[CANCER RESEARCH 62, 1020–1024, February 15, 2002]

Advances in Brief

Early Placenta Insulin-like Growth Factor (pro-EPIL) Is Overexpressed and Secreted by c-erbB-2-positive Cells with High Invasion Potential[1]

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Abstract

By differential-display PCR a subclone of the SKBR3 cell line with high *in vitro* transendothelial invasiveness was identified to express increased levels of the *INSL-4* gene. This new member of the insulin-like growth factor family encodes for a peptide, designated early placenta insulin-like (EPIL), being expressed in the so-called "invasive" phase of the placental development. Immunohistochemistry on tissue microarrays revealed a heterogeneous expression of EPIL in breast cancer tissue and no expression in the surrounding stroma cells. A coexpression of pro-EPIL and c-erbB-2 could be observed predominantly in cell clusters at the infiltrating edge of the tumor. Our results give new suggestions for the presence of a signaling network of receptor tyrosine kinases underlying breast cancer invasion and metastasis.

Introduction

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 preexists within the heterogeneous primary tumor (1, 2). Early micrometastasis 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 type I receptor tyrosine kinase c-erbB-2, belonging to the erbB family, defined a more aggressive type of breast cancer with early onset of metastasis in several clinical studies (4–11). In a previous study, we observed that a high-risk group of breast cancer patients expressed c-erbB-2 within the primary tumor and on blood-borne epithelium-derived cell clusters (12). Moreover, c-erbB-2-positive cell subpopulations from the breast cancer cell line SKBR3 and cell clusters from fresh breast cancer tissue displaying high invasiveness could be selected by *in vitro* extravasation experiments based on the transendothelial penetration of an endothelial monolayer of HUVECs[3] by breast cancer cells followed by the invasion of an underlying basement membrane (13). Thus, we provide evidence that c-erbB-2 expression indicates cell populations with high extravasation capability. However, considerable numbers of cells expressing c-erbB-2 did not display this feature leading to the assumption that additional processes, implying yet unidentified molecules, have required the full invasive phenotype. In this context, several observations showed that the IGF family members can influence the key steps required for metastatic spread of breast cancer by contributing to extracellular matrix degradation, cellular chemotaxis, and angiogenesis (14). Particularly, these may act on c-erbB-2 phosphorylation through IGF receptors present on the surface of breast tumor cells (15, 16).

The present study was designed to identify molecules involved in the high invasion potential of a SKBR3 cell subclone by using differential display PCR. Our results showed that a member of the insulin superfamily identified recently, namely the *INSL-4* gene, was overexpressed in the invasive subclone in comparison to the parental clone (17). The *INSL-4* gene encodes for a peptide, designated EPIL, which is mainly detected in the trophoblastic cells of placenta during the first trimester of the gestation (18). Interestingly, INSL-4 expression behaves similar to that described for several growth factors, including transforming growth factor-α and epidermal growth factor, being expressed in the so-called invasive phase of the placental development. Finally, by using tissue array immunohistochemistry, we found that, in contrast to the IGF-I and -II expression pattern, EPIL peptides are produced by invasive breast cancer cells.

Materials and Methods

Cell Lines and Culture. Breast cancer cell line SKBR3 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM (ICN, Eschwege, Germany) supplemented with 2 mM L-glutamine, antibiotic drugs as described below, and 10% FCS. The invasive subclone was obtained from the SKBR3 cells, which could penetrate the extravasation model. The model system contained HUVECs. which 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 and Ham's F12 nutrient mixture (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10 μg/ml sodium heparine (Boehringer Ingelheim, Heidelberg, Germany), 5 μg/ml transferrin, 2.5 ng/ml basic fibroblast growth factor (Sigma Chemical Co., Deisenhofen, Germany), 5 μM β-mercaptoethanol, 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Sigma Chemical Co.), and 15% FCS (PAA Laboratories, Linz, Austria).

***In Vitro* Growth Rate Analysis.** The *in vitro* growth rates of the parental SKBR3 cell line and the invasive subclone were assessed by counting increases in cell number in a Neubauer hematocytometer after staining with trypan blue.

Invasion Assays. The assays were performed as described by Roetger *et al.* (13). In brief, cell culture inserts with 8-μm porous polyethylene terephthalate membranes were coated with ECM (Harbor Bio-Products, Norwood, MA) at a concentration of 125 μg/cm^2 by drying an appropriate ECM dilution overnight under a laminar flow hood. HUVECs were seeded onto the rehydrated coated membranes in a concentration of 2×10^5 cells/well. After confluent monolayer

Received 12/11/01; accepted 1/2/02.

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[1] Supported by grants Innovative Medizinische Forschung BÜ 2 2 00 18, Deutsche Forschungsgemeinschaft BR 1756/5–1.

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[3] The abbreviations used are: HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; EPIL, early placenta insulin-like; DD, differential-display PCR; IGF, insulin-like growth factor; ECM, basement membrane extracellular matrix; RPA, RNase protection assay; IRMA, immunoradiometric assay; TMA, tissue microarray, INSL-4, insulin-like 4; RLX, relaxin; APAAP, alkaline-phosphatase-antialkaline phosphatase method; pro-EPIL, early placenta insulin-like peptide.

formation 2×10^5 SKBR3 cells were placed onto the HUVEC monolayer on the ECM-coated membrane. The invasion assays of primary breast cancer cells were performed applying $\sim 10^6$ 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 h, and, thereafter, HUVEC monolayer and noninvading cells on the upper surface of the membrane were removed. Invading cells on the bottom side of the membrane were cultured in as described above.

Differential Display PCR and RNA Preparation. Total RNA from SKBR3 cell lines were isolated with RNAzol B reagent (Biotex Laboratories, Houston, TX). Two-base anchored oligodesoxythymidylate primers $HT_{11}G$, $HT_{11}A$ and $HT_{11}C$ were used to reverse transcribe RNA from SKBR3 cells into first-strand cDNAs, which were amplified subsequently by PCR using the arbitrary upstream primer 5′-$AAGCT_{11}C$-3′ of the RNAimage kit 1 and 2 (GenHunter, Nashville, TN). PCR conditions used were the same as described previously. PCR products were analyzed on a 6% DNA sequencing gel using 0.5 mM α-^{35}S-labeled dATP (1200 Ci/mmol). The bands on the cDNA ladder that were unique to SKBR3 parental cell line or to the invasive subclone were cut off the gel, eluted, and reamplified by PCR.

Cloning and Sequencing. The reamplified cDNA bands were cloned into plasmid PCRII using TA cloning kit (Invitrogen, San Diego, CA). Individual clones were sequenced using an automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany).

RPA. Differential expression of EPIL mRNA in SKBR3 parental cell line *versus* the invasive subclone was confirmed using PCRII plasmids carrying the specific sequence of EPIL as a probe in quantitative RPA. RPA was performed by nonradioactive HybSpeed RPA kit according to the manufacturer's instructions (Ambion, Austin, TX).

Immunofluorescence Studies. For immunofluorescence studies the cells were fixed and permeabilized, and blocked with 10% human AB-serum (AB-serum for serological reactions; Biotest, Dreieich, Germany) to inhibit nonspecific staining. The cells were additionally incubated with the following antibodies: rabbit polyclonal anti-c-erbB-2 antibody (0485; Dako, Hamburg, Germany), monoclonal mouse anti-EPIL antibody EPIL08 (19). The mouse antibody and the rabbit antibodies were visualized using polyclonal Alexa 594-conjugated sheep-antimouse IgG F(ab′) fragments and FITC-conjugated goat-antirabbit antiserum, respectively, which were applied for 30 min at room temperature. After rinsing, the slides with PBS staining was evaluated by a fluorescence microscope Laborlux S (Leica, Wetzlar, Germany).

Flow Cytometry. Fixed and permeabilized cells ($1–3 \times 10^6$) were incubated in a total volume of 100 μl with 2 μl of anti-EPIL mouse mAb for 30 min on ice. Anti-c-erbB-2 polyclonal rabbit antibody (0485; Dako) was used as a positive control. The mouse antibodies were visualized using polyclonal PE-conjugated pig-antimouse IgG F(ab′) fragments and FITC-conjugated sheep-antirabbit antiserum, which were applied for 30 min at room temperature, respectively. Nonspecific staining was controlled by using mouse and rabbit isotype control antibodies of the same IgG subclasses and concentrations.

Immunoassay for the Detection of pro-EPIL. EPIL peptide levels were measured in the culture supernatants using a two-site ≪sandwich≫ IRMA (19). Briefly, this IRMA is based on two mAbs raised against synthetic peptides analogous to two distinct regions of the pro-EPIL polypeptide: (*a*) mAb EPIL08, directed to the 88–98 C chain portion, is used as the capture antibody on a solid phase support; and (*b*) mAb EPIL02, directed to the 125–137 A chain portion, serves as the radiolabeled indicator. Thus, this assay detects all of the EPIL molecular forms including the connecting C peptide. The sensitivity of the IRMA is 2 ng/ml.

Tissue Array Construction and EPIL-Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue of 104 breast cancer cases were used for the production of a multitumor TMA. These consisted of 48 lymph node negative (N0) tumors, 29 T1, 50 T2, 11 T3, and 14 T4 carcinomas. Eighty-six were ductal invasive, 3 tubular invasive, 8 lobular invasive, 4 medullary invasive, and 3 mucinous invasive carcinomas, respectively. To gain an overview about the intratumoral heterogeneity of EPIL expression, three spots per tumor have been taken according to standard procedures using an H&E-stained section to guarantee representative tumor spots on the array (20). The spot diameter was 0.6 mm, and the distance between the spots was 1 mm. One spot originated from the center of the tumor, the two others were punched out of the infiltrative border of the tumor.

Immunohistochemistry was performed on 4-μm thick sections according to standard procedures. Antigen retrieval was done by use of a microwave (Epil 08 15′, citratbuffer; ER 30′ microwave, citratbuffer) and an autoclave respectively (Mib-1 10′autoclave, 120°C, Citratbuffer). No antigen retrieval procedure was required for c-erbB2. The primary antibodies (ER Ventana monoclonal ER Clon 6F11; Epil mouse mAb, kindly provided by J. Bidart; Ref. 19; c-erbB2 polyclonal Dako A0485; Mib-1 Dianova monoclonal DIA 505) were detected by the use of a 3,3′-diaminobenzidine detection kit (ER Ventana), and with the APAAP using a monoclonal APAAP complex (1:100 in RPMI 1640, 60′ in room temperature; DAKO).

Quantification of immunohistochemical staining was performed according to conventional standards (c-erbB2, "Dako-Score"). Because in the tumor spots either all or none of the cells showed an immunoreaction for Epil 08, only the staining intensity was evaluated in a semiquantitative manner (absent, weak, or strong). The proliferation rate (Mib-1) was subdivided into low, intermediate, and high (<10%, 10–25%, and >25% immunoreactive nuclei).

Results

Identification of the INSL-4 Gene. An *in vitro* model consisting of a porous polyethylene terephthalate membrane coated with extracellular matrix and a monolayer of HUVECs was used as a system mimicking the *in vivo* situation in blood capillaries (13). About 1% of the SKBR3 cells, expressing high c-erbB-2 levels, displayed transendothelial invasiveness on this three-layer-system. SKBR3 cells showed a rather high invasiveness compared with other cell lines expressing moderate or negligible levels of c-erbB-2 (MCF-7 and MDA-468; Ref. 13).

The invasive subclone was cultured, and RNA was isolated. Fig. 1*A* illustrates the mRNA differential display present in the invasive subclone and in the parental cell line. One band could be identified (arrow in Fig. 1), which presents a higher intensity in the invasive subclone in comparison to that in the parental cells. Reamplification of the cDNA, subcloning, and sequencing revealed the identity of the

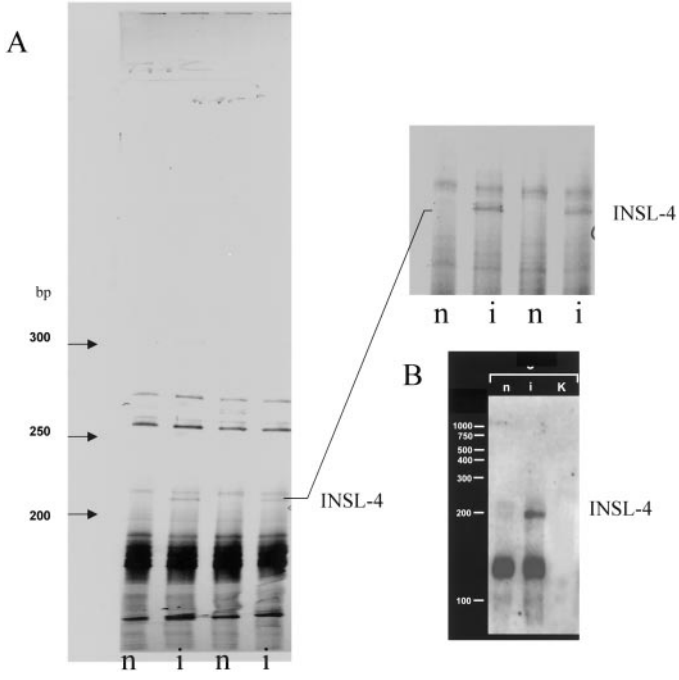

Fig. 1. Identification by DD of altered gene expression in parental SKBR3 cells and invasive subclone. *A*, DD was carried out using a 5′-arbitrary primer (5′-AAGCTTTTACCGC-3′) and a 3′-anchored primer (5′-$AAGCT_{11}C$-3′), and resolved by electrophoresis. *Lanes* showing the mRNA expression of the parental SKBR3 are labeled by *n* and those for the invasive subclone by *i*. *Top right inset*, magnification of the area of the gel containing the bands indicating INSL-4 mRNA expression. *B*, confirmation of DD pattern by RPA. Negative control is shown on *Lane K*.

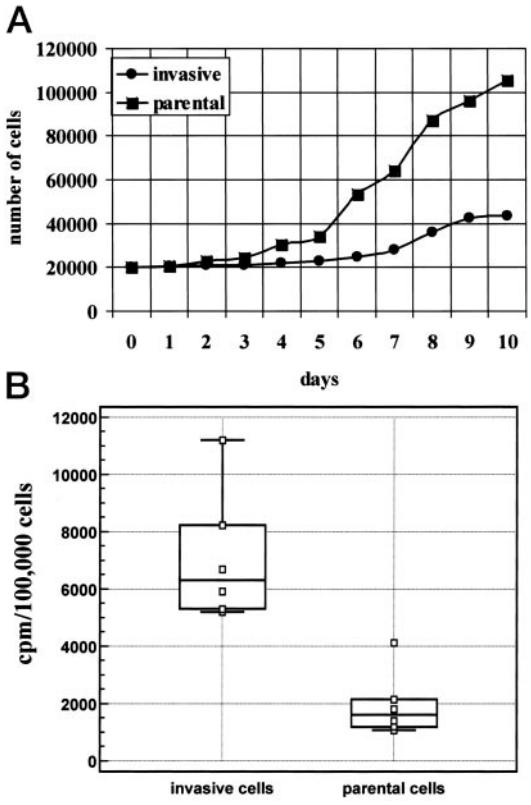

Fig. 2. Growth curves of the parental SKBR3 cell line, and the invasive subclone and pro-EPIL concentrations in the culture medium adjusted for cell number. *A*, cell numbers as determined in triplicate every 24 h by Neubauer hemocytometer. The mean coefficient of variation was 6.4% ± 5.1%. *B*, pro-EPIL concentration in the culture medium adjusted to 100,000 cells measured at the exponential growth phase of the cells through days 5 and 10. A significant difference in pro-EPIL expression was estimated ($P < 0.001$).

mRNA as that of the *INSL-4* gene. To confirm that INSL-4 mRNA is differently expressed in the invasive subclone *versus* the parental cell line, a plasmid containing the cDNA sequence of the *INSL-4* gene was used as a probe in a quantitative RPA assay. As shown in Fig. 1*B*, up-regulation of this mRNA in the invasive subclone of SKBR3 was observed.

Detection of EPIL Peptides. Expression of mRNA does not imply that proteins are synthesized, as exemplified by HLA class I expression in placenta. Therefore, parental SKBR3 cells and the invasive subclone were stained with an antibody directed to pro-EPIL. Fluorescence immunocytochemistry and flow cytometry indicated that EPIL peptides are detectable in these cells with only a slight higher intensity in the invasive subclone (flow cytometry mean fluorescence 6.9) compared with that in the parental cell line (flow cytometry mean fluorescence 5.5). The differences in c-erbB-2 expression between the parental cell line and invasive subclone were not detectable (flow cytometry mean fluorescence invasive subclone 31.4 and parental cell line 33.2). Because IGFs are small peptides released extracellularly, cells were seeded at a number of 20,000 grown in six-well plates, and EPIL levels were measured in the culture medium when the log phase of growth was reached between day 5 and day 10. Low levels of EPIL peptides were detected with concentrations ranged from 2 to 20 ng/ml. Adjusted for the number of cells, a five-time higher secretion rate of pro-EPIL could be determined for the invasive subclone than for the parental SKBR3 cell line ($P < 0.001$; $n = 6$; Student's *t* test; Fig. 2*B*). Interestingly, the invasive subclone grew significantly slower than the parental cell line as shown in Fig. 2*A*.

Expression and Distribution of EPIL Peptides in Invasive Breast Cancer Tissues. A TMA block containing tissue samples collected from different representative regions of the 104 donor tissues was constructed. Applying antibodies to EPIL peptides revealed that 89 of 104 (86%) tumors were moderately stained (67%) or highly positive (19%) in at least one tumor area of the TMA. Heterogeneity of EPIL peptide expression was high in most of the tumors, which displayed highly positive staining areas adjacent to negative areas of the tumor. Normal breast epithelium in the array samples stained merely negative for EPIL peptides and, when observed, positive staining was always weak. In contrast to observations on IGF-I and -II but in agreement with those on RLF, EPIL peptides were exclusively expressed in the cancer tissue. Surrounding stroma (fibroblasts and lymph nodes) stained negative as shown in Fig. 3. Moderate combined with high simultaneous expression of one of both pro-EPIL and c-erbB-2 was observed in 45.4% of the tissue spots. A homogeneous (3+) c-erbB-2 and high EPIL expression (2+) staining in one spot was only observed in four cases. However, cancer clusters found at the infiltrating border of the tumor stained highly positive for both parameters (Fig. 3). Surprisingly, the isolated EPIL expression was significantly related to a high mitotic activity as indicated by a high staining for Ki-67 (Mib-1; $P < 0.01$; Table 1). A decrease occurred in

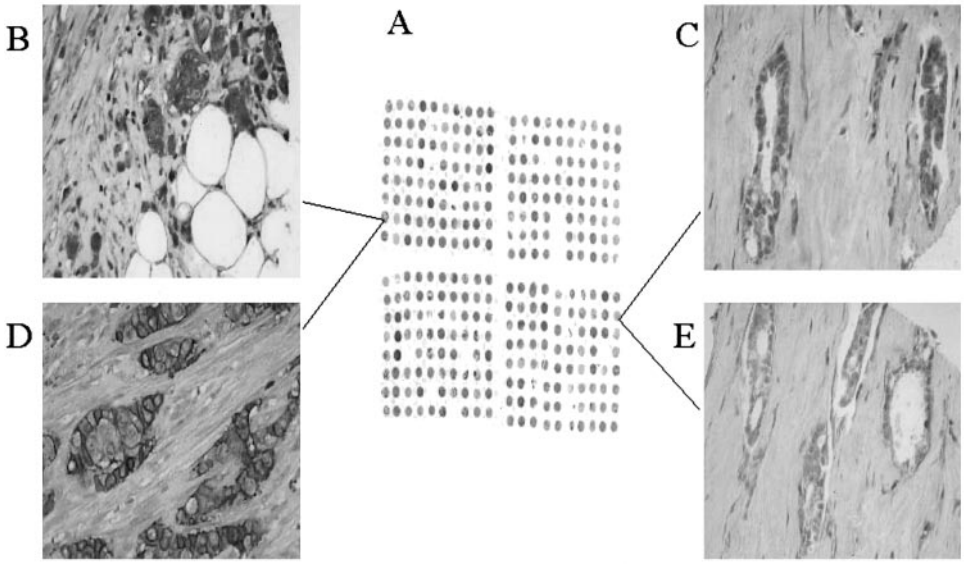

Fig. 3. Immunohistochemistry for pro-EPIL and c-erbB-2 on breast cancer TMAs. *A*, representative section of the TMA used containing 3 samples of 104 breast cancer cases, respectively (*HE*). *B*, immunohistochemical staining of EPIL in an invasive breast cancer case displaying a strong cytoplasmic staining reaction in the tumor cells invading the adjacent fat tissue (×20; APAAP). *D*, strong membrane bound immunoreactivity for anti-c-erbB-2 antibody in the more central part of the tumor shown in *B* (×20; APAAP). *C* and *E*, negative immunoreactivity for EPIL and c-erbB2, respectively, in a case of a tubular invasive carcinoma, which are characterized by a low invasive and metastatic potential (×20; APAAP).

Table 1 *Crosstabulation of pro-EPIL and/or c-erbB-2 expression with Ki-67 expression determined by immunohistochemistry*[a]

	EPIL (+)ve[b]/ c-erbB-2 (−)ve[c]	EPIL (+)ve[b]/ c-erbB-2(+)ve[c]	EPIL (−)ve[b]/ c-erbB-2(−)ve[c]
Ki-67 (−)ve[d]	57.7%	75.8%	81.4%
Ki-67 (+)ve[e]	42.3%	24.2%	18.6%

[a] $P < 0.01$ (χ^2 test).
[b] EPIL(+)ve = strong EPIL staining; EPIL(−)ve = absent to weak EPIL staining.
[c] c-erbB-2(−)ve = DAKO-score 0 to +1; c-erbB-2(+)ve = DAKO-score +2 to +3.
[d] Ki-67 (−)ve ≤ 25% immunoreaktive nuclei in one spot.
[e] Ki-67 (+)ve ≥ 25% immunoreaktive nuclei in one spot.

tumor areas coexpressing EPIL peptides and c-erbB-2. Furthermore, there was no statistical significant correlation with the estrogen receptor status of the tumor.

Discussion

Previously, we isolated clustered cells positive for c-erbB-2 from the peripheral blood of breast cancer patients (12). Therefore, we were interested in the metastatic behavior of cells, especially in the step of extravasation, which follows the tumor cell passage through the blood stream. We continued our work by developing an *in vitro* model consisting of a HUVEC monolayer growing on porous membranes coated with extracellular matrix (13). By applying several breast carcinoma cell lines to our extravasation assay, we found a strong positive correlation between the level of c-erbB-2 expression and the numbers of cells that had penetrated the barrier after 48 h (13). Nevertheless, the portion of the cells with the highest invasion potential, SKBR3, was ~1% of the total cell number applied to the extravasation assay for 48 h (13).

To discover genes additionally expressed in the invasive subclone of SKBR3 that contribute to the invasive phenotype we cultured the invasive cells. We isolated RNA from the subclone and the parental SKBR3 cells, and applied the powerful method of DD. DD is a rapid method for screening and identifying at the mRNA level altered gene expression between two or more cell populations. In the invasive subclone we identified by DD the up-regulated gene *INSL-4*, which was identified recently as a new member of the insulin-like family (17). The overexpression of the gene in the invasive subclone was confirmed by quantitative RPA using a plasmid containing the cDNA fragment that was reamplified by DD primers and identified by sequencing.

INSL-4 encodes a 139-amino acid polypeptide tentative named pro-EPIL. Like other members of the insulin-like superfamily, the peptide is synthesized as a preprohormone characterized by a signal peptide, a B chain, a connecting C peptide, and a terminal A chain (17). Experimental data showed that pro-EPIL is more closely related to RLX than to the other members of the insulin family, suggesting that, as RLX, EPIL would be involved in maintenance of endometrium decidualization during early pregnancy (21). This might imply that pro-EPIL belongs physiological to an autocrine mechanism that assures the survival of the embryo at the maternal uterus wall, a mechanism which would be also supporting for cancer cells invading on their migration through basal membranes and stromal tissue. Interestingly, pro-EPIL peptide levels were also found in significantly higher amounts in amniotic fluids from abnormal pregnancies, namely trisomy 21, than in amniotic fluid from chromosomally normal pregnancies (19).

To confirm that mRNA expression also leads to protein translation we stained parental SKBR3 cells and the invasive subclone with an specific mAb to the C peptide of pro-EPIL. By fluorescence immunocytochemistry and, prominently, by the quantitative method of flow cytometry we obtained no significant result for the difference in pro-EPIL expression between the invasive subclone *versus* the parental cell line. Secretion of the peptide by the cells might prevent accumulation of cytoplasmic pro-EPIL. Therefore, and because immunocytochemistry and flow cytometry do not enable an absolute measurement of concentration, we determined the pro-EPIL levels in the culture medium of the invasive and the parental cell lines in exponential growth phase.

As shown in Fig. 2 the cell number adjusted levels for pro-EPIL in the growth medium of the invasive subclone exceeded those of the parental cell line significantly ($P < 0.001$). The total levels are in a range below 20 ng/ml. Therefore, the efficacy of the autocrine loop might depend on the expression of attainable receptors for pro-EPIL. Of note, the growth rate of the invasive subclone was much lower then that of the parental cell line. This gives additional support for the hypothesis that the imbalance of growth regulation, leading to uncontrolled proliferation, does not, by itself, result in metastasis; separate molecular mechanisms for carcinogenesis and metastasis have been postulated (2).

TMAs containing multiple samples from individual tumor specimens are an ideal tool for a systematic analysis of protein expression and estimation of tissue heterogeneity (20). The more heterogeneous the distribution of a particular tissue characteristic, such as pro-EPIL expression, in a tumor, the less likely it is that the alteration will be detected in all of the arrayed samples of one tumor. In our study, 83% of the tumors investigated showed at least moderate pro-EPIL expression, but the heterogeneity of expression was high, one tumor presented with array samples of high and no detectable expression. Furthermore, pro-EPIL is expressed by the tumor cells themselves and not by stromal cells like fibroblasts as observed for IGF-I and -II. Normal breast epithelium revealed only in a few cases a very weak positive staining reaction for pro-EPIL. *INSL-3*, another member of the insulin-like superfamily, encoding RLF, also known as Ley-II, was observed also to be expressed in normal breast epithelium and not expressed in stromal cells (22). The RLF expression is maintained in invasive breast cancer and lymph node metastasis but, contrary to pro-EPIL, on significantly lower levels then in the normal tissue (22). In summary, the mentioned data might lead to the conclusion that a subtle coordinated network of insulin-like peptides mediating autocrine and paracrine signals promote breast tumor growth and invasion.

IGF-I/II signaling via their IGF receptor tyrosine kinases correlated to good prognosis and well-differentiated hormonally responsive tumors indicating a function of those molecules in earlier stages of breast cancer. The observation that invasive breast cancer is frequently positive for pro-EPIL and a simultaneous pro-EPIL, and c-erbB-2 highly positive cancer cell clusters in the invasive front of the tumor (Fig. 3*B*) support the assumption that pro-EPIL is related to advanced stages of breast cancer. Nevertheless, pro-EPIL function might be considered as a part of a signaling network of receptor tyrosine kinases. This is indicated by the fact that mitotic activity expressed by Ki-67 immunohistochemical staining was at its highest in the subgroup of tumors solely expressing pro-EPIL and decreases with coexpression of the c-erbB-2 receptor. Nevertheless, up- and down-regulation experiments will clarify how pro-EPIL contributes to growth rate control and invasiveness of c-erbB-2-expressing breast cancer cell lines. Furthermore, pro-EPIL as a therapeutic target might be evaluated from those studies.

Coincidential high staining for pro-EPIL and c-erbB-2 in cell clusters of the invasive front leads to the assumption that both molecules support a highly selective process, which favors the survival and growth of subpopulations in the neoplasm. Clinical follow-up studies have to be performed to figure out the prognostic relevance of this interesting observation.

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Cancer Res 2002;62:1020-1024.