Selection of Potentially Metastatic Subpopulations Expressing c-erbB-2 from Breast Cancer Tissue by Use of an Extravasation Model

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Overexpression of the c-erbB-2 gene-encoded p185^{c-erbB-2} is correlated with early onset of metastasis in breast cancer patients. Furthermore, the detection of blood-borne epithelium-derived clustered cells expressing p185^{c-erbB-2} was related to advanced stages in breast cancer. To further elucidate the receptor's function in the metastatic process of human breast cancers, we analyzed disaggregated cells and cell clusters from freshly dissected breast cancer tissues. We studied whether their capability of extravasation is correlated with their expression of c-erbB-2. A model for the venular wall was constructed by growing human umbilical vein endothelial cells (HUVECs) on porous membranes coated with basement membrane extracellular matrix. In four control breast cancer cell lines (SK-BR-3, MCF-7, MDA-MB-468, and MDA-MB-468, the latter transfected with a full-length c-erbB-2 cDNA vector) producing different levels of the c-erbB-2 receptor, the expression level correlated positively with the invasiveness of the cells. The invasive, predominantly clustered cells from 14 of 23 tumors were positively stained for p185^{c-erbB-2} by immunocytochemistry. Furthermore, we show that the invasive cell populations express the metastasis-associated proteins matrix metalloproteinase MMP-2, CD44, and integrins $\alpha v\beta 3$ and $\alpha 6$. In this first study on the behavior of cells and cell clusters from disaggregated operated cancers in an extravasation model, we could demonstrate the presence of c-erbB-2-expressing cell subpopulations within the individual breast cancers that are presumably of high metastatic potential. (Am J Pathol 1998, 153:1797-1806)

Breast cancer metastasis is the major cause of death for patients with breast carcinomas. Metastasis is viewed as a highly selective competition, favoring the survival of a

subpopulation of metastatic tumor cells that pre-exists within the heterogeneous primary tumor.^{1,2} Early micro-metastasis in patients with small resectable cancer poses a great problem for the treatment of cancer.³ Hence, it is of major importance to understand the molecular mechanisms of cellular processes essential for cancer metastasis as a basis for new therapeutic approaches.

The c-erbB-2 (HER-2/neu) proto-oncogene encodes a receptor tyrosine kinase, p185^{c-erbB-2} or p185^{neu}, that shares extensive sequence homology with epidermal growth factor receptor (EGFR).⁴ Like EGFR, c-erbB-2 is expressed in various fetal and adult epithelia and is believed to play an important role in growth and development.^{5,6} In cancer, oncogenic amplification and/or overexpression of c-erbB-2 is found in many different human primary tumors.⁷ In human breast cancer, numerous studies have reported c-erbB-2 amplification and overexpression,⁸ and some of them found a positive correlation with earlier relapse and poorer overall survival of the patient.⁹⁻¹¹ In addition to the reported clinical correlations, experimental approaches using animal and *in vitro* models have provided evidence that the c-erbB-2 oncogene plays an important role in cancer metastasis.⁸ Yu and co-workers found that expression of c-erbB-2 promotes the invasion steps in the metastatic cascade in human lung and breast cancer cells, such as increased motility, migration through the extracellular matrix, and secretion of enzymes degrading the basement membrane.^{12,13} In a recently published study by Verbeek et al, overexpression of c-erbB-2 was related to random cell migration.¹⁴ It was also shown that p185^{c-erbB-2} interacts with members of the laminin receptor family ($\alpha 6\beta 4$ and $\alpha 6\beta 1$ integrins) and that this interaction might also contribute to generate a locomotive phenotype of carcinoma cells.¹⁵

In a previous study in our laboratory we observed that a high-risk group of breast cancer patients expressed p185^{c-erbB-2} within the primary tumor and on blood-borne epithelium-derived cells.¹⁶ The onset of progressive dis-

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ease was related to the occurrence of blood-borne epithelium-derived cells positively stained for *c-erbB-2* receptor protein. Here we report on the evaluation of the biological features of p185^{*c-erbB-2*}-positive cells and cell clusters from fresh breast cancer tissue by *in vitro* extravasation experiments. For our study, we designed an *in vitro* model for the venular wall that consists of an endothelial monolayer of human umbilical vein endothelial cells (HUVECs) growing on porous membranes covered with extracellular matrix basement membrane. Thus, the transendothelial penetration by breast cancer cells followed by invasion of the underlying basement membrane can be examined. We evaluated our *in vitro* model using four breast cancer cell lines expressing different levels of p185^{*c-erbB-2*}. One of them was transfected with the human *c-erbB-2* cDNA. We then tested cells from disaggregated surgical breast tissue in our model to explore the invasion capacity of cells from benign and malignant breast tissues and the role of *c-erbB-2* for their invasive potential. For the breast cancer cell lines we found that the level of *c-erbB-2* expression correlated positively with the cells' extravasation potential. Our results for fresh breast cancer tissues provide evidence that *c-erbB-2* expression is characteristic of cell populations with high locomotive capability that pre-exist within the primary tissues. Further immunocytochemical analysis of the invasive cell populations revealed the expression of proteins that are likely to be involved in the metastatic invasion process (matrix metalloproteinase MMP-2, CD44, and integrins $\alpha v \beta 3$ and $\alpha 6$). We conclude that we can select cell subpopulations in breast cancer tissues that are presumably of high metastatic potential.

Materials and Methods

Cell Lines and Culture

HUVECs were isolated from human umbilical cord veins as described by Gimbrone et al¹⁷ with modifications according to Friedl et al.¹⁸ The cells were grown on gelatin-coated flasks and passaged four to six times in a medium containing equal volumes of Iscove's modified Dulbecco's medium (IMDM) and Ham's F12 nutrient mixture (Life Technologies, Eggenstein, Germany) supplemented with 10 μ g/ml sodium heparin (Boehringer Ingelheim, Heidelberg, Germany), 5 μ g/ml transferrin, 2.5 ng/ml basic fibroblast growth factor (bFGF; Sigma, Deisenhofen, Germany), 5 μ mol/L β -mercaptoethanol, 2 mmol/L L-glutamine (Life Technologies), 100 U/ml penicillin, 100 μ g/ml streptomycin, 250 ng/ml amphotericin B (Sigma), and 15% fetal calf serum (FCS; PAA Laboratories, Linz, Austria).

Breast cancer cell lines MCF-7, SK-BR-3, and MDA-MB-468 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM; ICN, Eschwege, Germany) supplemented with 2 mmol/L L-glutamine, antibiotic drugs as above, and 10% FCS. For culture of transfectants, cells were grown under the same condi-

tions except for addition of 400 μ g/ml Geneticin (G418) (Sigma) to the culture medium.

Cell Transfection and Selection

MDA-MB-468 cells were stably transfected with the plasmid vector pCVN/HER-2 (generously provided by A. Ullrich, Martinsried, Germany) using the LIPOFECTIN-Reagent (Life Technologies) as described in the technical protocol. For control experiments, cells were transfected with plasmid vector pCVN lacking the *c-erbB-2* cDNA insert. Two days after transfection, cells were harvested and selected with 400 μ g/ml G418. For a second selection step, G418-resistant cells were positively sorted using immunomagnetic beads (Dynabeads, Dynal, Hamburg, Germany) coated with p185^{*c-erbB-2*}-specific antibody c-neu (Ab-5, Oncogene Research Products, Cambridge, MA). Sorted MDA-MB-468/HER-2 cells were >99% p185^{*c-erbB-2*}-positive as detected by FACS analysis (FACScalibur, Becton-Dickinson, Heidelberg, Germany).

Patients, Tissue Collection, and Disaggregation

Human mammary tissue specimens were received from the operating room in sterile tubes containing DMEM with 10% FCS and antibiotic drugs and were kept on ice until disaggregation. We examined 20 primary breast carcinomas (5 stage I and 15 stage IIA/IIIB) and 3 lymph node metastases (2 stage IIIB and 1 stage IV). Tumor histology was classified according to conventional criteria, and all identifiable lymph nodes were histologically examined. Mammary tissues from five patients with benign breast diseases (four with cystic mastopathia and one with fibroadenoma) were chosen as negative controls. Breast tissue was mechanically disaggregated by means of automated tissue disaggregator Medimachine (DAKO, Hamburg, Germany). For this purpose, tumor tissue was cut into small pieces and placed into a disposable disaggregation chamber (Medicon; DAKO) together with 1.5 ml of serum-free invasion medium (DMEM with 2 mmol/L L-glutamine, antibiotic drugs, and 0.1% bovine serum albumin (BSA; Sigma)). The Medicon was inserted into the motor unit of the machine and run for 2 minutes at a pre-fixed rotation speed of approximately 80 rpm. An aliquot of the cell suspension containing single cells and cell clusters up to 30 cells was examined microscopically for cell viability by Trypan blue exclusion. Cells were counted in a Neubauer hemacytometer.

Invasion Assays

Originally, our *in vitro* invasion assay was based on the procedure of Albini et al.¹⁹ Cell culture inserts with 8- μ m porous polyethylene terephthalate (PET) membranes were placed in 12-well plastic tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NY). The membranes were coated with basement membrane extracellular matrix (ECM; Harbor Bio-Products, Norwood, MA) at a concentration of 125 μ g/cm² by drying an appropriate

ECM dilution overnight under a laminar flow hood. Dried ECM was rehydrated with 500 μ l of HUVEC culture medium (see above) for 1 hour. HUVECs were seeded onto the coated membranes in a concentration of 2×10^5 cells/well. After culturing for 2 days at 37°C in a humidified atmosphere of 5% CO₂, HUVECs formed confluent monolayers, which was verified by panoptic staining (Diff-Quik; Baxter Health Care Co., Miami, FL). Cell culture inserts were used for up to 3 days after endothelial cells reached confluence.

For the invasion assays of breast cancer cell lines, cells were harvested with 0.25% trypsin/2 mmol/L EDTA (Life Technologies) and adjusted to a density of 2×10^5 cells/ml with serum-free invasion medium (see above), and 2×10^5 cells were placed onto the HUVEC monolayer on the ECM-coated membrane. The invasion assays of primary breast cancer cells were performed applying approximately 10^6 disaggregated cells to the membrane. The invasion medium was placed into the wells under the bottom sides of the membranes as well. Invasion assays were incubated for 48 hours at 37°C in 5% CO₂.

At the end of the invasion assay, the HUVEC monolayer and noninvading cells on the upper surface of the membrane were removed by cotton swabs and thorough rinsing with phosphate-buffered saline (PBS; pH 7.4). Invading cells on the bottom side of the membrane were fixed in 4% paraformaldehyde and characterized using double immunocytochemistry (see below).

Immunocytochemistry

Membranes with invasive cells were double stained by applying a combined immunogold-enzymatic technique according to Riesenberget al²⁰ with slight modifications. All antibodies were diluted in PBS containing 10% AB serum (Biotest, Dreieich, Germany) and 0.1% acetylated BSA (BSA-C; Aurion, Wageningen, The Netherlands), and each incubation step was followed by three 3-minute washes with PBS.

After fixation (see above), cells were permeabilized by incubation in 0.1% Triton X-100 in PBS for 10 minutes. Cells were blocked for 20 minutes in 10% AB serum with 0.1% BSA-C. Cells were incubated with rabbit polyclonal antibody *c-erbB-2* oncoprotein (DAKO), followed by incubation with 5-nm-gold-conjugated goat anti-rabbit antibody (Paesel & Lorei, Hanau, Germany). After a second fixation step with 2% glutaraldehyde in PBS and a second blocking step, mouse monoclonal biotinylated antibody to human cytokeratin 8 (Progen, Heidelberg, Germany) was applied. Samples were then incubated with alkaline-phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA).

For detection of metastasis-associated proteins, mouse monoclonal antibodies to matrix metalloproteinase MMP-2 (Oncogene Research Products), to CD44 (phagocytic glycoprotein-1 or hyaluronic acid receptor; Chemicon, Temecula), and integrin $\alpha v \beta 3$ (vitronectin receptor; Chemicon), and to integrin $\alpha 6$ (component of the laminin receptors; Novocastra Laboratories, New Castle,

UK) were applied. Samples were then incubated with rabbit anti-mouse immunoglobulins (DAKO) as a link antibody, followed by the next incubation step with a complex of calf intestinal alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (APAAP; DAKO).

The antibody binding to cytokeratin, MMP-2, CD44, and integrins $\alpha v \beta 3$ and $\alpha 6$, respectively, was visualized immunoenzymatically by using the DAKO Newfuchsin substrate system (DAKO) according to the manufacturer's protocol. Silver enhancement of colloidal gold particles was performed with a silver enhancement kit (IntenSE) purchased from Amersham (Braunschweig, Germany). After rinsing the samples with distilled water, freshly prepared silver enhancement mixture was applied for ~20 minutes while monitoring the reaction under the microscope. To abrogate the enhancement reaction, membranes were rinsed with distilled water. Samples were counterstained using Mayer's hematoxylin (Merck, Darmstadt, Germany). Finally, membranes were separated from the insert assembly and mounted with Aquamount improved (BDH Laboratory Supplies, Poole, UK) on microscope glass slides. To exclude nonspecific staining, unrelated rabbit IgG (Sigma) and mouse myeloma proteins (MOPC21; Sigma) served as a negative control for immunocytochemistry. Enzymatic and immunogold staining with silver enhancement was viewed by light microscopy and epipolarization using a fluorescent microscope (Laborlux S, Leica, Wetzlar, Germany) with an IGS filter (Leica).

Transmission Electron Micrography

For transmission electron microscopy (TEM), PET membranes with HUVEC monolayers were washed once with PBS and fixed with 2% glutaraldehyde. The samples were embedded in Epon 812, and ultra-thin sections were mounted on 200-mesh copper grids. The specimens were examined with a Philips EM at 60 kV.

Western Blot Analysis

For preparation of cell lysates, confluent cell monolayers were washed three times with PBS and scraped from 75-cm² tissue culture flasks in ice-cold lysis buffer containing 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Tris/HCl (pH 7.2), 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, and protease inhibitors (cocktail tablets Complete; Boehringer, Mannheim, Germany). Breast tissue was homogenized in the same lysis buffer. After 15 minutes on ice, the lysate was centrifuged at 4°C and $12,000 \times g$ for 1 hour. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL), and defined amounts of protein were electrophoresed on a 7.5% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (Roth, Karlsruhe, Germany), and nonspecific binding sites were blocked with 10% nonfat dry milk (De-Vau-Ge Gesundheitswerk, Lüneburg, Germany) in PBS with 0.1% Tween 20. Blots were probed with antibody *c-neu* (Ab-3) for detection of p185^{neu} (On-

cogene Research Products), followed by incubation with horseradish-peroxidase-conjugated goat anti-mouse antibody (Amersham). Both antibodies were used at a concentration of 0.1 $\mu\text{g/ml}$. Bands were visualized with the enhanced chemiluminescence (ECL) system (Amersham) with an exposure time of 3 minutes.

Results

Evaluation of an in Vitro Extravasation Model Using Breast Cancer Cell Lines with Different c-erbB-2 Expression

As a system that mimics the *in vivo* situation in blood capillaries, we used an *in vitro* model consisting of a porous PET membrane coated with extracellular matrix and a monolayer of HUVECs (Figure 1, A–C). Confluence of HUVEC monolayers was verified by panoptic staining (Figure 1A). The size proportions of the HUVEC monolayer (1), basement membrane layer (2), and PET membrane (3) are revealed by TEM (Figure 1C).

To evaluate the selection capacity of the model, a panel of breast cancer cell lines (SK-BR-3, MCF-7, and MDA-MB-468) expressing different levels of p185^{c-erbB-2} was tested for their extravasation potential. As shown in Figure 2, MDA-MB-468 cells express no detectable levels of p185^{c-erbB-2}, and only very few cells penetrated the HUVEC monolayer and the basement membrane after incubation for 48 hours. SK-BR-3 cells expressing high p185^{c-erbB-2} levels showed high transendothelial invasiveness, and MCF-7 cells expressing moderate amounts of p185^{c-erbB-2} showed increased invasiveness compared with MDA-MB-468 cells. To ensure that differences in the extravasation capacity were due to differences in the c-erbB-2 expression levels, and not to a different genetic background, we transfected low-invasive MDA-MB-468 cells with the plasmid vector pCVN/HER2, containing the full-length wild-type human c-erbB-2 cDNA,²¹ to generate the MDA-MB-468/HER-2 transfectants. As shown in Figure 2B, their c-erbB-2 expression level is between the one of MCF-7 and SK-BR-3. We used the MDA-MB-468/*neo* cell line, which was established by transfecting MDA-MB-468 cells with the empty pCVN vector, as a control. As for parental MDA-MB-468 cells, p185^{c-erbB-2} is not detectable in MDA-MB-468/*neo* cells. Again, we compared the c-erbB-2 expression level with the number of cells on the bottom side of the membrane and found significantly more cells from the MDA-MB-468/HER2 cell line with high c-erbB-2 expression, than MDA-MB-468/*neo* cells, which exhibited the same low extravasation capacity as the parental cells (Figure 2A).

For visualization of c-erbB-2 expression in the highly motile SK-BR-3 and MDA-MB-468/HER-2 cells, we characterized the cells on the bottom side of the porous membrane by double immunocytochemistry for epithelial marker cytokeratin 8 and p185^{c-erbB-2} (Figure 3). In the case of cells from disaggregated breast cancer tissue (see below), cytokeratin analysis was useful to characterize invasive cells with regard to their histological origin. As shown in Figure 3, A and C, for both c-erbB-2-over-

Figure 1. The model for extravasation. **A:** Density of the HUVEC monolayer grown for 48 hours on the upper side of the ECM-coated membrane is demonstrated by panoptic staining. Scale bar, 100 μm . **B:** Schematic drawing of the model. **C:** Cross section of the model vessel wall observed by transmission electron microscopy. Scale bar, 10 μm . 1, HUVEC monolayer; 2, extracellular matrix basement membrane; 3, PET membrane.

expressing cell lines (wild-type SK-BR-3 and MDA-MB-468/HER-2), double immunocytochemistry revealed an accumulation of p185^{c-erbB-2} in membrane protrusions (arrows) indicated by the black silver stain, whereas cytokeratin (red color) was mainly localized in the core of the cells. In Figure 3, B and D, the same cells are viewed by epipolarization, which enhances the sensitivity of the

Figure 2. Extravasation potential of breast cancer cell lines and expression of *c-erbB-2*. **A:** A total of 2×10^5 tumor cells were placed onto the HUVEC monolayer on the ECM-coated membrane. The columns show numbers of invasive cells of indicated breast cancer cell lines after a 48-hour incubation. Each column represents an average counting from five separate experiments. **B:** Immunoblot analysis for the *c-erbB-2*-encoded p185 proteins in the cell lysates of the indicated cell lines. Protein (20 μ g) from each sample was electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with a monoclonal anti-human *c-neu* antibody (Ab-3). The position of p185^{c-erbB-2} (p185) is indicated.

silver staining and makes the stain shine with a fluorescent-like bluish glow against a dark background.

Extravasation Capacity and *c-erbB-2* Expression of Cells from Disaggregated Surgical Breast Tissues

To investigate whether cells from fresh breast cancer tissues are capable of extravasation in our model and whether *c-erbB-2* plays an important role for this phenomenon, we tested 23 mechanically disaggregated malignant tumor tissues (20 primary breast cancers and three lymph node metastases; Table 1). Using double immunocytochemistry (see above), invasive cells were characterized with regard to their epithelial origin and *c-erbB-2* expression; 12 of 16 primary breast cancer tissues contained *c-erbB-2*-positive, predominantly clustered cells capable of invasion after 48 hours. There was no tumor that contained only single invasive cells. We never observed invasive cell clusters that were positive

for p185^{c-erbB-2} and negative for cytokeratin. In case a tumor contained *c-erbB-2*-expressing invasive cell clusters, all of the cells within the invasive clusters were positive for p185^{c-erbB-2}.

In Figure 4, invasive cells/cell clusters from surgical breast cancers characterized by double immunocytochemistry are shown. Figure 4A illustrates a single cell on the bottom side of the PET membrane. In Figure 4, C and E, typical invasive cell clusters are shown. The black silver stain on the membrane visualizes the *c-erbB-2* expression. In Figure 4, B, D, and F, the cells are viewed with epipolarization (see above). In some cases we observed that the invasive cells detached from the bottom of the membrane, attached to the plastic surface of the culture well, and proliferated for several days (not shown).

All carcinoma tissues that expressed detectable p185^{c-erbB-2} (eight primary breast carcinomas and two lymph node metastases) as determined by Western blotting on a piece of the tissue we also used for disaggregation (see Figure 5 and Table 1) contained invasive cell clusters or cells. Each of the three lymph node metastases contained invasive cells, and in two cases invasive cells/cell clusters were positive for p185^{c-erbB-2}, too. Initially, it seems to be paradoxical that four mammary carcinoma tissues, which were negative for the *c-erbB-2* protein, presented p185^{c-erbB-2}-positive invasive cells/cell clusters in the extravasation assay. This phenomenon can be explained by the presence of only very few *c-erbB-2*-expressing cell populations within a heterogeneous tumor, not detectable by Western blotting, that were capable of transendothelial migration. Interestingly, four breast carcinomas that did not contain invasive cells/cell clusters were pathologically staged lymph node negative. Those carcinomas did not express *c-erbB-2*. For several reasons, quantification of invasive cells from fresh breast tissue was substantially more difficult than for the cell lines (see previous section). First, there were remarkable differences in cell viability after disaggregation (between 10% and 60%). Second, tumors consist of varying proportions of carcinoma cells and surrounding stromal cells, eg, fibroblasts. And third, we observed the vast majority of the invasive cells to be organized in dense clusters (see Figure 4, C and E), which made determination of cell numbers very difficult. In a semiquantitative analysis, we classified the tissues we had tested in the three categories: (+), representing 1 to 10; +, representing 10 to 100; and ++, representing >100 invasive entities, considering a cell cluster or single cell expressing the epithelial marker cytokeratin as one entity (see Table 1). Regarding the correlation between *c-erbB-2* expression and motility, as observed using the cell lines (Figure 2), a similar trend was also evident for the tumor tissues applied to our extravasation model. There were five tumors with a high expression of p185^{c-erbB-2} as determined by Western blot analysis (breast carcinomas 10, 17, 18, and 20 and lymph node metastasis 21 in Figure 5), and four of them contained >100 invasive entities. The five tumors expressing medium or low levels of the receptor (breast carcinomas 3, 6, 13, and 14 and lymph node metastasis 23 in Figure 5) contained 10 to

Figure 3. Transendothelial invasive cells from breast cancer cell lines stained by double immunocytochemistry to cytokeratin and p185^{c-erbB-2}. **A:** An invasive SK-BR-3 cell was fixed on the bottom side of the membrane. Double immunocytochemistry was performed using a mouse monoclonal biotinylated antibody to human cytokeratin 8 and a rabbit polyclonal antibody to *c-erbB-2* oncoprotein. **C:** An invasive MDA-MB-468/HER-2 cell stained as described above. Membrane protrusions of both cell types contain less cytokeratin (red color from the Newfuchsin dye reaction) but accumulations of the *c-erbB-2* receptor visualized by the dark brown staining from the silver enhancement procedure (arrows). **B** and **D:** When viewing the same cells by epipolarization, the silver granules appear in a fluorescent-like bluish glow against a dark background. Scale bars, 10 μ m.

100 invasive entities, whereas 1 to 10 invasive entities occurred only in tumors without detectable expression of p185^{c-erbB-2}.

We used cells from five benign tissues (four cystic mastopathias and one fibroadenoma tissue), which should not have an invasive potential as a control. Unexpectedly, there were two single cells in one of the five

control benign breast tissues that were found on the bottom side of the PET membrane. This could be explained by a leakage in our model vessel wall or by the presence of a few premalignant cells in this tissue, which was histologically classified as a fibroadenoma. The first explanation is more likely, because the two invasive entities were single cells, which should pass a membrane

Table 1. Extravasation after 48 Hours and *c-erbB-2* Expression of Invasive Cells and Fresh Breast Tissues

Tissue samples	Number of samples			
	Invasive entities	<i>c-erbB-2</i> expression of invasive entities	p185 ^{c-erbB-2} immunoblot (tissue samples positive)	
Primary breast carcinomas (<i>n</i> = 20)	16	4 ++ 9 + 3 (+)	12	8
Lymph node metastases (<i>n</i> = 3)	3	1 ++ 1 + 1 (+)	2	2
Controls, tissues from benign breast diseases (<i>n</i> = 5)	1	1 (+)	0	0

Invasive entities = invasive clustered or single cells that stained positive for epithelial marker cytokeratin 8. (+), 1 to 10; +, 10 to 100; ++, >100 invasive entities.

Figure 4. Transendothelial invasive cells and cell clusters from disaggregated breast cancer tissues stained by double immunocytochemistry to cytokeratin and p185^{c-erbB-2}. **A:** Typical single double-positive cell on the bottom side of the membrane. Scale bar, 10 μ m. **C and E:** Double-positive cell clusters. Scale bars, 20 μ m. Co-localization of antibody binding to p185^{c-erbB-2} and cytokeratin is visualized by the presence of black grains on the background of a red-colored product. **B, D, and F:** The same cells are viewed using an epipolarization filter, which enables the discrimination of the specific luminous silver stain from nonspecific background staining.

leakage more easily than a cluster. This is also supported by the fact that neither of the cells expressed *c-erbB-2*. In addition, all benign samples were p185^{c-erbB-2}-negative as shown in Figure 5. As mentioned above, in contrast to this benign tissue, there was no malignant tumor that contained only single invasive cells, but at least a com-

bination of single and clustered cells or solely clustered cells that managed to migrate through the model vessel wall.

For further analysis of the invasive *c-erbB-2*-expressing entities from fresh breast cancer tissue with regard to their biological relevance in the metastatic process, we

Figure 5. Western blot analysis of expression of p185^{c-erbB-2} in fresh breast tissues. Protein (20 μ g) from a piece of the tissue we also used for disaggregation was analyzed with a monoclonal anti-human *c-neu* antibody (Ab-3). The position of p185^{c-erbB-2} (p185) is indicated. **Lanes 1 to 20,** protein from primary breast cancers; **lanes 21 to 23,** protein from lymph node metastases; **lanes 24 to 28,** protein from benign breast tissues; **lane 29,** protein from control MDA-MB-468/HER-2 cells. Breast carcinomas 3, 6, 10, 13, 14, 17, 18, and 20 and lymph node metastases 21 and 23 expressed detectable levels of p185^{c-erbB-2} after a 3-minute exposure using a chemiluminescence immunoblot procedure.

Figure 6. Transendothelial invasive cell clusters from breast cancer tissues stained by double immunocytochemistry to p185^{c-erbB-2} and MMP-2, CD-44, α v β 3, and α 6 integrins, respectively. The red color from the Newfuchsin dye reaction indicates the expression of the respective metastasis-associated protein, whereas expression of p185^{c-erbB-2} is visualized by the brown/black staining from the silver enhancement reaction. Scale bars, 20 μ m. **A:** Double-positive cell cluster for MMP-2 and p185^{c-erbB-2}. **B:** Double-positive cell cluster for CD44 and p185^{c-erbB-2}. **C:** Double-positive cell cluster for α v β 3 integrin and p185^{c-erbB-2}. **D:** Double-positive cell cluster for α 6 integrins and p185^{c-erbB-2}. The small inset in the lower right corner represents a cell cluster that was incubated with unrelated rabbit IgG and mouse myeloma proteins as negative controls for double immunocytochemistry.

performed immunocytochemistry with monoclonal antibodies directed against matrix metalloproteinase MMP-2, CD44 (hyaluronic acid receptor), and integrins α v β 3 and α 6, which are suggested to be involved in the metastatic invasion process.^{2,22-25} In four of four tumors with p185^{c-erbB-2}-expressing invasive cell clusters, the invasive entities also expressed CD44; in three of four, they expressed α v β 3 integrin; and in two of four, they expressed MMP-2 and α 6 integrins.

In Figure 6, A–D, examples of transendothelial invasive cell clusters characterized by double immunocytochemistry to p185^{c-erbB-2} and MMP-2, CD44, and α v β 3 and α 6 integrins, respectively, are shown. Again, the brown/black silver stain visualizes the *c-erbB-2* expression, whereas the red color reveals the expression of the respective metastasis-associated protein.

Discussion

It is widely accepted that metastasis is not a random, but a highly selective, process that favors the survival and growth of a few subpopulations of cells that pre-exist within the parent neoplasm.²⁶ As discussed by Liotta et al,² estimating the size of the metastatic subpopulation

has clinical significance, as a prognostic assay based on a sample of the primary tumor would be highly inaccurate if the aggressive subpopulation was only a small proportion of the total number of tumor cells. In previous studies in our laboratory, we isolated clustered cells positive for cytokeratin/p185^{c-erbB-2} from the peripheral blood of breast cancer patients.¹⁶ Therefore, we were interested in the metastatic behavior of cells from fresh tumor tissue, especially in the step of extravasation, which follows the tumor cells' passage through the bloodstream. We developed a model consisting of a HUVEC monolayer growing on porous membranes coated with extracellular matrix, which mimics the venular walls *in vitro*. By using fresh tumor tissue in this model, we were also able to examine whether the previous findings about the role of *c-erbB-2* in breast cancer metastasis using breast cancer cell lines that had been cultivated extensively *in vitro* could be extrapolated.

By applying several breast carcinoma cell lines to the model, our extravasation assay was evaluated. We found a strong positive correlation between the level of p185^{c-erbB-2} expression and the numbers of cells that had penetrated the barrier after 48 hours. It should be noted that all cell lines used for our experiments derive

from metastatic breast carcinomas and that increased expression of *c-erbB-2* augmented the invasive capacity in our model. This was clearly demonstrated by the *c-erbB-2* cDNA transfection of the low-invasive MDA-MB-468 cell line. Simultaneously, we demonstrated that our *in vitro* model is suitable to identify cancer cells that are likely to succeed extravasation. Furthermore, our results from testing the breast cancer cell lines support the data of Yu et al¹² and Tan et al¹³ who found that the introduction of the human *c-erbB-2* gene into lung and breast cancer cells promotes the invasion steps in the metastatic cascade, such as increased motility, migration through extracellular matrix, and secretion of basement-membrane-degrading enzymes. We extended their results in that we demonstrated that the migration through an endothelial monolayer grown on extracellular matrix is also enhanced by the increased expression of *c-erbB-2*.

Our finding that all tumor tissues that express *c-erbB-2* contained invasive entities (Table 1) and that the invasive cell number tends to be related to the level of *c-erbB-2* expression underlines the important role of the *c-erbB-2* receptor for the transendothelial invasiveness of the cells and correlates with our results for the breast carcinoma cell lines. We observed a high ratio of p185^{c-erbB-2}-positive, highly motile cells and cell clusters that derived from disaggregated breast cancer tissues. Thus, we assume an important functional role of the *c-erbB-2* receptor in the metastatic steps that require cell migration, eg, the extravasation process.

Recent studies have shown that overexpression of *c-erbB-2* is sufficient to induce cell migration.¹⁴ It was reported that a critical level of p185^{c-erbB-2} seems to be necessary to achieve transformation,²⁷ which can be explained by a model in which there is an equilibrium between monomeric and dimeric forms of *c-erbB-2*.²⁸ As the quantity of p185^{c-erbB-2} increases by overexpression, the equilibrium is shifted to the dimeric state resulting in constitutive activation of the tyrosine kinase and inappropriate cellular signaling, subsequently leading to a locomotive phenotype.²⁸ Thus, it was not necessary to add an agonist of the *erbB* receptors (eg, epidermal growth factor, EGF) to our extravasation assay to stimulate cell motility.

The process of cell migration requires the coordinated activation of both growth factor and adhesion receptor signaling.¹⁴ It has been described that signals downstream of *c-erbB-2* can modulate integrin-mediated processes.²⁹ The reported interaction of p185^{c-erbB-2} with members of the integrin family¹⁵ may also contribute to generate a more invasive phenotype in carcinoma cells.

This is the first study of the behavior of cells and cell clusters from disaggregated fresh breast cancers in an extravasation model. Our finding of predominantly clustered carcinoma cells that managed to migrate through our model vessel wall is consistent with data from Friedl et al³⁰ that suggests that locomoting highly polarized clustered cells are prime candidates for precursors of metastasis formation. It also backs up our data that mainly cyokeratin/p185^{c-erbB-2}-positive clustered cells are found in the peripheral blood of breast cancer pa-

tients, which we assumed to be the metastasis-forming entities.¹⁶

Our discovery of *c-erbB-2* expression in the majority (14 of 19, Table 1) of transendothelial invasive cell clusters and single cells from surgical breast cancers supports the assumption that p185^{c-erbB-2} indicates even small subpopulations with high invasion potential within the primary tumor. This is substantiated by our finding that four tumors with no detectable expression of *c-erbB-2* by Western blotting contained cells/cell clusters with high locomotive potential that expressed p185^{c-erbB-2}. Moreover, the immunocytochemical detection of several presumably metastasis-associated proteins (MMP-2, CD44, and integrins $\alpha v \beta 3$ and $\alpha 6$),^{2,22-25} which are expressed by the invasive cells/cell clusters selected in our extravasation model, further supports this view.

As mentioned above, metastasis results from the preferential survival and growth of a few subpopulations of cells that pre-exist within the parent tumor.²⁶ Clinical data support this view.³¹ Overexpression of *c-erbB-2* even on a focal basis in cancer tissues has been taken as indicating patients who are poorly responsive to adjuvant chemotherapy.³¹ The detection of those entities in a primary tumor that are less sensitive to chemotherapy may be achieved by our assay. In addition, the more detailed characterization of the metastatic subpopulations could be essential for devising new therapeutic approaches.

Our extravasation assay allows an analysis of individual breast tumors *in vitro* and may help to identify those patients who are prime candidates for more aggressive chemotherapeutic regimens, whereas other patients can be protected from the harm of an aggressive chemotherapy. Thus, by creating this assay we made a contribution to the individualization of the clinical management of breast cancer.

Thus, our findings may close a gap in that we selected the same cells with regard to their extravasation capacity we would have later isolated from the patients' bloodstream. Moreover, our experimental results complement the findings of Pantel et al³² and Niehans et al³³ who reported that p185^{c-erbB-2}-positive cells from breast cancer patients lodged in the bone marrow or spread to distant organs. Our view is also supported by the finding that the four breast tumors that did not contain invasive entities in our model were derived from patients without detectable lymph node metastasis. Nevertheless, because of the small sample number in this study and the short follow-up time, we were not able to detect further correlations between the tumor staging and/or prognosis and invasive potential of the cells from the disaggregated tumors.

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