2. Cell lines and frozen sections were fixed in a manner similar to ICAM-1 staining. After fixation, the cultured cells and frozen sections were treated for 20 min with 10% goat serum. The N24 monoclonal antibody was then applied for 30 min. Next, biotinylated goat anti-mouse Ig was used as the linking antibody and applied for 20 min. Finally, the labeling reagent alkaline phosphatase-conjugated streptavidin was applied for 15 min. The resulting localized Neu was visualized with CAS red chromogen, and DNA counterstaining was accomplished with the CAS DNA stain kit. All incubations took place at room temperature. The average amount of Neu protein staining was quantitated using sparsely growing AU-565 cells for calibration of the image analysis system. Their level of staining was defined as 100% of Neu protein content. All measurements of Neu antigen were related to this value. The level of stained protein included the surface as well as the cytoplasmic staining.

α-Lactalbumin. Anti-α-lactalbumin immunostaining was accomplished in a manner similar to that of the aforementioned ICAM-1 staining except that the DNA counterstaining preceded the α-lactalbumin immunostaining.

Lipid Visualization

A modified "Oil Red O in propylene glycol" method was used to visualize neutral lipids as previously described (32).

DNA Staining

The CAS DNA stain kit (Cell Analysis Systems) was used to stain the DNA of cell lines in a Feulgen-based reaction as previously described (32).

DNA Probes

β-Actin and tubulin probes were provided by Dr. Lester Lau. The Neu probe was provided by Dr. Dihua Yu, and the ICAM-1 complementary DNA clone was provided by Dr. Jeffrey M. Greve (Miles Pharmaceutical Division, West Haven, CT).

Histoblots

Histoblots were performed as previously described (36). Briefly, cells were grown to 70% confluency on tissue culture dishes and then washed with phosphate-buffered saline. After the removal of phosphate-buffered saline, the cell monolayer was transferred to nitrocellulose filters (0.45 μm; Schleicher and Schuell) by placing the filters on the cells and pressing lightly over the entire filter surface to attach the cells to the membrane. The filters were completely air dried and baked at 80°C for 2 h. Prehybridization was carried out in a solution containing 5× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0); 50% formaldehyde; 0.2% SDS; 5× Denhardt's solution; 0.2 mg/ml salmon sperm DNA, for at least 4 h at 42°C. A blank section of nitrocellulose was carried through the prehybridization and hybridization steps to determine background levels. The hybridization solution was the same as the prehybridization solution except for the addition of the [^{32}P]-dCTP-labeled probe. Probes were labeled to high specific activity and used at approximately 5×10^6 cpm/ml of hybridization solution. Hybridization was carried out at 42°C for 12–16 h. Filters were first washed three or four times, for 10 min each, at room temperature in 1× SSC; in 0.5% SDS, followed by washing twice in 0.2× SSC; and in 0.2% SDS at 60–65°C, for 10 min each. The filters were then dried and exposed to x-ray film or quantitated by Betascope (Betagen, Inc.) analysis.

Slot Blots

RNA was isolated from cell cultures by using a standard guanidine isothiocyanate procedure. RNA quality was checked by electrophoresis in a formaldehyde-agarose gel. Slot blots were prepared using 0.3, 1.0, and 3.0 μg of RNA on nylon membranes (Zetaprobe, Bio-Rad, Inc.) and hybridized with ^{32}P-labeled DNA probes (1×10^9 cpm/μg) using the Multiprime labeling kit (Amersham, Arlington Heights, IL). Hybridization was carried out in 5× SSC, 0.5% SDS, 50% formamide, 5 μg/ml of ^{32}P-DNA probe, and 500 μg/ml of salmon sperm DNA at 42°C for 24 h, after 2 h of prehybridization at the same

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conditions without labeled probe. Membranes were washed in 0.2× SSC with 0.5% SDS at 65°C and subjected to quantitative Betascope (Betagen, Inc.) analysis.

Polymerase Chain Reaction

RNA was extracted from 20-μm frozen sections by a standard SDS/ proteinase K method. RNA was converted to complementary DNA and then amplified basically as described (37). Primers corresponding to base pairs 257–281 and 589–570 of the sequence of human heregulin-α (24) were used for polymerase chain reaction, as well as a pair that amplified β_2-microglobulin as an internal control. The two DNA fragments were amplified separately and run on a polyacrylamide gel. Tumors were scored as either positive or negative for NDF expression. Only samples that scored positive for β_2-microglobulin were considered.

Statistical Analysis

Differences of proportions were tested by the Fisher exact test. Common odds ratio calculations in stratified populations were done by both the Mantel-Haenszel test (38) and by an appropriate logistic model (39).

RESULTS

NDF Up-regulates the Expression of ICAM-1 in Cultured Mammary Tumor Cells. It has been previously reported that NDF (20), gp30 (40), certain monoclonal antibodies to Neu (17), and low-molecular-weight compounds, such as retinoic acid and PMA (Ref. 32) can induce the differentiation of certain cultured human breast tumor cells. In addition to growth arrest at the late G_2 phase, the treated cells synthesize and secrete milk components (casein and lipids) and undergo morphological alterations that include an increase in the nuclear area, cell flattening, and an abundance of cytoplasm. In order to identify cytoskeletal and surface molecules that participate in the establishment of the mature phenotype, we analyzed the expression of a series of proteins in NDF-treated AU-565 and SKBR-3 human mammary tumor cells by using immunocytochemistry. Limited or no prominent changes were observed in the levels and patterns of distribution of the cytoskeletal proteins actin, tubulin, and cytokeratins, as well as several cell adhesion molecules (e.g., endothelial leukocyte adhesion molecule ELAM-1, H-CAM, integrins, and E-cadherin). However, NDF-treated cells, unlike control untreated AU-565 cells, displayed strong staining with antibodies to ICAM-1 (Fig. 1). Staining appeared over all of the cellular processes, and most of the cells were ICAM-1-positive after 4 days of incubation with NDF. Other mammary cell lines that express Neu (e.g., MDA-MB453, SKBR-3, and UACC-2116 cells) were also induced to synthesize ICAM-1 after NDF treatment. However, mammary cells that do not express Neu (i.e., MDA-MB468 cells) and ovarian carcinoma cells that overexpress it (e.g., SKOV-3 cells) did not respond to the factor (data not shown). It was therefore concluded that the induction of ICAM-1 is cell type specific. Concomitant with the appearance of ICAM-1, the Neu protein translocated from the cell surface, partially disappeared, and assumed diffuse cytoplasmic staining (Figs. 1 and 2). Interestingly, cultured ovarian cells did not respond to NDF, but their
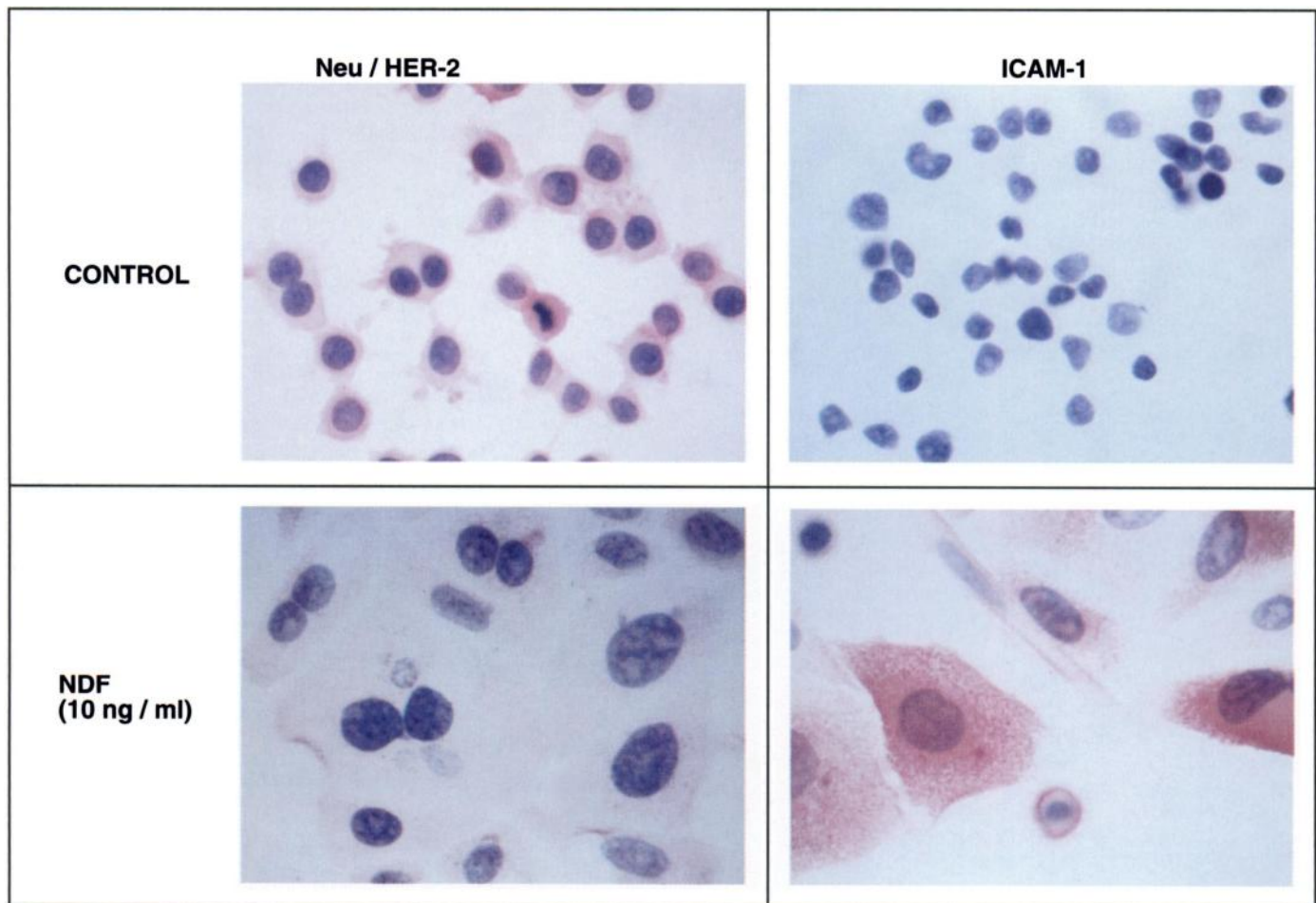

Fig. 1. NDF-induced changes in Neu and ICAM-1 expression. AU-565 human mammary carcinoma cells were plated in 8-well Lab Tek (Nunc) chamber slides at 1×10^4 cells in 1 ml of medium. After 1 day the cells were treated with NDF (10 ng/ml) or left untreated. Following 4 additional days of incubation the cells were stained with either a mAb to Neu (N24; Ref. 16) or with a mAb to human ICAM-1 (Becton Dickinson, San Jose, CA). Biotinylated goat anti-mouse antibodies or biotinylated rabbit anti-mouse antibodies were used to visualize the stained proteins (*red stain in the photomicrographs*). The magnification used in all panels is ×400.
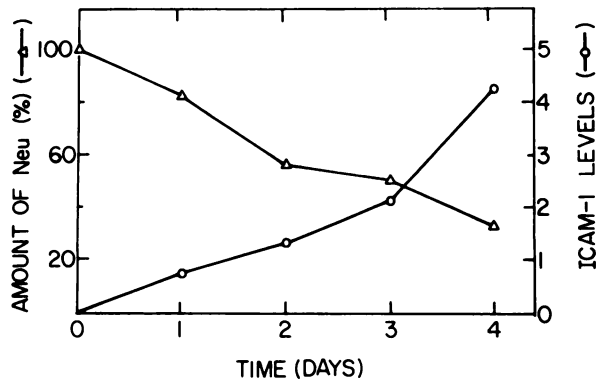
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Fig. 2. Quantitation of the effect of NDF on the expression of ICAM-1 and Neu. AU-565 cells were treated with NDF and stained for Neu ($\triangle$) or ICAM-1 ($\bigcirc$) as described in Fig. 1. Computerized image analysis was used to quantitate the level of each antigen at the indicated time points. Arbitrary units were used to express the level of ICAM-1.

incubation with IFN-γ was followed by simultaneous up-regulation of ICAM-1 and down-regulation of Neu (data not shown and Ref. 41). This behavior was also seen with IFN-γ-treated AU-565, which also underwent growth arrest (Table 1 and Fig. 3). The observation that both NDF and IFN-γ inhibited cell growth and elevated the expression of ICAM-1 raised the possibility that inhibition of cellular proliferation by other treatments will similarly induce the appearance of ICAM-1. However, incubation of cells with tamoxifen, retinoic acid, cytochalasin B, or mitomycin C exerted limited, if any, effect on ICAM-1 expression, although all of these agents inhibited the proliferation of AU-565 cells (Table 1).

The combined effect of NDF and tamoxifen was studied in more detail (Figs. 3 and 4 and Table 1). Tamoxifen alone exerted no effect on ICAM-1, cellular morphology, or disappearance of Neu from the cell surface (Fig. 3). However, it reduced the rate of cell growth, because of a cell cycle arrest at the late G_1 phase (Fig. 4). When cotreated with NDF and tamoxifen, AU-565 cells displayed no induction of polyploidy, and the distribution of their DNA content reflected growth arrest at the late G_1 phase, rather than at late G_2, which is the step that is blocked by NDF (Fig. 4). Taken together, the results obtained with tamoxifen and other growth-inhibitory molecules suggest that up-regulation of ICAM-1 is independent of growth arrest or a specific phase of the cell cycle. This conclusion was further supported by the ability of NDF to induce ICAM-1 in serum-starved cells; incubation of AU-565 cells in 0.5% fetal calf serum for 6 days increased the fraction of ICAM-1-expressing cells from 4% to 14%. However, treatment with NDF resulted in ICAM-1 expression in 85% of the serum-starved cells.

In order to determine the mode of regulation of ICAM-1, total cytoplasmic RNA was extracted from NDF-treated AU-565 cells and from control untreated cells. Slot blot analysis with a DNA probe of the ICAM-1 gene confirmed that the induction of increased expression was at the mRNA level (Fig. 5A). In addition, NDF induced a small decrease in the level of Neu transcripts (Fig. 5A), in agreement with the reduction observed at the protein level (Fig. 2). The induction of ICAM-1 transcripts by NDF was also made evident by using an alternative assay, e.g., histoblotting hybridization (36). In situ hybridization analysis using an ICAM-1 probe showed that IFN-γ exerted a maximal effect on ICAM mRNA, whereas NDF and PMA induced smaller stimulatory effects (Fig. 5B).

ICAM-1 Induction Involves the Action of Protein Kinase C. It has been previously shown that PMA, an activator of protein kinase C, is a potent inducer of differentiation of cultured mammary tumor cells (32). Treatment with PMA strongly induced the synthesis of the ICAM-1 protein (Table 1 and Fig. 6) and mRNA (Fig. 5B). However, the effect of PMA exhibited kinetics different from the responses that were induced by NDF and by IFN-γ; whereas the peptide factors induced a gradual increase in ICAM-1 level over a period of 3 days, the effect of PMA peaked after 1 day, with 78% of the cells staining positively for ICAM-1, and declined in a time-dependent manner (Fig. 6). It is possible that PMA down-regulates its receptor, e.g., protein kinase C, thus preventing further induction of the cell adhesion molecule. Indeed, continuous treatment of AU-565 cells for 7 days with NDF resulted in 84% of the cells staining positively for ICAM-1, whereas only 10% of PMA-treated cells were positive after 7 days. However, when the cells were first treated with 20 nM PMA for 3 days and NDF (10 ng/ml) was added for an additional period of 3 days, only 20% of the cells exhibited ICAM-1 expression. On the other hand, when PMA was removed before adding NDF, up to 70% of the cells stained positively with antibodies to ICAM-1. It thus appears that down-regulation of protein kinase C by sustained stimulation with PMA prevents the effect of NDF on ICAM-1 and suggests that this protein kinase mediates the action of NDF. To further test this possibility, we attempted to inhibit protein kinase C by using calphostin C (42). The inhibitor itself had no effect on ICAM-1 synthesis, but at 1 nM concentration it partially blocked the effect of both PMA and NDF on the expression of ICAM-1 (Table 2). Based on the induction of ICAM-1 by PMA, its specific inhibition by an inhibitor of protein kinase C, and the absence of ICAM-1 induction in protein kinase C-depleted cells, it was concluded that the elevated expression of ICAM-1 by NDF is probably mediated, at least in part, by protein kinase C.

Table 1 *Cellular and differentiation parameters of factor-treated AU-565 cells*

Cultures of human mammary carcinoma cells, AU-565, were treated for 4 days with the indicated agents. The numbers of cells were then determined in a hemocytometer and the cells were stained with antibodies specific to lactalbumin, Neu, or ICAM-1. Lipid visualization was carried out according to the Oil Red O in propylene glycol method (32). DNA quantitation was performed on cells stained by the Feulgen method. Nuclear area and quantitation of immunostaining was determined by using an image analysis system.

| Treatment of AU-565 cells	Concentration	ICAM-1	Cell no. ($10^4/cm^2$)	Cell stained for lipids (%)	Lactalbumin levels	Neu levels	Nuclear area (μ^2)	Average DNA (pg/cell)
None		1	6.2	15	1	1	79	18.3
NDF	10 ng/ml	2.9	3.6	75	2.8	1.2	191	26.01
Prolactin	10 μg/ml	1.2	7.0	20	1.8	0.7	78	18.08
Estrogen	10 nM	0.3	8.2	15	1.2	0.5	115	18.07
Tamoxifen	1 μM	1	4.7	41	1.7	1.1	124	17.80
Prolactin + NDF	10 μg/ml + 10 ng/ml	1.8	6.4	40	2.1	1.3	121	23.73
Tamoxifen + NDF	1 μM + 10 ng/ml	3.1	2.3	80	2.8	2.4	207	17.98
Estrogen + NDF	1 nM + 10 ng/ml	2	4.8	70	3.4	1.4	127	22.29
γ-Interferon	800 u/ml	3	3.6	>90	1	1.2	119	23.00
Retinoic acid	2.5 μM	1.3	3.1	80	4	1.1	188	17.13
Phorbol ester (PMA)	1.6 nM	2	2.8	>90	1.7	1.2	220	17.21
EGF	30 nM	2	2.5	60	1.8	2.5	180	17.36
Cytochalasin	0.125 μg/ml	1.1	2.5	34	0.6	1.5	121	25.24
Mitomycin C	50 ng/ml	1.7	1.7	30	1	2.8	124	35.45
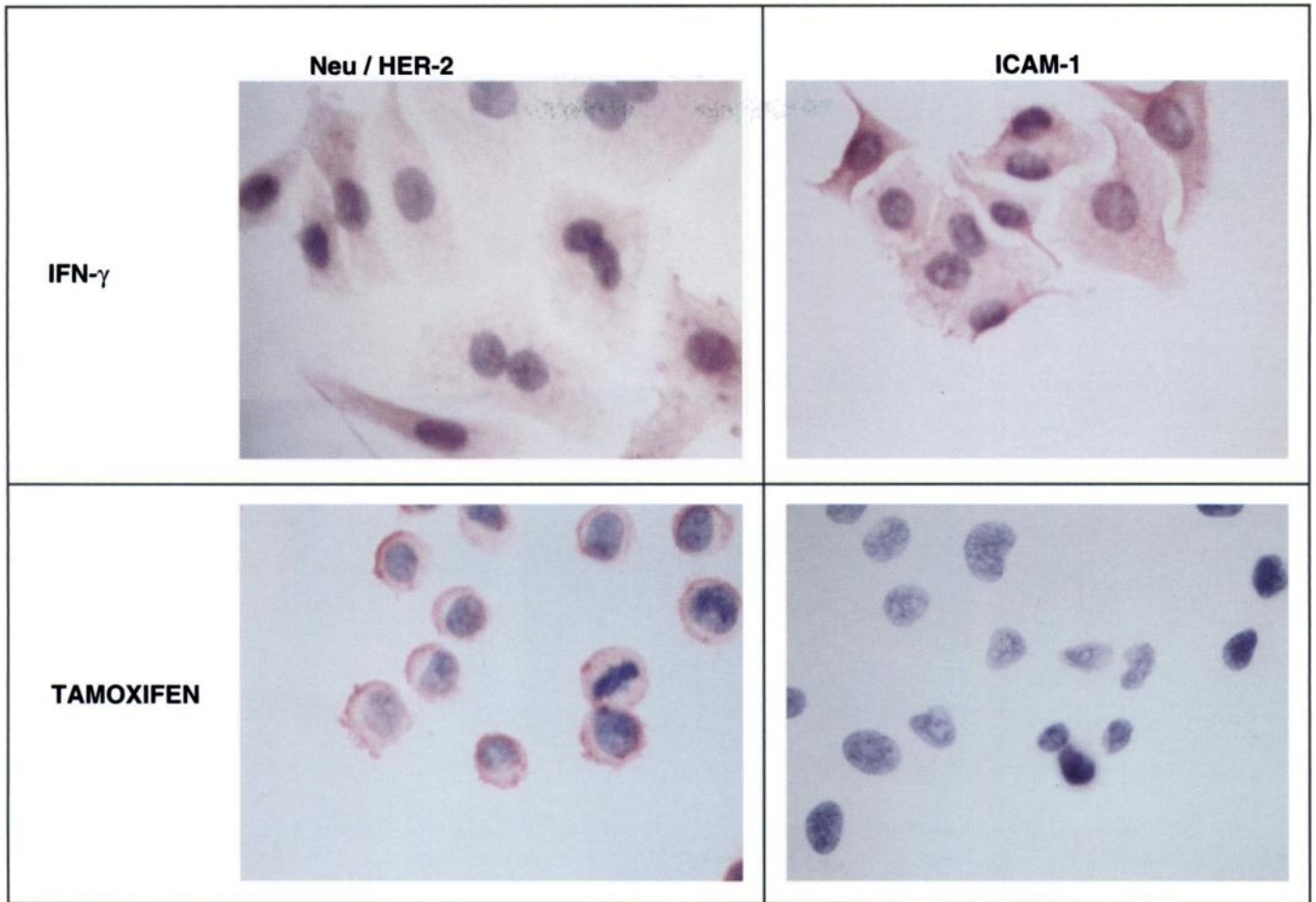
Fig. 3. The effects of IFN-γ and tamoxifen on the expression of Neu and ICAM-1. Sparse cultures of AU-565 cells were treated with either IFN-γ (800 units/ml) or with tamoxifen (1 μM). Following 4 days of incubation the cells were immunostained with mAbs to Neu or to ICAM-1 as described under "Materials and Methods" and in Fig. 1. The magnification used is ×400, and the same magnification is shown for control and treated cells.

Coexpression of Neu and ICAM-1 in Human Mammary Tumors Characterizes Invasive Ductal Carcinomas with a Prominent CIS Component and Correlates with the Absence of Nodal Metastases. The observation that ICAM-1 expression in cultured mammary tumor cells can undergo up-regulation by NDF in a manner that involves down-regulation of Neu raised the possibility that coexpression of the two receptors may have implications for tumor biology. Elevated expression of ICAM-1 may increase the susceptibility of a tumor to the immune system, whereas down-regulation of Neu may reduce the tumorigenic potential of certain adenocarcinoma cells (43). In order to examine these possibilities we used a bank of breast tumor specimens that were collected in one of our laboratories over the past 3 years. All of the tumor samples were obtained either by needle biopsy or by surgical tumor excision, snap frozen, and stored at −70°C.

The expression of NDF was analyzed in a series of 17 tumor specimens by polymerase chain reaction. RNA was extracted from the specimens and used in combination with reverse transcriptase, a thermostable DNA polymerase, and a pair of primers to amplify an approximately 300-base pair-long DNA segment of the NDF transcript. Detectable amplified DNA was found in eight specimens (Table 3). Interestingly, no lymph node metastasis was observed in seven of the corresponding patients, but most of the NDF-positive tumors contained a prominent CIS component and expressed ICAM-1. However, the level of expression of Neu in the NDF-positive tumors displayed wide variation (Table 3). It is worthwhile to mention that high expression of NDF has been previously reorted in several human tumor
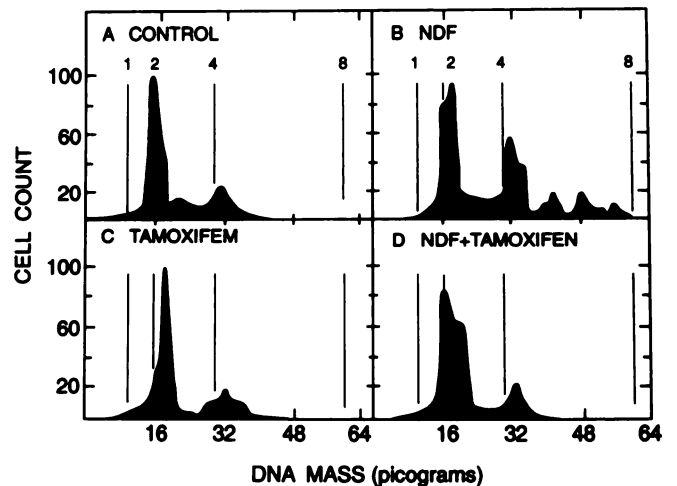

Fig. 4. Distribution of DNA content in cells treated with NDF, tamoxifen, or with their combination. Cultures of AU-565 breast tumor cells were incubated for 4 days with either NDF (10 ng/ml) (B), tamoxifen (1 μM) (C), or a combination of NDF and tamoxifen (D) or were left untreated (A). DNA content in individual cells was then determined by using the Feulgen reaction and a DNA staining kit. Quantitation was performed with a CAS 200 image analyzer. The numbers at the top of each vertical line represent the DNA index. The index was calculated by dividing the amount of cellular DNA in each cell that was examined by the content in a reference human diploid cell at the G_0-G_1 stage of the cell cycle.
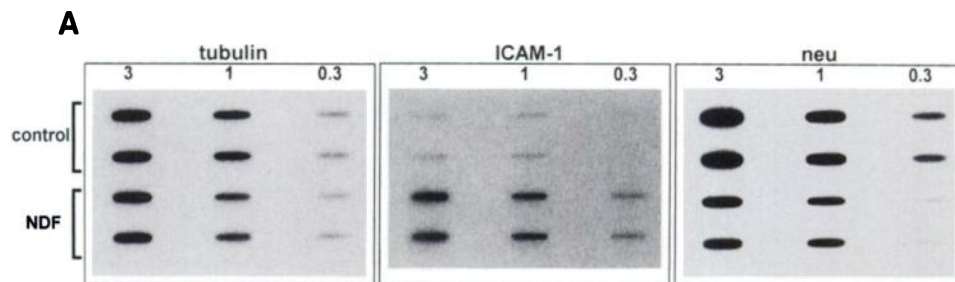
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Fig. 5. Changes in mRNA levels of ICAM-1. AU-565 cells were treated with NDF (10 ng/ml), IFN-γ (800 units/ml), or PMA (1.6 nM) or were left untreated, as indicated. Following 4 days of incubation the amount of ICAM-1 mRNA was determined by RNA extraction and slot blot analysis (A) or by transfer of the cells to nitrocellulose filters followed by *in situ* hybridization (B). Denatured ^{32}P-labeled DNA probes of human ICAM-1 and *neu*/HER-2 were used in parallel with a tubulin-specific probe (for control). The amounts of RNA (in micrograms) that were applied to the slot blots are indicated in A. Hybridization signals that were obtained in the *in situ* analysis (B) were quantitated, and their relative intensities after subtraction of the background radioactivity are shown.
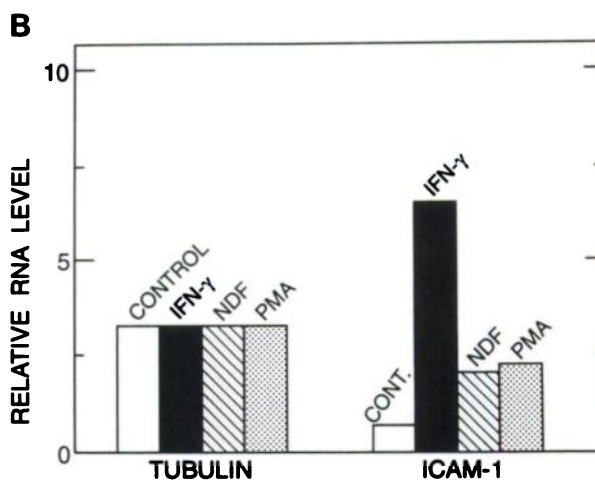
cell lines, and it may be correlated with transformation by the *ras* oncogene (21). Independent of the exact mechanism of transcriptional activation of NDF in the mammary tumors, the data presented in Table 3 suggest that some, but not all, breast tumors express the factor, and this may be correlated with the absence of metastasis to the lymph nodes.

We next concentrated on tumors that stained positively with antibodies to Neu/HER-2 and analyzed their level of expression of ICAM-1 by using immunohistochemical staining. Histologically the majority of ICAM-1-expressing tumors displayed features of invasive ductal carcinoma that are characterized by a prominent component of CIS. These morphological features included large cells, abundant

Table 2 *Inhibition of ICAM-1 induction by calphostin C*

The expression of ICAM-1 in AU-565 cells was determined by immunohistochemical staining and image analysis. Sparse monolayers of cells were treated for 3 days with the indicated agents or their combinations. The experiment was repeated three times.

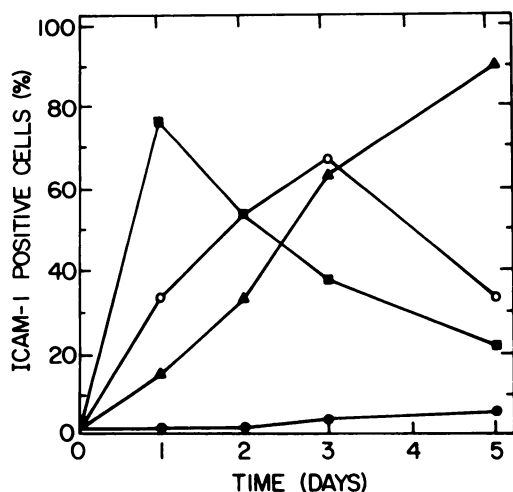| Treatment of AU-565 cells	ICAM-1-positive cells (%)
Control	2–5
Calphostin C (1 nM)	2–4
PMA (1.6 nM)	74
PMA + calphostin C	25
NDF (10 ng/ml)	79
NDF + calphostin C	37

Fig. 6. Kinetics of ICAM-1 induction. The numbers of ICAM-1-positive cells were determined by immunostaining of AU-565 cell cultures that were treated for 1–5 days with NDF (10 ng/ml; ▲), PMA (1.6 nM; ■), or IFN-γ (800 units/ml; ○) or were left untreated (●). The numbers of ICAM-1-positive cells were determined as the averages obtained from 10 microscope fields, and they are expressed as fractions of the averages of the total number of cells in a field.

Table 3 *NDF expression in mammary tumors*

The expression of NDF in 17 human breast tumors was analyzed by using PCR. The level of expression of Neu in each tumor was determined histochemically and quantitatively analyzed by using an image analyzer. Values lower than 0.1 were considered negative. The number of lymph nodes that showed metastasis are given together with the total number of lymph nodes that were examined in each patient. Also indicated are the expression of ICAM-1, which was determined histochemically, and the presence of a prominent CIS component.

| Tumor	NDF	Neu	Positive lymph nodes	ICAM-1	Morphology
1	–	+	0/12	+	Infiltrating + CIS
2	–	–	0/6	–	Infiltrating
3	–	–	?	–	Infiltrating
4	–	+	0/12	+	Infiltrating + CIS
5	–	+	6/30	–	Infiltrating
6	+	–	0/8	+	CIS + acinar formation
7	–	+	T2/N1	+	Infiltrating
8	+	+	0/14	+	CIS + acinar formation
9	+	+	0/7	+	CIS
10	+	–	0/8	+	CIS + acinar formation
11	+	–	0/11	+	Acinar formation
12	+	+	0/16	+	Infiltrating + CIS
13	–	+	0/7	+	Infiltrating + CIS
14	–	–	0/17	–	Infiltrating + CIS
15	+	–	0/17	–	Infiltrating
16	–	–	3/16	–	Infiltrating
17	+	–	16/23	+	CIS + acinar formation

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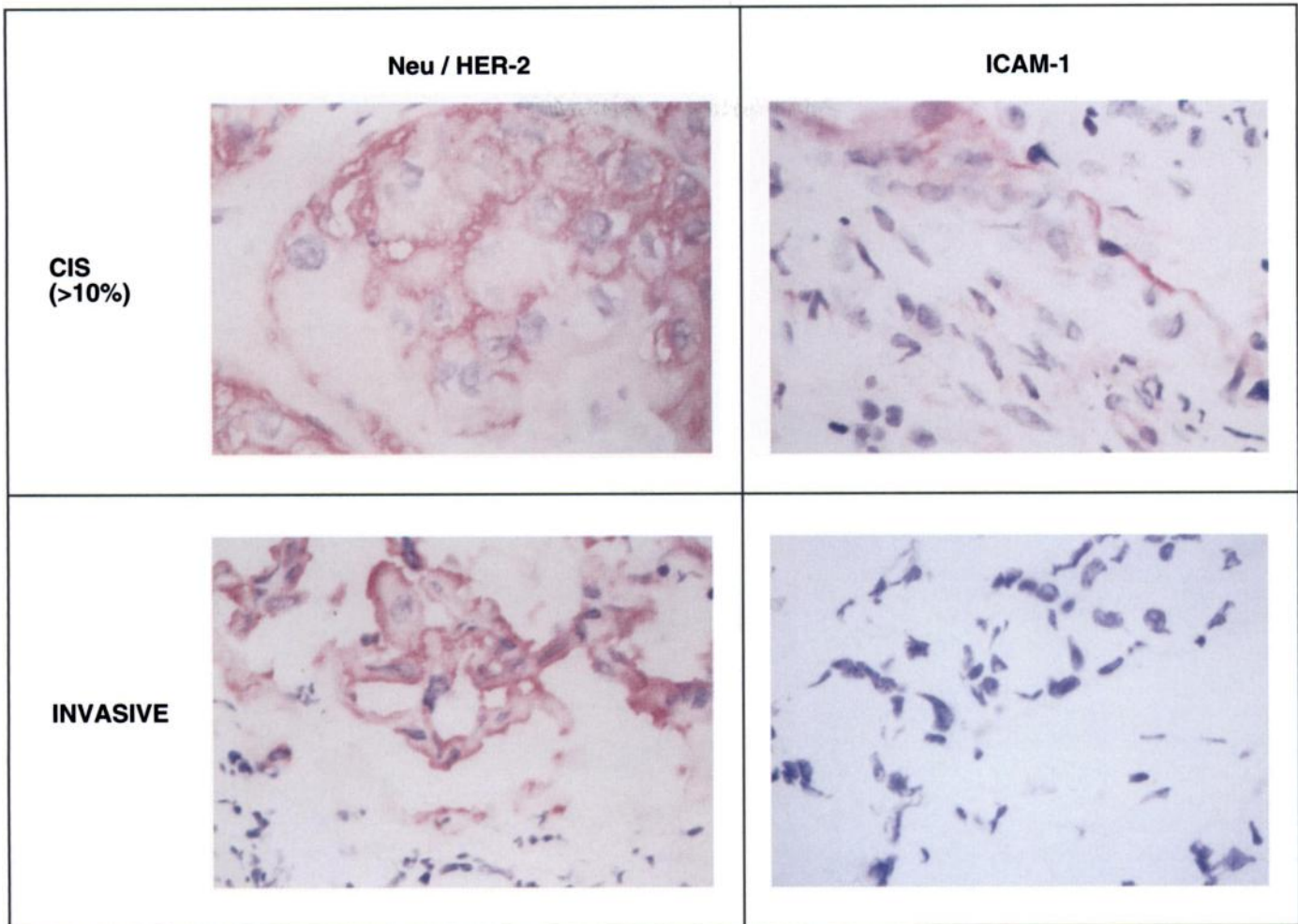
Fig. 7. Immunohistochemical staining of thin sections of two mammary carcinomas. Monoclonal antibodies to Neu and ICAM-1 were used to visualize the corresponding proteins (*red stain in the micrographs*). *Top two panels*, sections of an ICAM-1-positive invasive ductal carcinoma that contained a prominent *in situ* component. The sectioned tissue was a small (<1 cm) T_1N_0 tumor. The photomicrographs depict only the *in situ* component. Note the apical staining of ICAM-1. *Bottom two panels*, an ICAM-1-negative invasive ductal carcinoma of stage T_4N_1 that contained no *in situ* component. Magnification, ×400.

mitotic figures, and in most tumors prominent central necrosis that characterizes the comedo type of CIS (Fig. 7). Both the invasive and the *in situ* components displayed expression of Neu and ICAM-1 and may therefore share a common origin. In addition, the identified subset of tumors displayed desmoplastic reaction, apical ductal localization of ICAM-1, and some lymphoplasmocytic infiltration (Fig. 7). The infiltrating cells appeared to be helper T-cells, since they stained positively with antibodies to CD4 but not with antibodies to CD8 (data not shown). In contrast with the ICAM-1-expressing tumors, the majority of Neu-positive tumor specimens that showed no expression of ICAM-1 displayed morphological characteristics of pure invasive ductal carcinoma with minimal, if any, CIS component (Fig. 7). Consistent with this observation, ICAM-1-negative tumors displayed a proliferation index that was higher (21 versus 15) than ICAM-1-positive tumors when analyzed with antibodies to Ki-67. The marked differences in ICAM-1 immunostaining were also reflected in the corresponding mRNA levels (Fig. 8), indicating that transcriptional control of ICAM-1 expression is involved.

The distinct morphological characteristics of the ICAM-1-positive and ICAM-1-negative tumors were also preserved *in vitro*. Primary cultures of ICAM-1-negative tumors formed sparse monolayers with no distinct cellular pattern (Fig. 9). By contrast, cells from tumors that coexpressed Neu and ICAM-1 formed dense three-dimensional clusters when cultured *in vitro* (Fig. 9). The latter formed domelike structures, the cores of which were empty of living cells (Fig. 9). This pattern is reminiscent of the necrotic centers of the parental invasive carcinoma that contained a comedo-type *in situ* component.

Table 4 summarizes the analysis of ICAM-1 expression in all of the Neu-positive specimens of mammary invasive ductal carcinoma ($n = 39$). Statistical analysis indicated that the association between the status of ICAM-1 expression and the histological type of a tumor was highly significant ($P < 0.001$; Fisher's exact test). This correlation suggests that the CIS-rich tumors that coexpress Neu and ICAM-1 may share with pure CIS low invasiveness and metastasis. To test this predication we analyzed clinical data that were available for 34 of the 39 patients. Indeed, lack of expression of ICAM-1 in Neu-positive tumors was found to be a statistically reliable ($P = 0.002$) predictor of metastasis to adjacent lymph nodes (Table 4). This correlation was independent of the histological type of the tumor, as shown by both the Mantel-Haenszel test and by a logistic model. Both tests yielded an odds ratio of 5 ($P = 0.05$).

DISCUSSION

Aimed at characterization of the NDF-induced mature phenotype of mammary tumor cells, the present study identified the cell adhesion molecule ICAM-1 as a component of the *in vitro* differentiation-like process. Since we previously correlated the induction of differentiation by certain monoclonal antibodies to Neu with a tumor-inhibitory function (17), the present study also attempted to relate the induction
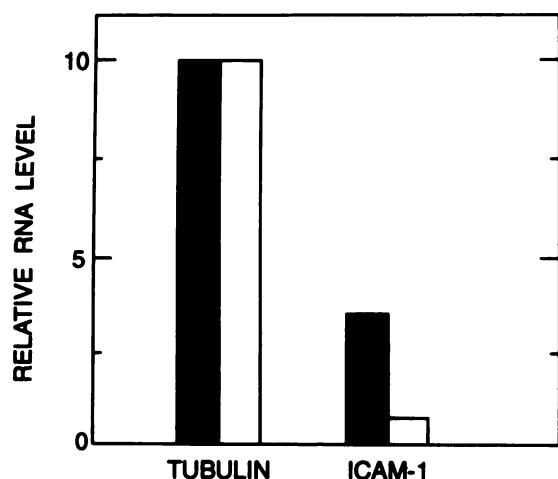
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Fig. 8. Histoblotting *in situ* analysis of mRNA levels of ICAM-1 in two mammary tumors. *In situ* hybridization of an ICAM-1 DNA probe was used to compare the levels of the corresponding mRNA molecule in two tumor specimens. Both sections were prepared from Neu-expressing invasive ductal carcinomas that contained either a large *in situ* component (■) or no CIS fraction (□). The latter tumor metastasized to 6 out of 16 lymph nodes. A DNA probe of tubulin was similarly used to allow quantitative comparison of the tumors. The background hybridization of each probe to a nitrocellulose filter was separately subtracted from the signals that were obtained.

of ICAM-1 to tumor development. Like other polypeptide growth factors, NDF exerts pleiotropic cellular effects that may depend on the exact cellular context. For example, *in situ* hybridization analysis indicated that the factor is synthesized predominantly in the central and peripheral nervous systems (22), where it participates in the formation of the neuromuscular junction (44). In addition to inducing elevated expression of the acetylcholine receptor by the postsynaptic muscle, the neuronal factor, called ARIA, also increases the number of voltage-gated sodium channels (45). However, NDF, as well as some of its alternatively spliced forms, is not only a differentiation factor but also a mitogen for other cells such as Schwann cells (46) and certain mammary tumor cell lines (Ref. 24 and our unpublished observations). It thus appears that the induction of ICAM-1 by NDF is only one of a series of cell-specific biological and biochemical effects of the pleiotropic factor. This property is not unique to NDF, since ICAM-1 expression is regulated in a wide variety of cells by inflammatory cytokines including interleukin 1, IFN-γ, and tumor necrosis factor α, as well as by retinoic acid (30, 47). Although these agents, like NDF, induce the growth arrest of many different cells, our results with tamoxifen and other cytostatic compounds (Fig. 3 and Table 1) indicated that the induction of ICAM-1 in AU-565 mammary cells is not simply an outcome of inhibition of cell proliferation.

The mechanism of induction of ICAM-1 in AU-565 cells appears to involve at least two protein kinases. These are a receptor tyrosine kinase, and the Ca^{2+}- and phospholipid-dependent protein kinase C. Although NDF elevated the tyrosine phosphorylation of Neu (20), the tyrosine kinase that undergoes activation is not necessarily that of Neu. Based on a cell type-specific dependency of interaction between NDF and target cells, we concluded that a still unknown receptor, or a coreceptor, mediates the interaction of NDF with Neu and the consequent biochemical effects (23). These effects include the induction of ICAM-1 since NDF, unlike IFN-γ, does not affect expression of the cell adhesion molecule in ovarian cells that overexpress Neu, probably because of the absence of the still unknown receptor. Predictably, the putative receptor is a member of the EGF-receptor family, which includes at least four receptor tyrosine kinases (48) that may form heterodimeric complexes (49). Codimerization of Neu with the putative receptor of its family presumably establishes high-affinity binding sites for NDF and enables the extensive down-regulation of

Neu that occurs simultaneously with elevated expression of ICAM-1 (Fig. 2). The observation that PMA, an agonist of protein kinase C, induces ICAM-1 expression (Table 1) implicates this kinase in the underlying biochemical pathway. However, the kinetics of the effects of PMA and NDF on ICAM-1 differ significantly (Fig. 6); PMA induces a transient elevation, whereas the effect of NDF increases slowly and persists for a longer time. Potentially, this pattern of NDF action could suggest a late-acting effect of the factor that is mediated by the inactivation of protein kinase C, rather than by its stimulation at long (>24 h) time periods. Indeed, studies of IFN-γ action on a human glioma cell line implicated such a mechanism in the induction of ICAM-1 (50). In AU-565 cells, however, prolonged inactivation of protein kinase C led to the inhibition of the effect of NDF on ICAM-1, as did a specific inhibitor of the kinase (Table 2). Possibly, rapid stimulation of protein kinase C is necessary, but late processes are also crucial for NDF action. It is worth noting that Neu-mediated activation of protein kinase C is probably due to rapid tyrosine phosphorylation and stimulation of phospholipase-Cγ (51), which generates an activator of the serine and threonine kinase.

Besides understanding basic cellular phenomena, the discovery of ligands that affect the function of Neu (20, 21, 24, 44, 46) may have clinical implications. Extensive analysis of human tumor specimens has established a prognostic value of overexpression of Neu in certain adenocarcinomas (reviewed in Ref. 52). However, the cellular and biochemical bases of this correlation have not been addressed directly, because of the inability to activate Neu by a specific ligand. The availability of NDF may open the way for such studies. With regard to breast cancer, a major open issue is the significance of the remarkably wide occurrence of overexpression of Neu in ductal CIS (up to 65% of tumors), which is in contrast to its moderate distribution in infiltrating ductal carcinomas (20–25% of all tumors; Refs. 7, 53–56). Invasive carcinomas that include an *in situ* histological component also tend to show Neu overexpression more frequently than pure infiltrating carcinomas (57). By analysing a relatively small ($n = 39$) series of tumors the referral pattern of which may be subject to a selection bias, we found that the expression of ICAM-1 in Neu-positive tumors correlates not only with the presence of a prominent *in situ* component but also with a statistically significant reduction in metastasis to lymph nodes (Table 4). In contrast, patients with tumors that overexpress Neu but do not express ICAM-1 frequently show lymph node involvement. Presumably, expression of ICAM-1 is associated with a restriction of the ability of a tumor to invade the lymphatic system and may thus behave as a protecting factor. The mechanism of protection could involve the recruitment of cytotoxic T-cells and macrophages. Such a mechanism was demonstrated with IFN-γ-treated ovarian cancer cells that lost their resistance to lymphokine-activated killer cell cytotoxicity as a result of overexpression of ICAM-1 (58). Another mechanism that could be involved in the

Table 4 *Histology and lymph node involvement of mammary tumors in relation to the expression of ICAM-1*

Specimens of Neu-positive invasive ductal carcinomas were analyzed for the presence of ICAM-1 and grouped histologically on the basis of the presence of an *in situ* component. The association between ICAM-1 status and histological type was determined by the Fisher's exact test (*P* values are indicated on the bottom line). Data regarding nodal involvement in metastatic disease were available for 34 of 39 patients. The association between ICAM-1 status and the nodal status was similarly determined.

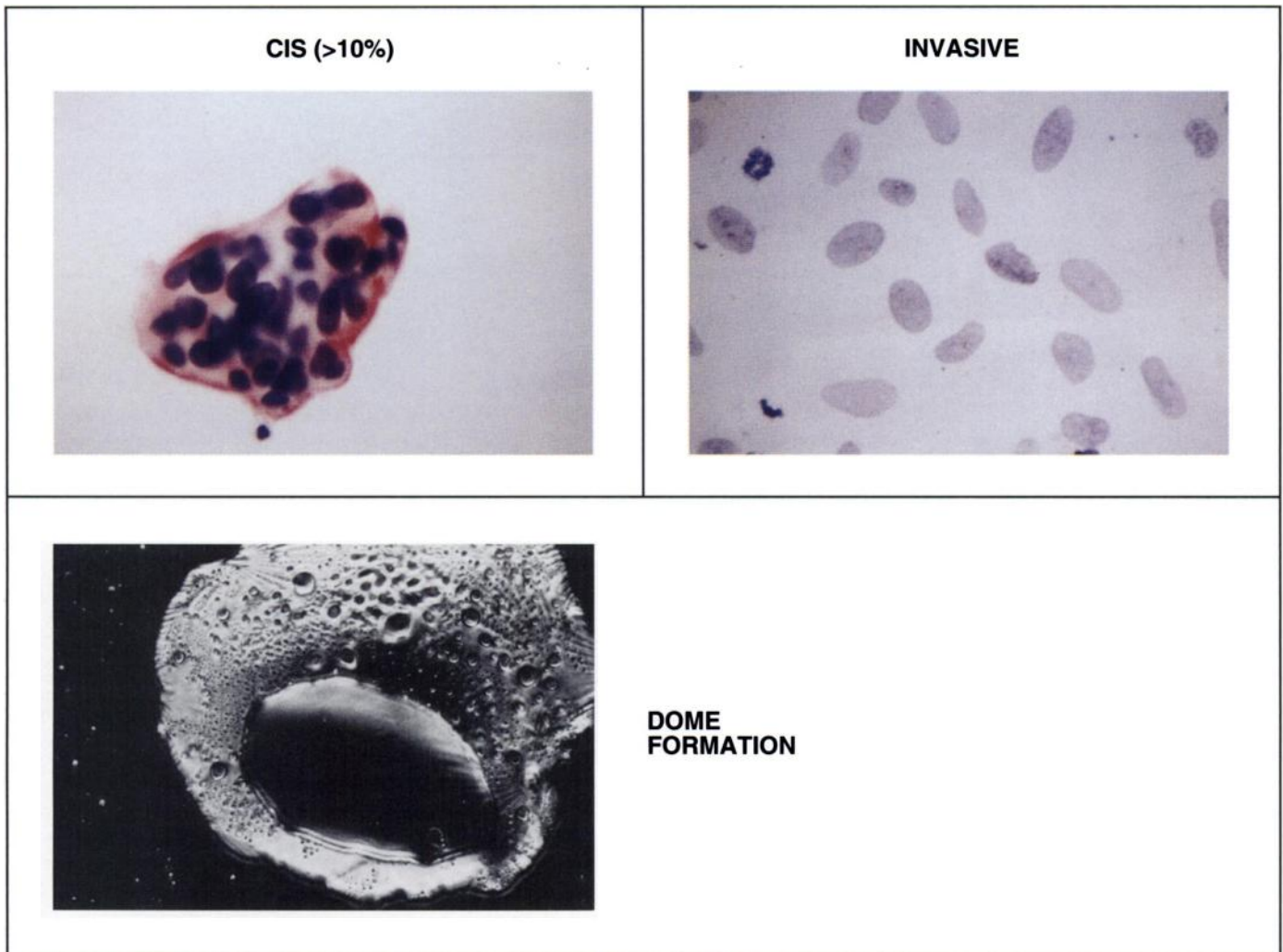|	Histology (39 patients)		Metastasis to lymph nodes (34 patients)	
	CIS > 10%	CIS ≤ 10%	Negative	Positive
ICAM-1 positive	9	6	11	3
ICAM-1 negative	2	22	5	15
Total	11	28	16	18
	$P < 0.001$		$P = 0.002$	

TISSUE CULTURE

Fig. 9. Primary cell cultures of Neu-positive invasive ductal carcinomas. Fresh specimens of two infiltrating carcinomas were dissociated, and their cells were cultured *in vitro*. *Top left*, Neu staining of cells from an infiltrating carcinoma that contained a prominent *in situ* component (*CIS*); *top right*, Neu staining of cells that were derived from a pure invasive ductal carcinoma (*INVASIVE*). Magnification, ×400. Note that the CIS-rich invasive carcinoma formed ICAM-1-positive three-dimensional structures. *Bottom*, the appearance of the latter culture as viewed by inverted phase microscopy (*DOME FORMATION*). Pictures of the primary cultures were taken at 450-fold magnification on an American Optical Series 1820 Biostar inverted scope. A Reichert Phostostar System was attached to the inverted scope's camera.

protection effect is the reduction of Neu expression that occurs in NDF-treated mammary cells *in vitro* (Fig. 2). Down-regulation of Neu was shown to reduce the rate of cellular proliferation and tumorigenicity in several cellular systems (14, 43, 59), whereas its overexpression induces resistance to tumor necrosis factor and to cytotoxic macrophages (60). It is also possible that overexpression of ICAM-1 directly leads to the down-regulation of Neu. This can be tested by transfection of the ICAM-1 gene into Neu-expressing mammary cells. Independent of the exact mechanism, the presence of ICAM-1 on a Neu-positive tumor apparently does not prevent invasiveness, and thereby it probably exposes the tumor cells to infiltrating lymphocytes, which often surround ICAM-1- and Neu-positive tumors.

It is relevant to consider the question of whether or not NDF is the factor that determines the level of expression of ICAM-1 in breast tumors. Although our limited analysis of NDF expression in mammary tumors (Table 3) is consistent with the possibility that different breast tumors may differ in the level of expression of the factor, more extensive analysis will be required to relate it to histological and clinical aspects of the disease. Potentially, other polypeptide factors, such as IFN-γ and tumor necrosis factor, may also up-regulate ICAM-1 expression. At least in the case of IFN-γ a concomitant

down-regulation of surface Neu is expected. Nevertheless, the absence of correlation between ICAM-1 expression and lymph node involvement in Neu-negative tumors (our unpublished results) supports the possibility that Neu and NDF affect the expression of ICAM-1 *in vivo*. Considering this possibility, it is worthwhile to examine the question of why most CIS tumors overexpress Neu while Neu-overexpressing infiltrating tumors account for only approximately 20% of all invasive mammary carcinomas. One possibility attributes the "dilution effect" to changes that reduce the level of expression of Neu during the progression of an *in situ* carcinoma to an invasive carcinoma (61). Accordingly, Neu is important in the initiation of an *in situ* carcinoma, perhaps because it accelerates the rate of cellular proliferation. However, tumors that express Neu may be more susceptible to ICAM-1 induction by NDF and, thereby, will be more accessible to the host immune system before they reach the lymph nodes. By contrast, tumors that do not overexpress Neu or have gradually lost the expression of this receptor rapidly transit through the CIS state, infiltrate, and metastasize into the lymph nodes. The third group consists of tumors that overexpress Neu in the absence of ICAM-1. These malignancies are expected to rapidly metastasize into the lymph nodes, because they lack the protecting effect of the cell adhesion molecule.

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Moreover, the presence of an overexpressed Neu may confer on them an accelerated rate of cellular proliferation, so that this type of tumor is predicted to be the most aggressive of the three groups.

While this model may explain the "dilution" of Neu-positive infiltrating tumors and the correlation between poor prognosis and Neu overexpression in patients with axillary node involvement, its validity should be examined by using larger tumor banks and appropriate experimental model systems. In addition, the relationships between the expression of ICAM-1, NDF, and Neu should be compared with other prognostic factors, including steroid hormone receptors, tumor size, multicentrity, and cellularity. Other open questions relate to the identity of the putative receptor that participates in NDF binding and its expression in various mammary tumors. Despite the unanswered issues, the present work is expected to be followed by additional studies that will try to approach the functions of Neu *in vitro* and *in vivo* and will correlate them with clinical observations.

ACKNOWLEDGMENTS

REFERENCES

1. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell, *45:* 649–657, 1986.
2. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. Similarity of protein encoded by the human c-*erb*B-2 gene to epidermal growth factor receptor. Nature (Lond.), *319:* 230–234, 1986.
3. Coussens, L., Yang, F. T., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science (Washington DC), *230:* 1132–1139, 1985.
4. Di Fiore, P. P., Pierce, J., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science (Washington DC), *237:* 178–182, 1987.
5. Hudziak, R. M., Schlessinger, J., and Ullrich, A. Increased expression of the putative growth factor receptor p185HER-2 causes transformation and tumorigenesis of NIH-3T3. Proc. Natl. Acad. Sci. USA, *84:* 7159–7163, 1987.
6. Suda, Y., Aizawa, S., Furuta, Y., Yagi, T., Ikawa, Y., Saitoh, K., Yamada, Y., Toyoshima, K., and Yamamoto, T. Induction of a variety of tumors by c-*erb*B2 and clonal nature of lymphomas even with the mutated gene val659-Glu659. EMBO J., *9:* 181–190, 1990.
7. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science (Washington DC), *235:* 177–182, 1987.
8. van de Vijver, M., van de Bersselaar, R., Devilee, P., Cornelisse, C., Peterse, J., and Nusse, R. Amplification of the *neu* (c-*erb*B-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erb*A oncogene. Mol. Cell. Biol., *7:* 2019–2023, 1987.
9. Varley, J. M., Swallow, J. E., Brammar, W. J., Whittaker, J. L., and Walker, R. A. Alterations to either c-*erb*B-2(neu) or c-*myc* proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. Oncogene, *1:* 423–430, 1987.
10. Venter, D. J., Tsui, N. L., Kummar, S., and Gullick, W. J. Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: Immunohistological assessment correlated with gene amplification. Lancet, *ii:* 69: 72, 1987.
11. Berger, M. S., Greenfield, C., Gullick, W. J., Haley, J., Downward, J., Neal, D. E., Harris, A. L., and Waterfield, M. D. Evaluation of epidermal growth factor receptors in bladder tumours. Br. J. Cancer, *56:* 533–537, 1987.
12. Zhou, D., Battifora, H., Yokota, J., Yamamoto, T., and Cline, M. J. Association of multiple copies of the c-*erb*B-2 oncogenes with spread of breast cancer. Cancer Res., *47:* 6123–6125, 1987.
13. McKenzie, S. J., Marks, P. J., Lam, T., Morgan, J., Panicali, D. L., Trimpe, K. L., and Carney, W. P. Generation and characterization of monoclonal antibodies specific for the human *neu* oncogene product, p185. Oncogene, *4:* 543–548, 1989.
14. Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M., and Ullrich, A. p185HER2 monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol., *9:* 1165–1172, 1989.
15. Tagliabue, E., Centis, F., Campiglio, M., Mastroianni, A., Martignone, S., Pellegrini, R., Casalini, P., Lanzi, C., Menard, S., and Colnaghi, M. I. Selection of monoclonal antibodies which induce internalization and phosphorylation of p185HER2 and growth inhibition of cells with HER2/NEU gene amplification. Int. J. Cancer, *47:* 933–937, 1991.
16. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y., and Sela, M. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc. Natl. Acad. Sci. USA, *88:* 8691–8695, 1991.
17. Bacus, S. S., Stancovski, I., Huberman, E., Chin, D., Hurwitz, E., Mills, G. B., Ullrich, A., Sela, M., and Yarden, Y. Tumor-inhibitory monoclonal antibodies to the HER-2/Neu receptor induce differentiation of human breast cancer cells. Cancer Res., *52:* 2580–2589, 1992.
18. Yarden, Y., and Weinberg, R. A. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA, *86:* 3179–3183, 1989.
19. Yarden, Y., and Peles, E. Biochemical analysis of the ligand for the *neu* oncogenic receptor. Biochemistry, *30:* 3543–3550, 1991.
20. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben-Levy, R., and Yarden, Y. Isolation of the *neu*/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell, *69:* 205–216, 1992.
21. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Luo, Y., and Yarden, Y. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell, *69:* 559–572, 1992.
22. Orr-Urtreger, A., Trakhtenbrot, L., Ben-Levi, R., Wen, D., Rechavi, G., Lonai, P., and Yarden, Y. Neural expression and chromosomal mapping of Neu diferentiation factor to 8p12-p21. Proc. Natl. Acad. Sci. USA, *1952:* 1746–1750, 1993.
23. Peles, E., Ben-Levy, R., Tzahar, E., Naili, L., Wen, D., and Yarden, Y. Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2. EMBO J., *12:* 961–971, 1993.
24. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, M., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. Identification of heregulin, a specific activator of p185*erb*B2. Science (Washington DC), *256:* 1205–1210, 1992.
25. Simmons, D., Makgova, M. W., and Seed, B. ICAM, an adhesion ligand of LFA-1 is homologous to the neural cell adhesion moleule NCAM. Nature (Lond.), *331:* 624–627, 1988.
26. Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell, *52:* 925–933, 1988.
27. Barton, R. W., Rothlein, R., Ksiazek, J., and Kennedy, C. The effect of anti-intercellular adhesion molecule-1 on phorbol-ester-induced rabbit lung inflammation. J. Immunol., *143:* 1278–1282, 1989.
28. Johnson, J. P., Stade, B. G., Holzmann, B., Schwable, W., and Riethmuller, G. *De novo* expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc. Natl. Acad. Sci. USA, *86:* 641–644, 1989.
29. Makgova, M. W., Sanders, M. E., Luce, G. E. G., Dustin, M. L., Springer, T. A., Clark, E. A., Manhoni, P., and Shaw, S. ICAM-1 a ligand for LFA-1 dependent adhesion of B, T and myeloid cells. Nature (Lond.), *331:* 86–88, 1988.
30. Rothlein, R., Czajkowski, M., O'Neil, M. M., Martin, S. D., Mamolf, E., and Merluzzi, V. J. Induction of intracellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. J. Immunol., *141:* 1665–1669, 1988.
31. Vogetseder, W., Feichtinger, H., Schulz, T. F., Schwaeble, W., Tabaczewski, P., Mitterer, M., Bock, G., Marth, C., Dapunt, O., Mikuz, G., and Dierch, M. P. Expression of 7F7-antigen, a human adhesion molecule identical to intercellular adhesion molecule-1 (ICAM-1) in human carcinomas and their stromal fibroblasts. Int. J. Cancer, *43:* 763–773, 1989.
32. Bacus, S. S., Kiguchi, K., Chin, D., King, C. R., and Huberman, E. Differentiation of cultured human breast cancer cells (AU-565 and MCF-7) associated with loss of cell surface HER-2/*neu* antigen. Mol. Carcinog., *3:* 350–362, 1990.
33. Kischer, C. A., Leibovitz, A., and Pindur, J. The use of a transport medium (L15M15) for bulk tissue storage and retention of viability. Cytotechnology, *2:* 181–185, 1989.
34. Leibovitz, A. Development of tumor cell lines. Cancer Genet. Cytogenet., *19:* 9–11, 1986.
35. Macklis, D., Sidman, L., and Shine, D. Cross-linked collagen surface for cell culture that is stable, uniform and optically superior to conventional surfaces. In Vitro Cell. Dev. Biol., *21:* 189–194, 1985.
36. Gudkov, A. V., Kashkin, K. N., Zatterskaya, T. E., and Troganovsky, S. M. Histoblotting hybridization *in situ* detection of specific RNAs on tissue sections transferred on nitrocellulose. Int. J. Cancer, *44:* 1052–1056, 1989.
37. Noonan, K. E., Beck, C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., Von Hoff, D. D., and Rononson, I. B. Quantitative analysis of MDR1 (Multidrug Resistance) gene expression in human tumors by polymerase chain reaction. Proc. Natl. Acad. Sci. USA, *87:* 7160–7164, 1990.
38. Mantel, N., and Haenszel, W. Statistical aspects of the analysis of data from retrospective studies of disease. J. Natl. Cancer Inst., *22:* 719–748, 1959.
39. Steel, R. G. D., and Torrie, J. H. Principles and Procedures of Statistics. New York: McGraw-Hill Book Co., 1980.
40. Bacus, S. S., Huberman, E., Chin, D., Kiguchi, K., Simpson, S., Lippman, M., and Lupu, R. A ligand for the *erb*B-2 oncogenic product (gp30) induces differentiation of human breast cancer cells. Cell Growth Differ., *3:* 401–411, 1992.
41. Marth, C., Muller, H. E., Greiter, E., Cronauer, M. V., Zeimet, A. G., Doppler, W., Eibl, B., Hynes, N. E., and Daxenbichler, G. γ-Interferon reduces expression of the protooncogene c-*erb*B-2 in human ovarian carcinoma cells. Cancer Res., *50:* 7037–7041, 1990.
42. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun., *159:* 548–553, 1989.
43. Kasprzyk, P., Song, S., Di Fiore, P., and King, C. Therapy of an animal model of human gastric cancer using a combination of anti-*erb*B-2 monoclonal antibodies. Cancer Res., *52:* 2771–2776, 1992.
44. Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. ARIA, a

5260

protein that stimulates acetylcholine receptor synthesis, is a member of the Neu ligand family. Cell, 72: 801–815, 1993.

45. Corfas, G., and Fischbach, G. D. The number of Na$^+$ channels in cultured chick muscle is increased by ARIA, an acetylcholine receptor-inducing activity. J. Neurosci., 73: in press, 1993.

46. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Denehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBury, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. Glial growth factors are alternatively spliced erbB-2 ligands expressed in the nervous system. Nature (Lond.), 362: 312–318, 1993.

47. Pober, J. S., Gimbrone, M. A., Lapierre, I. A., Mendrick, D. L., Fiers, W., Rothlein, R., and Springer, T. A. Overlapping pattern of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. J. Immunol., 137: 1883–1896, 1986.

48. Plowman, D. G., Culouscou, J-M., Withney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA, 90: 1746–1750, 1993.

49. Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry, 29: 11024–11028, 1990.

50. Bouillon, M., and Audette, M. Transduction of retinoic acid and γ-interferon signal for intercellular adhesion molecule-1 expression on human tumor cell lines: evidence for the late-acting involvement of protein kinase-C inactivation. Cancer Res., 53: 826–832, 1993.

51. Peles, E., Levy, R. B., Or, E., Ullrich, A., and Yarden, Y. Oncogenic forms of the neu/HER2 tyrosine kinase are permanently coupled to phospholipase C gamma. EMBO J., 10: 2077–2086, 1991.

52. Stancovski, I., Sela, M., and Yarden, Y. Molecular and clinical aspects of the Neu/ErbB-2 receptor tyrosine kinase. In: M. Lippman and R. Dikson (eds.), Regulatory Mechanisms of Breast Cancer. Boston: Kluwer Academic Publishers, 1993.

53. Ali, I. U., Campbell, G., Lidereu, R., and Callahan, R. Lack of evidence for the prognostic significance of the c-erbB-2 amplification in human breast carcinoma. Oncogene Res., 3: 139–146, 1988.

54. van de Vijver, M., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O., and Nusse, R. Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N. Engl. J. Med., 319: 1239–1245, 1988.

55. Ramachandra, M., Ashley, M., and Gusterson, B. Immunohistochemical distribution of c-erbB-2 in in situ breast carcinoma, detailed morphological analysis. J. Pathol., 161: 7–14, 1990.

56. Lodato, R. F., Maguire, H. C. J., Greene, M. I., and Li Voisi, V. A. Immunohistochemical evaluation of c-erbB-2 oncogene expression in ductal carcinoma in situ and atypical ductal hyperplasia of the breast. Mod. Pathol., 3: 499–454, 1990.

57. Allred, D. C., Clark, G. M., Tandon, A. K., Molina, R., Tormey, D. C., Osborne, C. K., Gilchrist, K. W., Mansour, E. G., Abeloff, M., Eudey, L., and McGuire, W. L. HER-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. J. Clin. Oncol., 10: 599–605, 1992.

58. Fady, C., Gardner, A. M., Gera, J. F., and Lichtenstein, A. Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells: selective effects on HER2/neu-overexpressing cells. Cancer Res., 52: 764–769, 1992.

59. Marth, C., Cronauer, M. V., Doppler, W., Ofner, D., Ullrich, A., and Daxenbichler, G. Effects of interferons on the expression of the proto-oncogene HER-2 in human ovarian carcinoma cells. Int. J. Cancer, 50: 64–68, 1992.

60. Hudziak, R. M., Lewis, G. D., Shalaby, M. R., Eassalu, T. E., Aggarwal, B. B., Ullrich, A., and Shepard, H. M. Amplified expression of the HER-2/erbB-2 oncogene induces resistance to tumor necrosis factor in NIH-3T3 cells. Proc. Natl. Acad. Sci. USA, 85: 5102–5106, 1988.

61. Allred, D. C., Clark, G. M., Molina, R., Tandon, A. K., Schnitt, S. J., Gilchrist, K. W., Osborne, C. K., Tormey, D. C., and McGuire, W. L. Overexpression of HER-2/Neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. Hum. Pathol., 23: 974–979, 1992.

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