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Medullary Carcinoma Is Associated with Expression of Intercellular Adhesion Molecule-1

Implication to Its Morphology and Its Clinical Behavior

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The histological hallmarks for the diagnosis of medullary breast cancer are circumscription, syncytial architecture, diffuse inflammatory infiltrate, and highly atypical nuclei. The biological and prognostic implication is a lower propensity to metastasize. We studied 19 medullary carcinomas for expression of the intercellular adhesion molecule-1 and lymphocyte-function-associated antigen-1, Neu differentiation factor, tumor necrosis factor-α, and the expression of HER-2/neu, HER-4, and HER-3 receptors. Our study revealed that all of the 19 medullary carcinomas expressed the intercellular adhesion molecule-1 and lymphocyte function associated antigen. Eighteen of 19 cancers expressed Neu differentiation factor and tumor necrosis factor-α. All medullary cancers expressed the HER-2/neu receptor, however, in the majority of the cases, the staining was confined to the cytoplasm. Only 4 of 12 cancers expressed HER-4 and none of the eight medullary cancers tested expressed HER-3. By comparison, in a control group of infiltrating ductal carcinomas, expression of intercellular adhesion molecule-1, lymphocyte function associated antigen-1, and Neu differentiation factor was positive in about 25 to 30% of the cases, HER-4 was expressed in 75% and HER-3 in 95% of the cases. Taken together, our observations suggest that the expression of intercellular adhesion molecule-1, lymphocyte function associated antigen, Neu differentiation factor, and tumor necrosis factor-α as factors that may affect the special morphology and the biological behavior that characterizes medullary carcinomas. (Am J Pathol 1994, 145:1337–1348)

Medullary carcinoma of the breast is considered a distinctive subtype with a relatively good prognosis. It is characterized by large cells, syncytial architecture, and infiltration by benign lymphocytes and plasma cells.[1–4] Patients with this type of breast carcinoma seem to have a better prognosis than those with the other histological subtypes.[5–8] It is believed that the better outcome is due at least in part to the presence of the lymphocytic infiltration that is closely associated with the tumor.[2,3] In this study, we tried to correlate the morphology and clinical behavior of medullary carcinomas with molecular markers that may affect the biological behavior of these cancers.

Adhesion molecules participate in the many stages of immune response. T cell immune recognition requires the contribution of the T cell receptor, as well as adhesion receptors, which promote attachment of T cells to antigen-presenting cells and transduce regulatory signals for T cell activation.[9,10]

Intercellular adhesion molecule-1 (ICAM-1) is a 90-kd integral membrane glycoprotein whose extracellular region is divided into five immunoglobulin-like domains that are followed by a transmembrane region and a short cytoplasmic tail.[10,11] ICAM-1 plays an important role in inflammation as well as in malignant diseases. Cytolytic conjugate formation between cytotoxic T lymphocytes and target cells, such as endothelial and epithelial cells, occurs between

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the ICAM-1 molecule and the integrin lymphocyte-function-associated antigen (LFA-1).[9–11] The release of certain cytokines, such as γ-interferon, interleukin 1, and tumor necrosis factor (TNF-α) at sites of inflammation and immune response causes cell activation and results in elevated expression of ICAM-1 on the cell surface.[12–14] However, the molecule is not restricted to hematopoietic cells; it is also expressed on endothelial cells, dermal fibroblasts, melanomas, and certain types of carcinomas.[15,16]

The HER-2/neu proto-oncogene (also called erbB-2) encodes a transmembrane tyrosine kinase that is structurally homologous to the receptor for epidermal growth factor.[17–19] Amplification and overexpression of the gene have been detected at a high frequency in human adenocarcinomas from several tissues. Moreover, overexpression of HER-2/neu appears to be associated with poor prognosis in breast cancer patients.[20–25]

A factor that can stimulate tyrosine phosphorylation of the HER-2/neu receptor was detected in conditioned medium of ras-transformed rat fibroblasts.[26,27] Complete purification showed that the activity corresponded to a heat-stable 44-kd glycoprotein that stimulated tyrosine phosphorylation of the HER-2/neu receptor in mammary cells. Native and recombinant forms of the rat protein were found to inhibit growth and induce mature phenotype with syncytial morphology in certain breast cancer cell lines (AU-565 and MDA-MB-453).[28,29] On the basis of these findings, the active molecule was named Neu differentiation factor (NDF). The human homolog of NDF was termed heregulin, and the recombinant version of it was found to be mitogenic to the breast cancer cell line SKBR-3.[30] Concomitant with the morphological changes, NDF elevates the expression of ICAM-1 at both the protein and mRNA levels, downregulates HER-2/neu transcription, and changes the subcellular localization of HER-2/neu receptor from the membrane to being diffuse cytoplasmic.[29] However, the interpretation of these results as an indication for NDF and HER-2/neu receptor interaction is complicated by the occurrence of heterodimers of HER-2/neu and other receptors.[31,32] Several lines of evidence raised the possibility that the interaction between NDF and HER-2/neu involves one of the other receptors.[33] NDF has been shown to be a specific ligand to HER-4[34,35] and induces cells that express HER-4 and HER-2/neu (MDA-MB-453) to acquire a mature phenotype.[36] NDF has been shown to also bind to HER-3.[37] Most likely, heterodimer formation between HER-2/neu and HER-4 or HER-3 receptors is the mechanism that allows NDF to induce its biological effect on the HER-2/neu receptor.[35,37]

Because medullary carcinomas exhibit many of the phenotypic changes associated with NDF treatment, we examined the expression of ICAM-1, HER-2/neu, HER-4, HER-3, NDF, and TNF-α in 19 medullary carcinomas. We also tested the cancers for the expression of estrogen and progesterone receptors, their proliferation index, and their DNA ploidy distribution. Seventy-seven infiltrating ductal carcinomas provided a control group for the expression of some of these markers. Here we provide evidence that ICAM-1 and LFA-1 were expressed in all 19 medullary carcinomas. HER-2/neu expression has been detected in 18 of 19 medullary carcinomas. Only 4 of 13 cancers tested expressed HER-4. None of 8 medullary carcinomas tested expressed HER-3.

TNF-α transcripts were detected in 8 of 14 medullary carcinomas tested by the polymerase chain reaction (PCR), and 18 of 19 stained positive with an antibody to TNF-α. NDF transcripts were detectable in 13 of 19 medullary cancers by PCR, and NDF protein was detected in 18 of 19 of the medullary cancers stained for NDF.

Our data suggest that the *in vivo* effect of these factors may be relevant to the biological and morphological features of medullary carcinomas.

Materials and Methods

Clinical Cases of Medullary Carcinomas

This study included pure medullary carcinomas as well as cases accepted as medullary variant, in which one or more features that are not present in completely pure medullary carcinomas were present. The rules and indications of Ridolfi[6] were used for the diagnosis of medullary carcinoma. Although the majority of the cases were pure medullary carcinomas with perhaps a small focus of invasion at the edge, a few of the cases had areas of tumor cell clumps that might stretch the rule specifying that 75% of the tumor be syncytial. Two of the tumors had an *in situ* component adjacent to the tumor, which may or may not exempt these cases from being regarded as pure medullary carcinomas. Cancers that had glandular formation in more than 50% of the area of the tumor with cell arrangements were diagnosed as ductal carcinoma no special type and were exempted from the study. Seventy-seven infiltrating ductal carcinomas served as controls.

The medullary carcinomas were obtained from three different laboratories and classified by different

pathologists. All of these cancers were reexamined by one of us. The control infiltrating ductal carcinomas were obtained from one laboratory and classified by three different pathologists for consensus.

Immunohistochemical Staining

Paraffin blocks were sectioned into 5-μ slices and mounted onto poly-L-lysine-coated slides in a flotation bath containing no gelatin. The sections were dried in a 58 to 60 C incubator for 1 hour, then deparaffinized and hydrated with distilled water.

The following primary antibodies were used: a monoclonal antibody to the HER-2/neu protein, N24 (Weizmann Institute, Rehovot, Israel);[38] a monoclonal antibody to ICAM-1 (Becton Dickinson, San Jose, CA); a monoclonal antibody to proliferating cell nuclear antigen (PCNA; proliferation staining kit II from Becton Dickinson, Cell Analysis Systems (CAS), Elmhurst, IL); two polyclonal antibodies to the human NDF (Weizmann Institute); a polyclonal antibody to human TNF-α (Endogen, Inc., Boston, MA); a monoclonal antibody to LFA-1α (Becton Dickinson, San Jose, CA); monoclonal antibodies to T cell-helper CD4 and T-cell suppressor CD8 (Dako Corp., Carpenteria, CA).

Secondary antibodies used were biotinylated rabbit anti-mouse IgG-2B for ICAM-1 (Zymed Laboratories, San Francisco, CA), biotinylated rabbit anti-mouse IgG-2A for LFA-1α (Zymed), biotinylated goat anti-rabbit IgG for NDF and TNF-α (Jackson Laboratories, West Grove, PA), biotinylated goat anti-mouse for HER-2/*neu*, (Jackson), and peroxidase-conjugated rabbit anti-mouse IgG for the PCNA (Becton Dickinson, Elmhurst, IL).

The labeling antibody used for all the markers except for PCNA was streptavidin-conjugated alkaline phosphatase (Jackson). Diaminobenzidine was the chromogen used for the PCNA assay. All other markers used the alkaline phosphatase chromogen CAS red (Cell Analysis Systems). The PCNA assay was counterstained with ethyl green and CAS DNA stain (both from Cell Analysis Systems) was the counterstain used for the alkaline phosphatase markers. Breast cancer sections stained by the same procedures using an unrelated antibody (mouse IgG) were used as negative controls. For staining specificity AU-565 and MCF-7 breast cancer cell lines were used as positive and negative controls for ICAM-1 expression and HER-2/neu. We also used as controls positive and negative breast cancer tissues that were known to express or not express these antigens.

DNA

Hydrated paraffin sections were stained for DNA according to the instructions of the CAS DNA stain kit as previously described.[39,40]

PCR

Two 50-μ sections (in duplicate) for each medullary carcinoma were deparaffinized for 5 minutes (two times) in xylene followed by removal of xylene with washes in 100% and 95% ethanol, each for 5 minutes. The sections were then placed in 2× RNA lysis buffer (ABI, Foster City, CA) and placed on ice for 5 minutes. An equal volume of RNA dilution buffer (ABI) and 500 μg of proteinase K were added. The sections were then capped with mineral oil, placed at 37 C, and digested for 5 days. RNA was extracted by an acid phenol method and converted to cDNA with random priming and reverse transcriptase. A portion of the cDNA was used to amplify a portion of the β-2-microglobulin cDNA as a control[41] to determine the viability of the cDNA. Only those samples that demonstrated the β-2-microglobulin PCR product were considered for PCR amplification of NDF, HER-4, HER-3, or TNF-α. NDF primers corresponding to bp 257 to 281 and bp 570 to 589 of the sequence of human heregulin-α,[30,42] TNF-α primers (Clontech Laboratories, Palo Alto, CA), HER-4 primers corresponding to bp 2310 to 2333[43] and 2811 to 2835,[35] and HER-3 primers corresponding to bp 1650 to 1661 and 1845 to 1828, all of which are specific for cDNA, were used for 30 to 35 cycles of amplification. MDA-MB231 cells and MDA-MB-453 cells were used as positive controls for expression of NDF[30,42] and HER-4 or HER-3[35,43] respectively. A positive control for TNF-α was supplied by Clontech.

Quantitative Analysis

DNA Quantitation

Quantitation of DNA relies on the Feulgen staining reaction to specifically and quantitatively stain DNA. Hydrochloric acid hydrolyzes the ribose-purine bonds in the DNA to give sugar aldehyde residues. The dye then couples via a Schiff reaction to the sugar aldehyde to give a blue color. This staining reaction is stoichiometric to the amount of DNA present in a cell. The DNA was quantitated on tissue sections with the CAS 200 image analysis system (Becton Dickinson, Cellular Imaging Systems, Elmhurst, IL) as previously described.[44] For DNA analysis on tissue sec-

tions 100 tumor nuclei were analyzed and compared with control cells that had been predeposited on the same slide. The interactive nature of an imaging system enables the pathologist to accept and reject cells for DNA quantitation according to cell morphology. Thus, the DNA content can be determined for the nuclei of tumor cells only.

Quantitation of Immunohistochemical Staining

To quantitate the immunohistochemical staining of a nuclear antigens such as PCNA, which stains only proliferating cells that synthesize DNA,[45,46] a microscope-based, two-color system, with two solid state image sensing channels is used. The image channels are specifically matched to two-component immunohistochemical staining to specifically enhance the image of one stain in each channel. One channel is used to identify all components in the tissue counterstained with ethyl green (ie, all of the nuclear components) and the other channel is used to identify the proportion of nuclear components in which specific nuclear proteins (antigens) are stained immunohistochemically. This imaging technique has been referred to as nuclear masking.[47] The spectra for the stains have been matched to the imaging filters so that the mask image of one channel is essentially transparent to the other channel. For each breast cancer specimen a minimum of five fields (x40 magnification) of tumor epithelium were randomly measured. The use of an interactive command for each field makes it possible to select cancer cells for the determination of the proportion of labeled nuclei and exclude any other areas containing normal tissue or inflammatory components. The report for the percent positive stained nuclei for PCNA consists of the proportion of the positively stained area in the total nuclear area.

In the quantitation of the HER-2/neu, NDF, and TNF-α protein, both solid state imaging channels are used.[40] One channel quantitates the DNA content of the field after Feulgen staining, and the second channel quantitates the total amount of HER-2/neu, NDF, or TNF-α antigen in the field as revealed by immunostaining. Because the total amount of DNA per cell is known (by previous DNA quantitation), the number of cells in the field is estimated and used to derive the average HER-2/neu, NDF, or TNF-α protein in cancer cells. Cells with known levels of HER-2/neu protein (AU-565 cells) were used for calibration of the image analysis system. The amount of HER-2/neu protein in AU-565 cells was independently determined by using enzyme immunoassay (ELISA) (human neu quantitative ELISA, Oncogene Science, Uniondale, NY) and was performed on 1×10^6 cells by the double monoclonal antibody to HER-2/neu protein capture assay. Calibration of the ELISA reader was based on the absorbance of purified neu proteins P185 and P105 (neu protein extracellular domain). The ELISA results indicated that AU-565 cells contain 1 pg of protein per cell and overexpress HER-2/neu (4×10^6 HER-2/neu receptors per cell). These cells were used to calibrate the HER-2/neu protein assay. Their level of staining was defined as 100% of HER-2/neu protein content; all other measurements of HER-2/neu antigen are related to this value. Normal expression of HER-2/neu was determined by measuring HER-2/neu protein on residual normal tissue or on cell lines that express normal amounts of HER-2/neu (MCF-7 cells).[48] 0.15 pg/cell was estimated as a cutoff point for overexpression. Breast cancer cells containing more than 0.15 pg/cell of HER-2/neu protein were considered to overexpress the HER-2/neu. Arbitrary units were used to quantitate the relative levels for the NDF and TNF-α.

Results

We studied the expression of several ICAM growth factors, and oncogene receptors on 19 medullary carcinomas and 77 infiltrating ductal carcinomas. We found that all 19 breast carcinomas, two of which are represented in Figure 1, that were diagnosed as medullary carcinomas, expressed ICAM-1 and LFA-1 as demonstrated by immunohistochemical staining (Table 1; Figure 2A-C). These 19 cancers also expressed the receptor for the HER-2/neu oncogene, 13 of them showing high levels of staining for HER-2/neu receptor (Figure 2D; Table 1) as measured by image analysis techniques. However, staining for the receptor revealed that in most cases the receptor localization was mainly diffuse cytoplasmic. Seventy-seven cases of infiltrating ductal carcinomas, which acted as controls, were divided according to their expression of HER-2/neu (Table 2). Fifty-two of the infiltrating no special type ductal carcinomas were negative for HER-2/neu overexpression. Of these 52 cancers, only 14 expressed ICAM-1. Twenty-five no special type cancers were positive for HER-2/neu overexpression, and only three of these cancers expressed ICAM-1. The high level of HER-2/neu staining was evident on the membrane in these 25 tumors.

All of the medullary carcinoma cases exhibited a very high percentage of proliferating cells as depicted by staining for PCNA, which recognizes proliferating cells only[45,46] (Tables 1 and 2; Figure 2E). In

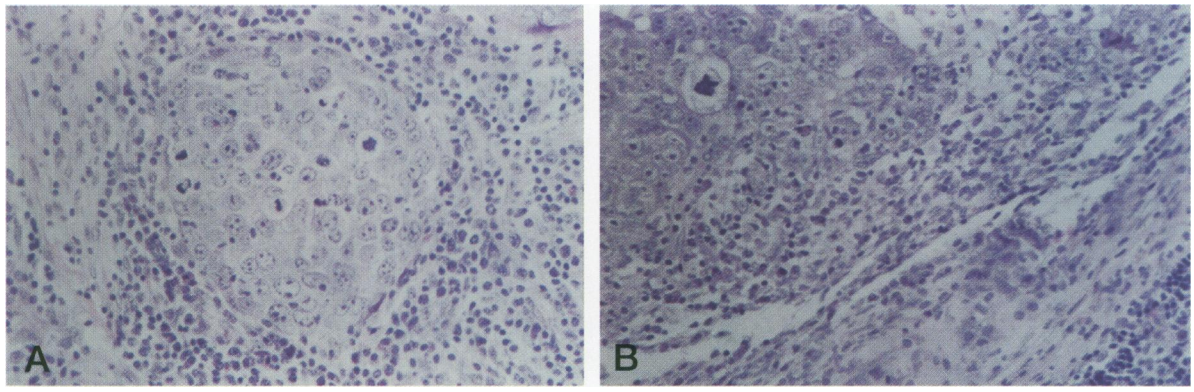

Figure 1. *Hematoxylin and eosin staining of paraffin sections* (**A** *and* **B**) *of medullary carcinoma depicting syncytial architecture, diffuse inflammatory infiltrating lymphocytes, and highly atypical nuclei* (×*400*).

Table 1. *Biological, Clinical, and Histological Profile of the Medullary Carcinomas*

Tissue*	Diffuse cytoplasmic HER-2/neu	ICAM-1	LFA-1	Lymph N	Outcome	ER/ PR†	DNA	PCNA	CD4	NDF	PCR NDF	PCR TNF-α	TNF-α	PCR HER-4
362 AM	0.30	+	+	(0/11)		–	1/1.6	24.03	+	1.21			0.18	
1109 MF	0.24	+	+	(0/23)	free-alive	–	1.23	58.25	+	0.15	–	+	0.47	–
1787 MF	0.06	+	+	(1/23)	expired		1.8	55.72		0.50	+	+	0.34	–
1591 AM	0.15	+	+	(0/11)	free-alive		1.81	58.25	+	0.18	–	+	0.25	–
1518 MF	0.19 membrane staining	+	+	(0/11)	unknown		1.63	45.98	+	0.15	+		0.18	–
1888 M	0.18	+	+	(0/11)	free-alive		1.56	10.36	+	0.3	+			–
3617 + DCIS AM	0.20	+	+				0.95/1.91	22.5	+	–				
5448 AM	0.68	+	+			–	0.96/1.59	48.7	+	0.33	+		0.26	–
2554B AM	0.21	+	+				1.01/1.6	74.7	+	0.34	+		0.17	
4001A M	0.13	+	+				1.6	31.3	+	0.50	+		0.14	
6262M	0.37	+	+				1.35	16.9	+	0.72	–	+	0.44	
2900A AM	0.30	+	+			–	1.07/1.4	38.0	+	0.29	+	+	0.17	–
10485 (3A) MF	0.13	+	+	1/23	alive-not free	–	1.01/1.75	27.4	+	0.12	–	+	0.34	+
24209 (B) MF	0.14	+	+	Biopsy	free-alive		1/1.7	73.4		0.51	+	–		+
24368Q MF	0.10	+	+	2/22	free-alive	–	2.27	79.2	+	0.15	+	–	0.26	+
10485 (3B) MF	0.12	+	+	1/23	alive-not free	–	1.01/1.75	46.8	+	0.09	+	–	0.46	–
24209 (D) MF	0.15	+	+	Biopsy	free-alive	–	1/1.7	65.9	+	0.61	–	–	0.33	–
21641 (E) AM	0.19	+	+	1/11	alive-not free		1/1.3	<1		0.35	+	–		–
21641 AM	0.19	+	+	1/11	alive-not free		1/1.3	<1		0.20				
12448 M	0.17	+	+				1.01/1.5	56.9	+	0.34	+	+	0.21	–
4948 M	0.27	+	+		alive	–	2.01	26	+	0.34	–	–	0.11	–
478 M	0.1	+	+	0/11	alive	–	1.69	25	+	0.04	+	+	0.3	+

* Histologic classification: M, medullary; AM, atypical medullary; MF, medullary features.
† Estrogen receptor/progesterone receptor.

most cases, more than 20% of the cells stained positive for the nuclear antigen, with an average proliferation of 45%. The average proliferation in the infiltrating carcinomas was 16% (Table 2).

Expression of NDF was analyzed by staining with two polyclonal antibodies that recognize a 15-amino-acid portion of the epidermal growth factor-like domain of human NDF. The expression of NDF was also analyzed in 19 medullary carcinomas by using PCR. RNA was extracted from the specimens and converted to cDNA by random priming. A thermostable DNA polymerase and two pairs of primers were used to amplify a 336-bp DNA segment of the NDF transcript or β-2-microglobulin as a control (see Materials and Methods). Detectable amplified NDF cDNA was found in 13 of the 19 specimens (68%) (Figure 3A). As a comparison, of the 77 infiltrating carcinomas, only 25% were positive for NDF expression by PCR. Stain-

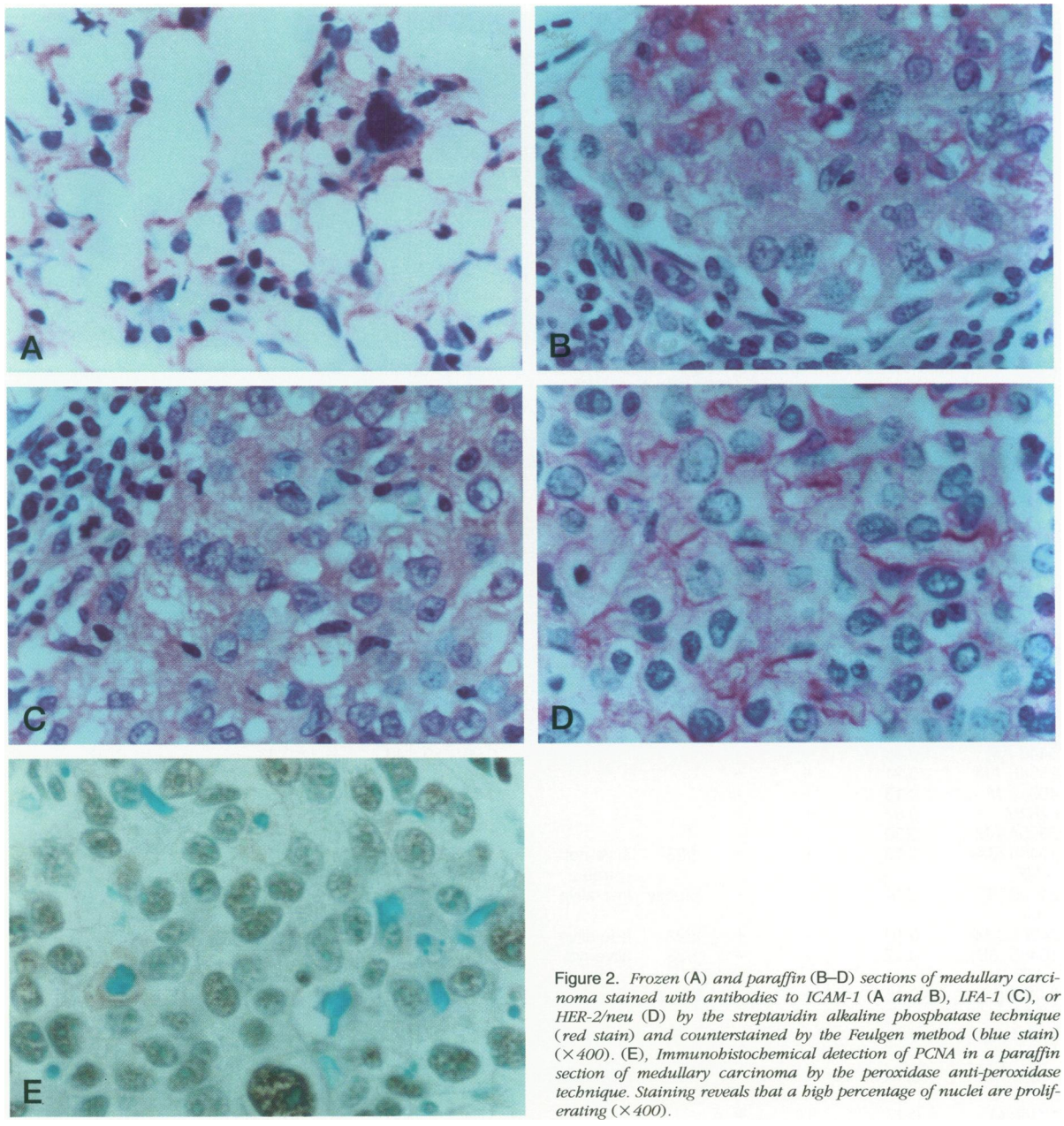

Figure 2. *Frozen* (A) *and paraffin* (B–D) *sections of medullary carcinoma stained with antibodies to ICAM-1* (A *and* B), *LFA-1* (C), *or HER-2/neu* (D) *by the streptavidin alkaline phosphatase technique* (*red stain*) *and counterstained by the Feulgen method* (*blue stain*) (×400). (E), *Immunohistochemical detection of PCNA in a paraffin section of medullary carcinoma by the peroxidase anti-peroxidase technique. Staining reveals that a high percentage of nuclei are proliferating* (×400).

ing of the medullary carcinomas with the two antibodies to NDF revealed that the cells of these cancers also expressed high levels of staining with the polyclonal antibodies to NDF (Figure 3B) as 18 of 19 cancers (95%) were positive for NDF staining. In the control group, only approximately 25% of the cases stained positive for NDF.

Expression of TNF, which also induces ICAM-1,[13] was tested by PCR in 14 medullary cancers. Eight of these cancers were positive for expression of TNF-α (Table 1; Figure 3C). The use of an antibody to TNF-α on 14 cases showed that TNF was expressed in all medullary carcinomas tested. Expression of HER-4 and HER-3 were also tested by the PCR technique. HER-4 is coexpressed with HER-2 in most breast cancer cell lines.[34] Testing of 16 medullary carcinomas that expressed ICAM-1 and HER-2/neu and were either positive or negative for NDF by PCR revealed that 12 of them lacked the HER-4 receptor (Table 1). By comparison, 75% of the infiltrating ductal carcinomas expressed HER-4. Testing of eight medullary carcinomas for HER-3, which is expressed in most breast

Table 2. *Expression of ICAM-1 and Proliferation Index in Infiltrating and Intraductal Carcinoma Compared with Medullary Carcinomas*

Proliferating	No. of cases	No. of cases positive for ICAM-1	% proliferating cells
Infiltrating ductal carcinomas negative for HER-2/*neu* overexpression	52	14	16
Infiltrating ductal carcinomas positive for HER-2/*neu* overexpression	25	3	21
Medullary carcinoma	19	19	45

cancer cell lines,[43] revealed that none of them expressed HER-3, whereas 95% of the control group was positive for HER-3 expression.

ICAM-1 is associated with the inflammatory reaction. Indeed, medullary carcinomas exhibit high levels of infiltrating lymphocytes, of which the majority have been shown in the past to be of T cell origin.[2,3] Therefore, we stained the tissue cancers for T lymphocyte markers such as CD4 and CD8. The majority of lymphocytes stained positive for CD4 (helper T lymphocytes; Figure 4A) but were negative for CD8 (suppressor T cells; Figure 4B).

Testing the DNA ploidy value of medullary cancer cells revealed that the large abnormal nuclei associated with medullary carcinoma (Figure 4C) were aneuploid and in some cases polyploid, which contained diploid and aneuploid stem lines (Table 1). The 52 cases of HER-2/neu-negative infiltrating ductal carcinomas showed DNA indices ranging from euploid to aneuploid, whereas the 25 cases of HER-2/neu overexpressing cancers had a polyploid DNA index with a main DNA peak of tetraploid (data not shown), confirming our previous results.[40]

Ten of the medullary cancers were tested for estrogen and progesterone receptors and exhibited negative staining (Table I). Again, in the control group, approximately 45% of the cases expressed estrogen and progesterone receptors.

Discussion

Medullary carcinoma of the breast is a form of infiltrating carcinoma characterized by sheets of large neoplastic cells that are associated with an intense lymphocytic infiltrate. Patients with this form of breast carcinoma have a better prognosis than do those with other types of mammary carcinoma. This better outcome has been attributed to the host's reaction to the tumor, which is evidenced by an intense inflammatory response (as well as the lack of stromal infiltration). Similarly, favorable prognoses are seen in other neoplasms that contain lymphocytes as a prominent histological feature, such as seminoma, gastric carcinoma, and cervical cancers.[2] Here we provide evidence that the adhesion molecules, namely ICAM-1 and its ligand LFA-1, which play a role in immune response, are present in medullary carcinomas.

Presumably, expression of ICAM-1 is associated with a restriction of the ability of a tumor to metastasize outside of the lymph nodes to other distant sites and may thus behave as a protecting factor. The mechanism of protection could involve 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 induction of expression of ICAM-1.[49] A similar phenomenon has been reported in which ICAM-1-negative melanoma cells that were resistant to T-cell-mediated cellular lysis became sensitive after induction of ICAM-1 by TNF-α.[13] Sensitization of neuroblastoma to natural killer cells after induction of ICAM-1 by γ-interferon was also reported.[14] Another mechanism, which could be involved in the protection effect, is that in medullary carcinomas, HER-2/neu receptors are expressed in the cytoplasm and not on the membrane. The exact role of the cytoplasmic HER-2/neu protein (or a component of the protein) is not clear. Subcellular translocation of the HER-2/neu receptor from the membrane to the cytoplasm is associated with terminal differentiation in some breast cancer cell lines overexpressing HER-2/neu.[28,29,50,51] Nesland et al[52] examined the localization of the HER-2/neu protein by means of immunoelectron microscopy and showed the presence of the HER-2/neu protein on the cell membrane, as well as in the cytoplasm, and in the rough endoplasmic reticulum in human cancer cells that overexpress HER-2/neu. One possible explanation for the differentiation effect of the cytoplasmic protein is that the induction of receptor endocytosis decreases the overall enzymatic activity of the constitutively active HER-2/neu kinase when the receptor is overexpressed on the membrane.[27] Downregulation and translocation of HER-2/neu receptor were shown to reduce the rate of cellular proliferation and tumorigenicity in several cellular systems,[50,53] whereas its overexpression on the cell membrane induces resistance to TNF-α and to cytotoxic macrophages.[54]

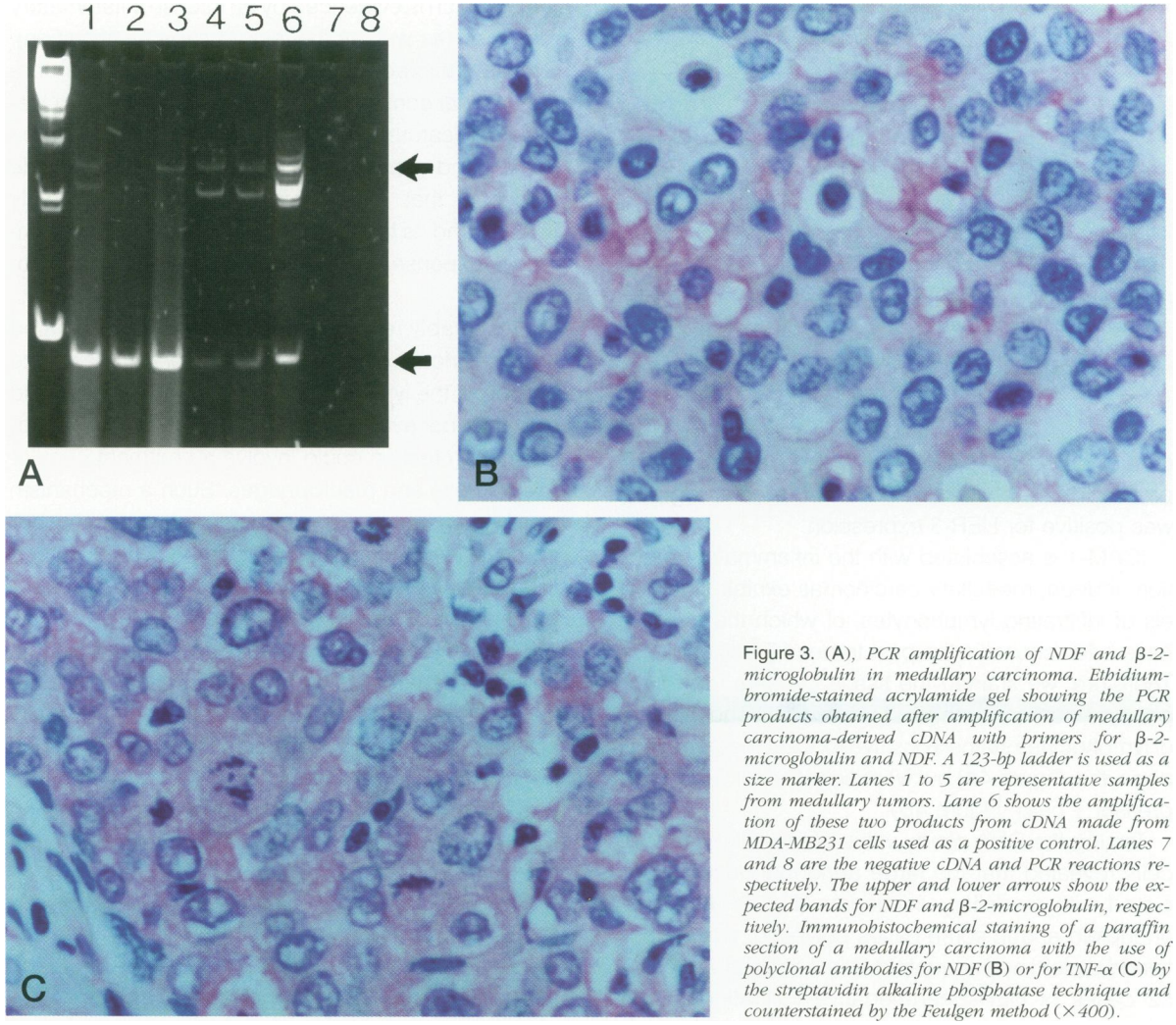

Figure 3. (A), *PCR amplification of NDF and β-2-microglobulin in medullary carcinoma. Ethidium-bromide-stained acrylamide gel showing the PCR products obtained after amplification of medullary carcinoma-derived cDNA with primers for β-2-microglobulin and NDF. A 123-bp ladder is used as a size marker. Lanes 1 to 5 are representative samples from medullary tumors. Lane 6 shows the amplification of these two products from cDNA made from MDA-MB231 cells used as a positive control. Lanes 7 and 8 are the negative cDNA and PCR reactions respectively. The upper and lower arrows show the expected bands for NDF and β-2-microglobulin, respectively. Immunohistochemical staining of a paraffin section of a medullary carcinoma with the use of polyclonal antibodies for NDF* (B) *or for TNF-α* (C) *by the streptavidin alkaline phosphatase technique and counterstained by the Feulgen method* (×400).

Interestingly, TNF-α has also been shown to downregulate HER-2/neu in breast and pancreatic tumor cells overexpressing HER-2/neu and upregulate P55TNF receptor[53,55,56] as well as induce ICAM-1 expression in other cell lines.[12] Independent of the exact mechanism, the presence of ICAM-1 on tumor cells exposes the tumor cells to infiltrating lymphocytes, which often surround ICAM-1-positive tumor cells in medullary carcinomas. In the past, we have shown that NDF (also known as heregulin) induced some breast cancer cells that overexpress HER-2/neu to acquire a mature phenotype (characterized by large nuclei, flat morphology, and formation of syncytial-like cells) as well as to upregulate the expression of ICAM-1. These changes were accompanied by HER-2/neu receptor translocation from the membrane to the cytoplasm.[28,29] Because medullary carcinomas expressed some of these acquired phenotypes, we asked whether NDF and TNF-α are the factors that determine the level of expression of ICAM-1 and are associated with the syncytial morphology and the specific HER-2/neu receptor localization in medullary tumors. Although our limited analysis of NDF and TNF-α expression in these mammary tumors was consistent with the possibility that these factors may play a role in the clinical behavior of these tumors, more extensive analysis will be required to relate it to histological and clinical aspects of this disease. Potentially, other polypeptide factors may also upregulate ICAM-1. In the case of NDF, IFN-γ, and TNF-α, a concomitant downregulation of surface HER-2/neu receptors and receptor translocation from the membrane to the cytoplasm is expected.[28,27,49,53,55,56]

The DNA sequence of the 336-bp PCR product amplified with the NDF pair of primers used in our study indicated that we amplified a region of NDF that has a high degree of homology to a large family of NDF.

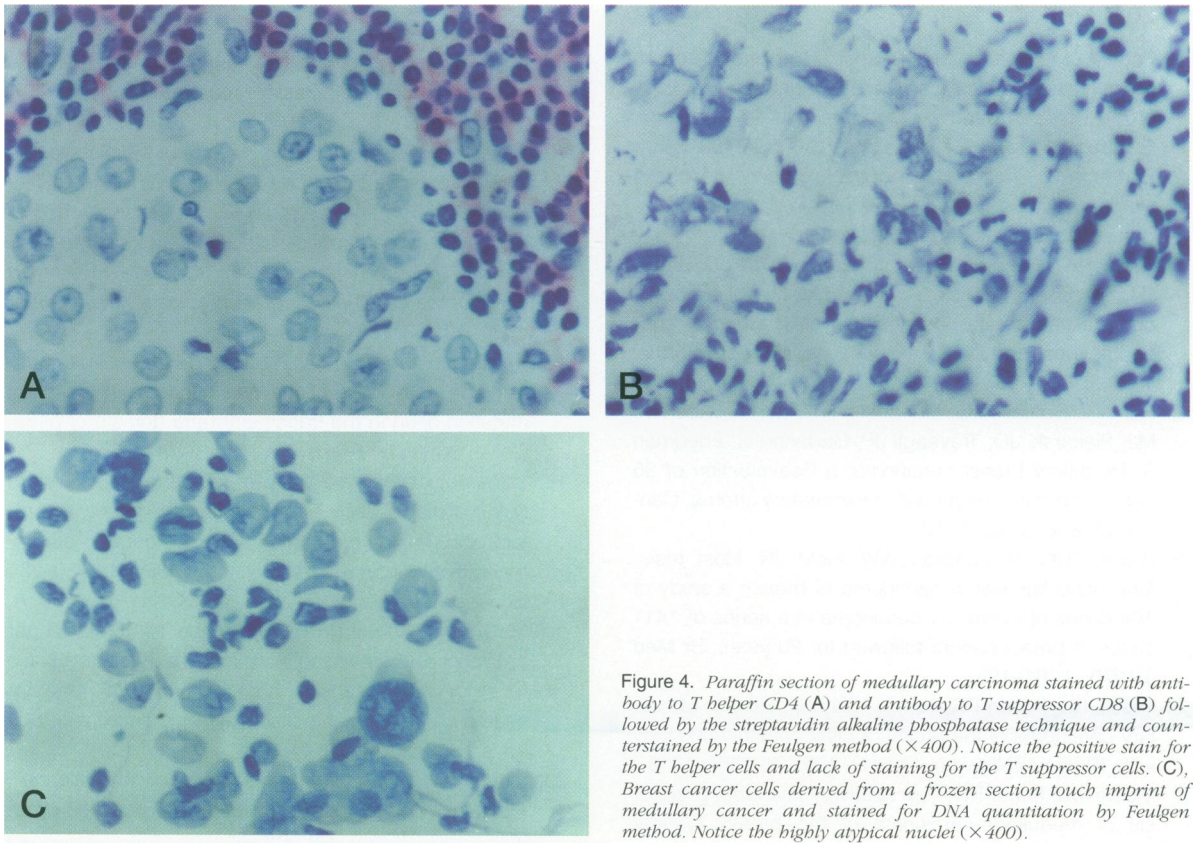

Figure 4. *Paraffin section of medullary carcinoma stained with antibody to T helper CD4* (A) *and antibody to T suppressor CD8* (B) *followed by the streptavidin alkaline phosphatase technique and counterstained by the Feulgen method* (×400). *Notice the positive stain for the T helper cells and lack of staining for the T suppressor cells.* (C), *Breast cancer cells derived from a frozen section touch imprint of medullary cancer and stained for DNA quantitation by Feulgen method. Notice the highly atypical nuclei* (×400).

This family includes alternatively spliced neuronal and mesenchymal growth factors that include NDF/heregulin, acetylcholine-receptor-induced activity, and glial growth factors,[30,42,55,57–59] some of which act as differentiation factors[34–36,42,55,57,58] or as mitogenic factors.[30,59] Interestingly, glial growth factor, which has been shown to be mitogenic to Schwann cells,[59] promotes glial cell differentiation.[58] The region amplified by our assay is not restricted to only NDF and could include all of these other factors. In addition to the expected 336-bp product that was amplified, we obtained two or three other PCR products in some tumors. We are currently analyzing these products to determine whether they are related to the NDF family.

Recently, NDF/heregulin has been identified as a specific ligand to another receptor tyrosine kinase, HER-4[34–36] (a member of the epidermal growth factor receptor family), and low affinity ligand to HER-3.[37] HER-4 forms heterodimeric complexes with HER-2, which establishes high affinity sites for NDF.[35] However, our analysis for HER-4 expression revealed that the majority of the medullary carcinomas in our study lacked HER-4 expression and none of the medullary carcinomas tested expressed HER-3, indicating that in medullary carcinoma, another receptor for NDF may be involved. Most likely the heterodimeric complexes formed between the different members of the erbB family, which includes at least four members (epidermal growth factor receptor, HER-2/neu, HER-3, and HER-4) and their ligands are responsible for the biological behavior of breast cancers. Inasmuch as 95% of infiltrating ductal carcinomas express HER-3, the lack of HER-3 in medullary carcinomas may serve as a molecular marker for these cancers. Finally, the presence of ICAM-1 and LFA-1 in medullary carcinomas may explain the extensive immunological response in these cancers and may explain the restrictive ability of these tumors to invade and metastasize. Thus, ICAM-1 and LFA-1 may behave as protective factors in these cancers.

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References

1. Page DL, Anderson TJ, Sakamoto G: Infiltrating carcinoma: major histological types. Diagnostic Histology of the Breast. Edited by DL Page and TJ Anderson. Edinburgh, Churchill Livingstone, 1987, pp 193–235
2. Ben-ezra J, Sheibani K: Antigenic phenotype of the lymphocytic component of medullary carcinoma of the breast. Cancer 1987, 59:2037–2041
3. Gaffey MJ, Frierson HF, Jr, Mills SE, Boyd JC, Zarbo RJ, Simpson JF, Gross LK, Weiss LM: Medullary carcinoma of the breast: identification of lymphocyte subpopulations and their significance. Modern Pathol 1993, 6:721–728
4. Rapin V, Contesso G, Mouriesse H, Bertin F, Lacombe MJ, Piekarski JD, Traveagli JP, Gadenne C, Friedman S: Medullary breast carcinoma: a Reevaluation of 95 cases of breast cancer with inflammatory stroma. Cancer 1988, 61:2503–2510
5. Bloom HJG, Richardson WW, Field JR: Host resistance and survival in carcinoma of breast: a study of 104 cases of medullary carcinoma in a series of 1411 cases of breast cancer followed for 20 years. Br Med J 1970, 3:181–188
6. Ridolfi RL, Rosen PP, Port A, Kinne D, Mike V. Medullary carcinoma of the breast: a clinicopathologic study with 10 years follow-up. Cancer, 1977 40:1365–1385
7. Moore OS, Foote FW: The relatively favorable prognosis of medullary carcinoma of the breast. Cancer 1949, July, 635–642
8. Richardson WW: Medullary carcinoma of the breast. Br J Cancer 1956, X:415–423
9. Makgeba MW, Sanders ME, Ginther Luce GE, Dustin ML, Springer TA, Clark EA, Mannoni P, Shaw S: ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. Nature 1988, 331:86–88
10. Simmons D, Makgova MW, Seed B: ICAM, an adhesion ligand of LFA-1 is homologous to the neural cell adhesion molecule NCAM. Nature 1988, 331:624–627
11. Staunton DR, Marlin SD, Stratowa C, Dustin ML, Springer TA: Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell 1988, 52:995
12. Rothlein R, Czajkowski M, O'Neil MM, Martin SD, Mamolf E, Merluzzi VJ: 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 1988, 141:1665–1669
13. Braakman E, Goedegebuur PS, Vreugdenhil RJ, Segal DM, Shaw S, Bolhuis RLH: ICAM-1-melanoma cells are relatively resistant to CD3-mediated T-cell lysis. Int J Cancer 1990, 46:475–480
14. Naganuma H, Kiessling R, Patarroyo M, Hansson M, Handgretinger R, Gronberg A: Increased susceptibility of IFN-γ-treated neuroblastoma cells to lysis by lymphokine-activated killer cells: participation of ICAM-1 induction on target cells. Int J Cancer, 1991 47:527–532
15. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmuller G: De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc Natl Acad Sci USA 1989, 86:641
16. Vogetseder W, Feichtinger H, Schulz TF, Schwaeble W, Tabaczewski P, Mitterer M, Block G, Marth C, Dapunt O, Mikuz G, Dierch MP: 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 1989, 43:763–773
17. Bergmann CI, Hung MC, Weinberg RA: Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell 1986, 45:649–657
18. Yamamoto T, Ikawa S, Akiyama T, Semba K, Normura N, Miyajima N, Saito T, Toyoshima K: Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. Nature 1986, 319:230–234
19. Coussens L, Yang FT, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985, 230:1132–1139
20. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987, 235:177–182
21. van de Vijer M, van de Bersselaar R, Devilee P, Cornelisse C, Peterse J, Nusse R: Amplification of the neu (c-erbB-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene. Mol Cell Biol 1987, 7:2019–2033
22. Varley JM, Swallow JE, Brammar WJ, Whittaker JL, Walker RA: Alterations to either c-erbB-2 (neu) or c-myc proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. Oncogene 1987, 1:423–430
23. Venter DJ, Tsui NL, Kummar S, Fullick WJ: Overexpression of the c-erbB-2 oncoprotein in human breast carcinomas: immunohistological assessment correlated with gene amplification. Lancet 1987, ii:67–72
24. Berger MS, Greenfield C, Gullick WJ, Haley J, Downward J, Neal DE, Harris AL, Waterfield MD: Evaluation of epidermal growth factor receptors in bladder tumours. Br J Cancer 1987, 56:533–537
25. Zhou D, Battifora H, Yokota J, Yamamoto T, Cline MJ: Association of multiple copies of the c-erbB-2 oncogenes with spread of breast cancer. Cancer Res 1987, 47:6123–6125
26. Yarden Y, Weinberg RA: Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene. Proc Natl Acad Sci USA 1989, 86:3179–3183
27. Yarden Y, Peles E: Biochemical analysis of the ligand for the neu oncogenic receptor. Biochemistry 1991,

30:3543–3550
28. Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG, Ben-Levy R, Yarden Y: Isolation of the neu/HER-2 stimulatory ligand: a 44-kd glycoprotein that induces differentiation of mammary tumor cells. Cell 1992, 69: 205–216
29. Bacus SS, Gudkov AV, Zelnick CR, Chin DM, Stern R, Stancovski I, Peles E, Ben-Baruch N, Farbstein H, Wen D, Sela M, Yarden Y: Neu differentiation factor (heregulin) induces expression of intercellular adhesion molecule 1: implication for mammary tumors. Cancer Res 1993, 53:5251–5261
30. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard M, Wood WI, Goeddel DV, Vandlen RL: Identification of heregulin, a specific activator of p185erbB2. Science 1992, 256:1205–1210
31. Goldman R, Levy RB, Peles E, Yarden Y: Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry 1990, 29:11045–11048
32. Dobashi K, Myers JN, Wada T, Brown VI, LeVea CM, Davis JG, Dobashi K, Greene MI: Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. Cell 1989, 58:287–292
33. Peles E, Ben-Levy R, Tzahar E, Liu N, Wen D, Yarden Y: Cell-type specific interaction of neu differentiation factor (NDF/heregulin) with neu/HER-2 suggests complex ligand-receptor relationships. EMBO J 1993, 12: 961–971
34. Plowman GD, Culouscou JM, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M: Ligand-specific activation of HER-4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci USA 1993, 90:1746–1750
35. Plowman GD, Green JM, Culouscou JM, Carlton GW, Rothwell VM, Buckley S: Heregulin induces tyrosine phosphorylation of HER4/p180^{erbB4}. Nature 1993, 336: 473–475
36. Culouscou J-M, Plowman GD, Carlton GW, Green JM, Shoyab M: Characterization of a breast cancer cell differentiation factor that specifically activates the HER-4/p180^{erbB4} receptor. J Biol Chem 1993, 268: 18407–18410
37. Akita RW, Schaefer GM, Carrawat KL, Lofgren JA, Fitzpatrick VD, Nuijens A, Vandlen RL, Cerione RA, Sliwkowski MX: A high affinity receptor for heregulin is formed from co-expression of erbB-2 and erbB-3. Am Assoc Cancer Res 85th annual meeting 1994, 35:43
38. Stancovski I, Hurwitz E, Leitner O, Ullrich A, Sela M: Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc Natl Acad Sci USA 1991, 88:8691–8695
39. Bacus SS, Ruby SG: Application of image analysis to the evaluation of cellular prognostic factors in breast carcinoma. Pathology Annual, part 1. Edited by PP Rosen, New York, and RE Fechner, Charlottesville, VA, 1993, pp 179–204
40. Bacus SS, Press MF, Bacus JW, Slamon DJ: HER-2/neu oncogene expression and proliferation in breast cancers. Am J Pathol 1990, 137:103–111
41. Noonan KE, Beck C, Holzmayer TA, Chin JA, Wunder JS, Andrulis IL, Gazdar AF, Willman CL, Griffith B, Von Hoff DD, Roninson IB: Quantitative analysis of MDR1 expression in human tumors by polymerase chain reaction. Proc Natl Acad Sci USA 1990, 87:7160–7164
42. Wen D, Peles E, Cupples R, Suggs SV, Bacus SS, Luo Y, Trail G, Hu S, Silbiger SM, Ben-Levy R, Luo Y, Yarden Y: Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell 1992, 69:559–572
43. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA: Isolation and characterization of ERBB-3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA 1989, 86:9193–9197
44. Bacus SS, Bacus JW, Bacus JV, Chin DM, Ortiz R, Stern RK, Morrow DW: Prostate adenocarcinoma: an image analysis technique for DNA ploidy determination. Lab Med 1993, 24:225–231
45. Kurki P, Ogata K, Tan EM: Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 1988, 109:49–59
46. Bravo R: Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. Exp Cell Res 1986, 163:287–293
47. Bacus S, Flowers JL, Press MF, Bacus JW, McCarty KS: The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. Am J Clin Pathol 1988, 90:233–239
48. Kraus MH, Popescu NC, Amsbaugh SC, King CR: Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms, EMBO J 1987, XX:605–610
49. Fady C, Gardner AM, Gera JF, Lichtenstein A: Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells: selective effects on HER-2/neu-overexpressing cells. Cancer Res 1992, 52:764–769
50. Bacus SS, Stancovski I, Huberman E, Chin D, Hurwitz E, Mills GB, Ullrich A, Sela M, Yarden Y: Tumor-inhibitory monoclonal antibodies to the HER-2/neu receptor induce differentiation of human breast cancer cells. Cancer Res 1992, 52:2580–2589
51. Bacus SS, Kiguchi K, Chin D, King CR, 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 Carcinogen 1990, 3:350–362
52. Nesland JR, Ottestad L, Heikilla R, Holm R, Tveit K, Borresen AL. C-erbB-2 protein and neuroendocrine

expression in breast carcinoma. Anticancer Res 1991, 11:161–168
53. Marth C, Cronauer MV, Doppler W, Ofner D, Ullrich A, Daxenbichler G: Effects of interferons on the expression of the proto-oncogene HER-2 in human ovarian carcinoma cells. Int J Cancer 1992, 50:64–68
54. Hudziak R, Lewis GD, Shalaby MR, Essalu TE, Aggarwal BB, Ullrich A, Shepard HM: Amplified expression of the HER-2/erbB-2 oncogene induces resistance to tumor necrosis factor in NIH-3T3 cells. Proc Natl Acad Sci USA 1988, 85:5102–5106
55. Kalthoff H, Roeder C, Gieseking J, Humburg IN, Schimiegel W: Inverse regulation of human ERBB-2 and epidermal growth factor receptors by tumor necrosis factor. Proc Natl Acad Sci USA 1993, 90:8972–8976
56. Kalthoff H, Roeder C, Henne-Bruns D, Kremer B, Schmiegel WH: Inverse regulation of ERBB-2 and epidermal growth factor receptor-expression by tumor necrosis factor-α in pancreatic tumor cells. Am Assoc Cancer Res 85th annual meeting 1994, 32:43
57. Falls DL, Rosen KM, Corfas G, Lane WS, Fishbach GD: ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell 1993, 72:801–811
58. Shah NM, Marchionni MA, Isaacs I, Stroobant P, Anderson DJ. Glial growth factor restricts mammalian neural crest stem cells in a glial fate. Cell 1994, 77: 349–360
59. Marchionni MA, Goodearl ADJ, Chen MS, Bermingham-McDonogh O, Kirk C, Hendricks M, Danehy F, Misumi D, Sudhalter J, Kobayashi K, Wroblewski D, Lynch C, Baldassare M, Hiles I, Davis JB, Hsuan JJ, Totty NF, Otsu M, McBurney RN, Waterfield MD, Stroobant P, Gwynne D: Glial growth factors are alternatively spliced erbB-2 ligands expressed in the nervous system. Nature 1993, 362:312–318