Role of Epidermal Growth Factor Receptor and STAT-3 Activation in Autonomous Proliferation of SUM-102PT Human Breast Cancer Cells

Carolyn I. Sartor, Michele L. Dziubinski, Chao-Lan Yu, Richard Jove, and Stephen P. Ethier

ABSTRACT

This report describes the isolation and characterization of a new human breast cancer cell line, SUM-102PT, obtained from a minimally invasive human breast carcinoma. SUM-102PT cells have a near diploid karyotype, and early-passage cells had minor chromosomal abnormalities including a 5, 12, and a 6, 16 reciprocal translocation. The cells were isolated and have been continually cultured in three defined media, one of which contains exogenous epidermal growth factor (EGF). SUM-102PT cells have also been carried in an EGF-free medium supplemented with progesterone. All SUM-102PT cells require EGF receptor (EGFR) activation for continuous growth, because incubation of the cells with EGFR-neutralizing antibodies or with EGFR kinase inhibitors blocks growth of these cells. Southern analysis indicates that the EGFR gene is not amplified in these cells; however, these cells express high levels of EGFR mRNA. Thus, SUM-102PT is representative of a class of human breast cancers characterized by high level EGFR expression in the absence of gene amplification. SUM-102PT cells cultured in EGF-free, progesterone-containing medium express high levels of constitutively active EGFR. Conditioned medium from SUM-102PT cells contains an EGF-like mitogen that binds to a heparin-agarose affinity matrix with high affinity. Northern analysis for various EGF family members indicates that SUM-102PT cells synthesize heparin binding (HB)-EGF mRNA. HB-EGF protein is detectable on the surface of these cells by immunohistochemistry, and SUM-102PT cells are killed by diphtheria toxin, which acts by binding to HB-EGF. Furthermore, HB-EGF antibodies partially neutralize the mitogenic activity of the conditioned medium. Thus, EGFR activation in SUM-102PT cells is mediated, at least in part, by autocrine/juxtacrine stimulation by HB-EGF. SUM-102PT cells also express constitutively active STAT-3 homodimers. Constitutively tyrosine-phosphorylated STAT-3 homodimers were also detected in another breast cancer cell line, MDA468, which has an EGFR amplification and also has constitutive EGFR activity. Thus, SUM-102PT is a new human breast cancer cell line that expresses activated EGFR as a result of an autocrine/juxtacrine interaction with HB-EGF which, in turn, results in activation of STAT-3.

INTRODUCTION

Over the past several years our understanding of the molecular biology of human breast cancer has improved steadily. Several oncogenes and tumor suppressor genes have been shown to play important roles in breast cancer progression including erbB-2, c-myc, p53, and others (1–4). Although it is clear that molecular changes associated with the development of breast cancer result in alterations in growth-regulatory mechanisms of the affected cells, the precise cellular phenotypes that result from these genetic changes are understood poorly. Thus, it remains to be elucidated how specific molecular alterations result in completely transform normal cells into cells with fully malignant properties.

One obstacle to a more clear understanding of the cellular consequences of molecular changes that occur in breast cancer has been the relative inability to culture primary breast cancer cells under well-defined conditions in vitro. This inability to isolate and culture primary human breast cancer cells is more reflective of a poor understanding of breast cancer cell biology than inadequacies of tissue culture technology, because one can routinely culture normal human mammary epithelial cells of both the luminal and basal/myoepithelial lineages (5–10). Furthermore, normal human mammary epithelial cells can be induced to express many differentiated functions in vitro by culturing them in appropriate matrices (11–13). Thus, normal human mammary epithelial cells can be induced to grow and differentiate in culture under well-defined conditions.

A major focus of our laboratory over the past several years has been to improve our understanding of the altered growth-regulatory pathways that distinguish human breast cancer cells from their normal counterparts. Accordingly, we have developed and continue to develop culture conditions for the growth of primary and metastatic human breast cancer cells (10, 14, 15). The purpose of these studies is not simply to isolate new human breast cancer cell lines but rather to isolate and culture human breast cancer cells under defined conditions to understand better how specific molecular alterations result in altered growth regulation of the cells. In this report, we describe a newly isolated breast cancer cell line, SUM-102PT, which was isolated from a minimally invasive primary human breast cancer. These cells have only minor karyotypic abnormalities, do not have amplifications of the known breast cancer oncogenes, but do overexpress EGFR3 to high levels at both the RNA and protein levels. SUM-102PT cells also synthesize HB-EGF, which acts as an autocrine/juxtacrine mitogen for these cells. SUM-102PT cells also express a constitutively activated STAT-3 complex. This complex was not observed in MCF-10A normal mammary epithelial cells, which also depend on EGFR activation for growth.

Thus, the SUM-102PT cell line is a representative of a large subset of human breast cancers characterized by EGFR overexpression. Several clinical studies have demonstrated EGFR overexpression, in the absence of gene amplification, to be a bad prognostic indicator in human breast cancer (16–23). Thus, SUM-102PT may be a good model cell line for early stages of this important subset of human breast cancer.

MATERIALS AND METHODS

SUM-102PT Isolation. One-half g of the primary tumor specimen was obtained by our laboratory. The tumor was minced into fine pieces using two scalpels in a cross-cutting manner. The pieces were then placed in a 50-ml centrifuge tube containing 20 ml of Medium 199 and vortexed by hand, allowing the smaller cell clusters to remain in suspension and the larger pieces to settle to the bottom of the tube. The smaller cell clusters and single cells in

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3 The abbreviations used are: EGFR, epidermal growth factor receptor; HB, heparin binding; DT, diphtheria toxin; SF, serum-free; SIE, serum-inducible element; STAT, signal transducer and activator of transcription.
suspension were centrifuged at 1000 rpm for 5 min and plated in 6-well culture plates in various culture media described below. The larger cell clusters were subjected to enzymatic dissociation by incubating the fragments in 15 ml of Medium 199 containing 1% Dispase (Boehringer Mannheim, Indianapolis, IN) and type III collagenase (Worthington Biochemical Corp., Freehold, NJ) at 200 units/ml overnight in a 65 cycles/min, 37°C shaking water bath. The cells were then washed three times with Medium 199, and nuclei were counted. Ten million cells from the enzymatic dissociation were then combined with 10^7 magnetic beads (Dynabeads; Dynal) conjugated to the monoclonal antibody MC-5 to further separate them from normal fibroblastic cells that are typically associated with a breast tumor specimen. The MC-5 antibody was kindly provided by Dr. J. Peterson (Cancer Research Fund of Contra Costa) and recognizes mucins expressed by human mammary epithelial cells. The cell-Dynabead mixture was allowed to agitate at room temperature for 2 h. The cells that bound to the MC-5-labeled Dynabeads were separated from the cell mixture using a magnetized tube holder. The purified mixture was then seeded onto 35-mm, 6-well culture dishes in media supplemented with different growth factors.

**Cells were isolated from the primary tumor by enzymatic dissociation.** The full-length EGFR probe was obtained from the American Type Culture Collection (Rockville, MD). The HB-EGF probe encompassed a 1.1-kb fragment beginning at bp 490 and was the kind gift of Dr. Stefan Stoll, as was the amphiregulin probe (900-bp 5' fragment). Transforming growth factor α probe was a gift from Genentech. Final washes were 0.1X SSC, 0.1% SDS at 65°C. Gels were exposed to autoradiography for 24–96 h.

**Immunocytochemical Analysis of HB-EGF and Cytokeratins.** SUM-102PT cells near confluence were fixed with cold methanol. Fixed cells were incubated for 30 min with 1 μg of polyclonal rabbit anti-HB-EGF (R&D systems, Minneapolis, MN), followed by incubation with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA). Alternatively, fixed cells were immunostained with anti-cytokeratin antibodies: anti-pankeratin, anti-keratin-18, anti-keratin 8 (Sigma Immunochromics, St. Louis, MO), and anti-keratin 19 (ICN Biomedical, Costa Mesa, CA). Cells were visualized using diaminobenzidine as a substrate for horseradish peroxidase according to the manufacturer’s instructions (Vector Laboratories, Inc.).

**Electrophoretic Mobility Shift Assay.** Nuclear extracts were prepared as described previously (24) by lysing cells under hypotonic conditions in buffer containing 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM sodium PP, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. Nuclei were isolated by centrifugation and lysed in the above buffer with 420 mM NaCl and 20% glycerol. Seven μg of nuclear proteins were incubated for 30 min at 30°C with end-labeled high-affinity SIE oligonucleotide (5'-AGCTTACATTCCGTTAAATCCTAAAGCT-3') with or without 100-fold excess of unlabeled competitor or unlabeled scrambled competitor (5'-AGCTTACATTCCGTTAAATCCTAA-3'). Complexes were separated through 7.5% nondenaturing PAGE. Gels were dried and autoradiographed 24–48 h. Supershifts were performed by incubating extracts for 20 min with 1 μg of anti-STAT-3 rabbit polyclonal antibody raised against peptides 750–769 of the COOH terminus (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Western Blot Analysis of Membrane Protein.** Confluent monolayers of cells were scraped from 60-mm tissue culture dishes in 20 ml HEPES containing 5 mM sodium orthovanadate, 10 mM sodium PP, and 1 mM phenylmethylsulfonyl fluoride. The lysate was dounce homogenized 50 times and centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 35 min, and the pellet was resuspended in a buffer containing 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM sodium orthovanadate, 10 mM sodium PP, and 1 mM phenylmethylsulfonyl fluoride. The lysate was assayed for protein content, and then defined amounts of membrane protein were loaded into individual wells and electrophoresed on a 7.5% SDS-polyacrylamide gel. Following electrophoresis, the proteins were electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA) and blocked with 3% nonfat dry milk in Iris-buffered saline with 1% Tween 20. The blot was probed with erbB-2 antibody (Pab 9.3, kindly provided by Dr. Beatrice Langton), anti-phosphotyrosine antibody (PY-20; ICN, Costa Mesa, CA), or EGFR antibody (Ciba-Corning, Alameda, CA). Protein bands were visualized by incubating blots with biotinylated secondary antibody and then with Vectastain ABC reagents (Vector Laboratories, Burlingame, CA) using diaminobenzidine as a substrate.

### RESULTS

**Isolation and Initial Characterization of SUM-102PT.** SUM-102PT was isolated from a 56-year-old woman with locally advanced left breast cancer. Fine-needle aspiration cytology of the lesion was positive for adenocarcinoma, and the patient underwent 6 months of neoadjuvant chemotherapy with cytoxan, Adriamycin, methotrexate, and 5-fluorouracil prior to definitive surgery. Chemotherapy was followed by modified radical mastectomy, and the neoplasm was diagnosed as a minimally invasive apocrine adenocarcinoma with extensive ductal carcinoma in situ. Immunohistological analyses were negative for both estrogen and progesterone receptors. The tumor was histopathological grade 1 on the Bloom-Richardson scale, and none of the 14 lymph nodes contained tumor.

Cells were isolated from the primary tumor by enzymatic dissociation and separated through 7.5% nondenaturing PAGE. Gels were dried and autoradiographed 24–48 h. Supershifts were performed by incubating extracts for 20 min with 1 μg of anti-STAT-3 rabbit polyclonal antibody raised against peptides 750–769 of the COOH terminus (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Heparin-Agarose Affinity Chromatography of SUM-102PT Conditioned Medium.** Forty-eight-hour conditioned medium was collected from near-confluent cultures of SUM-102PT cells in SF-HEM medium supplemented with 5% fetal bovine serum. PDGF was detected in conditioned SF-HEM medium using an antibody directed against murine PDGF-BB.

**Table 1 Media composition**

<table>
<thead>
<tr>
<th>Media Group</th>
<th>Growth factors/Supplements</th>
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<tbody>
<tr>
<td>Serum-containing media (5% fetal bovine serum)</td>
<td>Insulin, hydrocortisone</td>
</tr>
<tr>
<td>5% IH</td>
<td>Insulin, hydrocortisone, EGF</td>
</tr>
<tr>
<td>5% IHE</td>
<td>Insulin, hydrocortisone, EGF</td>
</tr>
<tr>
<td>SF media (0.1% BSA, ethanolamine, selenium, triiodothyronine, transferrin, and HEPES)</td>
<td>Insulin, hydrocortisone, progesterone</td>
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<tr>
<td>SF-I</td>
<td>Insulin, hydrocortisone</td>
</tr>
<tr>
<td>SF-IP</td>
<td>Insulin, hydrocortisone, progesterone</td>
</tr>
<tr>
<td>SF-IHP</td>
<td>Insulin, hydrocortisone, progesterone, estradiol</td>
</tr>
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*All media also contain fungizone and gentamicin.*
Cells were isolated using both 5% serum-containing and SF media. It was found that these cells were responsive to exogenous EGF. Cells cultured in human breast cancer cells, efforts were focused on cells cultured in the hormone- and growth factor-supplemented media listed in Table 1. From this specimen, proliferating cells were isolated using both 5% serum-containing and SF media that were supplemented additionally with insulin and hydrocortisone (IH medium), or with insulin, hydrocortisone, and progesterone (IHP medium). Interestingly, the cells cultured in the SF-IHP medium grew much more rapidly than the cells in the SF-IH medium, suggesting that progesterone had important effects on the growth of these cells.

Because of our interest in understanding growth regulation in human breast cancer cells, efforts were focused on cells cultured in the SF media. Since the early passages of SUM-102PT cells were cultured in EGF-free medium, experiments were carried out to determine if these cells were responsive to exogenous EGF. Cells cultured in SF-IH medium were cultured in the presence or absence of 10 ng/ml EGF. The data in Fig. 1 show that the addition of EGF to the culture medium increased the rate of proliferation of the SF-IH cells to a rate comparable to that of the cells cultured in SF-IHP medium. Thus, these cells, although not dependent on exogenous EGF for growth, are very responsive to this growth factor. From passage five onward, SUM-102PT cells were cultured continuously in three media: SF-IH, SF-IHP, and EGF-containing SF-IHE. SUM-102PT cells are immortal, have never exhibited signs of cellular senescence, and have been carried in culture for over 90 passages.

The isolation of immortal cell populations using media that do not support the growth of normal human mammary epithelial cells suggested that they represented the neoplastic component of the tumor specimen. To confirm this and to further characterize these cells, karyotype analysis was carried out. At passage five, SUM-102PT cells had a diploid karyotype with two clonal cytogenetic abnormalities: a reciprocal translocation involving chromosomes 5 and 12 and a second translocation involving chromosomes 6 and 16. The latter change was confirmed by fluorescence in situ hybridization using chromosome-specific probes (data not shown). Thus, the karyotype of these cells was consistent with their origin from an early-stage breast cancer that is only minimally invasive. Extended culture of SUM-102PT cells in the three different media resulted in further cytogenetic abnormalities, and these changes are summarized in Fig. 2. Despite the fact that these cells are cultured under slightly different conditions, all of the cells exhibited common marker chromosome abnormalities, indicating their origin from the same population of primary breast cancer cells.

Southern blot analysis of SUM-102PT cells for alterations in genes commonly amplified in human breast cancer did not reveal amplifications of the erbB-2, c-myc, Prad-1, FGFR-1, FGFR-2, and FGFR-4 genes (data not shown). These cells also did not overexpress nuclear p53 as examined by immunocytochemistry. However, exposure of these cells to 5 Gy of γ radiation resulted in the expression of nuclear p53 within 2 h of exposure, thus confirming the wild-type p53 status of these cells (data not shown).

Experiments were carried out to examine cytokeratin expression of the SUM-102PT cells. The vast majority of human breast cancers are of luminal cell origin (26, 27). Therefore, SUM-102PT cells were examined for expression of luminal cytokeratins by immunocytochemistry. SUM-102PT cells were negative for keratin-19 but were positive for the luminal cytokeratins keratin-8 and keratin-18 (Fig. 3). The neoplastic cells present in the biopsy specimen were also keratin-19 negative, whereas neighboring normal luminal cells stained positively for this keratin (data not shown). This pattern of luminal keratin expression indicates that the neoplasm arose from luminal cells. The expression of keratins 8 and 18 without expression of keratin 19 suggests that this tumor may have arisen in a duct rather than from a terminal duct lobular unit, because most luminal cells of the ducts are positive for keratins 8 and 18 but negative for keratin 19 (26, 27). This pattern of luminal cytokeratin expression may also be reflective of the apocrine metaplasia of the neoplastic cells of this specimen.

**Role of EGFR Activation in SUM-102PT Cell Growth.** To evaluate further the role of EGFR activation in the growth of the three SUM-102PT sublines, Western blots were prepared from membrane protein of these cells, and blots were probed with either an EGFR antibody or with the anti-phosphotyrosine antibody PY-20. Fig. 4 shows that all three sublines were positive for EGFR expression by Western blot. Interestingly, the highest levels of EGFR expression were detected in cells cultured in the SF-IHP medium. The lowest levels of expression, both at the message and protein levels, were in the subline cultured continuously in EGF-containing medium. Incubation of these cells in the absence of EGF for 24 h resulted in a dramatic increase in the level of cell surface EGFR (data not shown).

The phosphotyrosine Western blot also had a prominent M, 170,000 band, suggesting that EGFR is activated in these cells. As in the EGFR Western blot, the most intense p-Tyr-M, 170,000 band was detected in the SF-IHP cells. This result suggests that even the cells cultured continuously in the SF-IHP medium require EGFR activation for growth. To test this hypothesis further, all three sublines were cultured in their regular SF medium in the presence or absence of a neutralizing EGFR antibody. As expected, the EGFR antibody blocked growth of the cells cultured in the SF-IH and SF-IHE media. This antibody also blocked the proliferation of the cells cultured in the SF-IHP medium, consistent with the presence of constitutively tyrosine-phosphorylated EGFR in these cells (Fig. 5A). Similar experiments were then performed using an EGFR kinase inhibitor that has been shown to be a potent and specific inhibitor of the enzymatic activity of the EGFR (28). The EGFR kinase inhibitor, PD157665,
like the EGFR antibodies, potently inhibited the growth of SUM-102PT cultured in SF-IHP medium (Fig. 5B). By contrast, this inhibitor had no effect on growth of SUM-52PE human breast cancer cells, which do not express EGFR (15). Thus, we conclude that SUM-102PT cells, regardless of the media they are cultured in, require activation of the EGFR for growth in vitro under SF conditions.

**Autocrine/Juxtacrine Activation of EGFR.** The results presented above show that SUM-102PT cells require EGFR activation for growth. Therefore, experiments were performed to examine the EGFR gene copy number and expression levels in these cells. Southern blot analysis of genomic DNA from SUM-102PT cells indicated that the EGFR gene is not amplified in these cells (Fig. 6A). However, Northern blot analysis did show that EGFR is overexpressed at the message level in SUM-102PT cells cultured in SF-IHP, relative to MCF-10A normal human mammary epithelial cells (Fig. 6B). Interestingly, EGFR message levels were low, but detectable, in cells cultured continuously in the SF-IHE medium, suggesting that the
continuous presence of EGF in the culture medium influences the steady-state levels of EGFR message in these cells.

Given that the EGFR is not amplified in SUM-1O2PT cells, it is likely that the constitutive tyrosine phosphorylation of EGFR seen in the SF-IHP subline was due to an autocrine- or juxtacrine-acting growth factor synthesized by the cells. Therefore, experiments were carried out to determine if SUM-1O2PT cells were producing an EGF-like factor that was responsible for the EGFR activation. We have shown previously that MCF1OA cells, which are completely dependent on EGF for proliferation, can be used as sensitive indicator cells for EGF-like activity in conditioned media (29, 30). The data in Fig. 7 show that conditioned medium obtained from SUM-1O2PT cells, grown continuously in SH-IHP medium, stimulated growth of MCF-1OA cells under SF, EGF-free conditions.

Northern blot experiments were then carried out to screen for production of known EGF-like ligands by SUM-1O2PT cells. These experiments indicated that SUM-1O2PT cells produced low levels of transforming growth factor α message, as did the control MCF1OA cells (data not shown). By contrast, neither SUM-1O2PT nor MCF1OA cells expressed detectable levels of amphiregulin (data not shown). However, SUM-1O2PT cells cultured continuously in either the SF-IH or SF-IHP media expressed readily detectable levels of HB-EGF message, whereas MCF1OA cells and SUM-1O2PT cells grown in SF-IHE medium did not (Fig. 8A). The finding of HB-EGF expression in the SUM-1O2PT cells that showed high levels of constitutive EGFR activation suggests that this factor acts as an important autocrine/juxtacrine factor for SUM-1O2PT cells.

To confirm that HB-EGF protein was expressed by these cells, immunocytochemical analysis was carried out using an HB-EGF antibody. The results of this analysis are shown in Fig. 8B and indicate

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**Fig. 4.** EGFR and phosphotyrosine Western blot of SUM-1O2PT cells. One hundred μg of membrane protein from SUM-1O2PT cells that were grown in each of the three media were electrophoresed through a 7% SDS-polyacrylamide gel, transferred to Immobilon-P membranes, and probed with: anti-phosphotyrosine (α PY20); anti-EGFR (α EGF-R); or anti-Erb-B2 antibodies (α Erb-B2). Note the overexpression of the M, 170,000 protein in the anti-EGFR blot, which co-migrates with the band in the anti-phosphotyrosine blot but is distinct from the M, 185,000 Erb-B2 band.
positive cell-surface expression of HB-EGF in these cells. To extend this observation, we took advantage of the fact that HB-EGF, in its membrane-bound form, is the DT receptor (31, 32). Cells that contain HB-EGF bind DT in the EGF-binding domain with subsequent internalization and cytotoxicity. Fig. 9 shows that SUM-102PT cells were sensitive to DT and were killed in a concentration-dependent manner. This result indicates that HB-EGF is present on the surface of these cells.

To confirm that HB-EGF was responsible for the mitogenic activity of the conditioned medium, SUM-102PT cells were grown to near confluence, and 48-h conditioned medium was collected. The conditioned medium was passed over a heparin-agarose affinity column, and the flow-through fraction was collected and tested for mitogenic activity. The column was then eluted in three fractions containing increasing salt concentrations, and the eluates were desalted and tested for EGF-like mitogenic activity. The data in Fig. 9A show that the medium that passed through the column was devoid of mitogenic activity and that the 1.0 M salt eluate did contain EGF-like activity. Thus, the conditioned medium factor does have high affinity heparin-binding activity. Finally, an HB-EGF antibody, as well as a nontoxic analogue of DT (CRM-197), was tested for their ability to inhibit the growth of SUM-102PT cells. The data in Fig. 9C show that both of these reagents could, at least partially, block the proliferation of SUM-102PT cells. Thus, SUM-102PT cells synthesize, express on their cell surface, and secrete HB-EGF, which plays a role in the constitutive tyrosine phosphorylation of EGFR in SUM-102PT cells and the growth of these cells in EGF-free medium.

STAT-3 Is Constitutively Activated in SUM-102PT Cells. The STAT family of transcription factors has been shown to be a component of the signal transduction pathway activated by EGFR in some cells (33–36). We, therefore, investigated whether this pathway was activated in the SUM-102PT cell line. Electrophoretic mobility shift assays were performed using the high affinity SIE of the c-fos promoter as a probe for activated STAT proteins (24, 37, 38). When SUM-102PT nuclear extracts were incubated with SIE oligonucleotides, several protein-oligonucleotide complexes formed, which resulted in distinct bands seen in Lane 1 of Fig. 10. Competition with excess unlabeled SIE oligonucleotides, but not a scrambled oligonucleotide of the same base pair composition, abrogated complexes specific for the SIE, as seen in Fig. 10, Lanes 2 and 3. To determine

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**Fig. 5.** The effect of a neutralizing EGFR antibody (A) or an EGFR kinase inhibitor (B) on the growth of SUM-102PT cells in EGF-free media. Cells were seeded at 3.5 × 10⁴ cells per 35-mm well and cultured in SF-IH or SF-IHP medium. On days 1 and 4, one-half the wells in each group were also given 1 μg/ml of EGFR neutralizing antibody, Ab-1, or exposed to PD157655 at a 1 μM concentration. Values are the mean number of cells/well for triplicate wells after 7 days in culture; bars, SD.

**Fig. 6.** EGFR gene copy number and expression levels in SUM-102PT cells. A, Southern blot of SUM-102PT cells. DNA was isolated from SUM-102PT cells, digested with EcoRI or HindIII, separated electrophoretically through 0.8% agarose gels, transferred to nylon membranes, and probed with full-length EGFR cDNA. Lane 1, SUM-102PT digested with EcoRI. Lane 2, EcoRI-digested DNA obtained from cells known to contain no amplification of the EGFR gene (1315 M02). Lane 3, SUM-102PT digested with HindIII. Lane 4, HindIII digest of the control DNA. B, EGFR Northern blot of SUM-102PT cells. Total RNA was isolated from SUM-102PT cells grown in the three media or MCF-10A cells grown in SF media with insulin, hydrocortisone, and EGF. Twenty μg of RNA were electrophoresed through 1.5% agarose, transferred, and probed with the full-length EGFR cDNA. Lane 1, MCF-10A. Lane 2, SUM-102PT cultured in SF-IH medium. Lane 3, SUM-102PT cultured in SF-IHE medium. Lane 4, SUM-102PT cultured in SF-IHP medium. 36B4 was used to standardize relative amounts of RNA (bottom panel). Note the intensity of the EGFR band in the SUM-102PT cells grown in the absence of EGF relative to MCF-10A cells (which are known to have high levels of EGFR), despite overloading of the MCF-10A, as indicated by comparing the 36B4 signal.
EGFR-STERIULATED GROWTH OF SUM-102PT CELLS

SUM-102PT cells express constitutively activated STAT-3, which was not present in MCF10A cells grown under identical conditions.

DISCUSSION

In this report, we describe the isolation and characterization of a new human breast cancer cell line from a primary breast neoplasm. There are a number of characteristics of this cell line that distinguish it from other breast cancer cell lines isolated previously by ourselves and others. This cell line was isolated from a primary tumor specimen following neo-adjuvant chemotherapy. The patient was originally diagnosed with locally advanced disease based on both mammographic and ultrasound findings and was, therefore, given chemotherapy prior to definitive surgery. Interestingly, the diagnosis of the final tumor specimen after mastectomy indicated that the tumor consisted mostly of carcinoma in situ with a microinvasive component. The original size of the primary neoplasm was the result of a large cystic component of the mass. Thus, this cell line was isolated from an early stage breast cancer, and the karyotype of SUM-102PT cells was consistent with the histological pattern of the neoplasm. SUM-102 cells were near diploid at early passages and exhibited only two discernible chromosome abnormalities: a reciprocal translocation involving chromosomes 6 and 16; and a second translocation involving chromosomes 5 and 12. At later passages, the karyotype became slightly more complex, but even these karyotypes were near diploid and much more normal in appearance than karyotypes from other primary tumor and metastatic cell lines that we have isolated (10, 41). One unusual feature of this breast cancer cell line is that it is negative for keratin-19 while being positive for the luminal cytokeratins keratin-8 and keratin-18. It is clear that human breast cancer arises, almost exclusively, from luminal epithelial cells and most often from the terminal duct lobular unit. Most, but not all, of these luminal cells express keratin-19. However, keratin-19-negative luminal cells do express two other luminal cytokeratins, i.e., keratins-8 and 18, and the SUM-102PT cells are positive for these. Thus, although the keratin-19 negativity of these cells is unusual, it is not without precedent in human breast cancer (26, 27).

SUM-102PT cells were first isolated using two selective media that do not support growth of normal human mammary epithelial cells. One of these media was supplemented with insulin and hydrocortisone, while the other medium also contained progesterone. The SF-IHP medium yielded cells with a more compact morphology that grew more rapidly than cells isolated in the SF-IH medium. The fact that the

Fig. 7. Bioassay of EGF-like activity in SUM-102PT conditioned medium. MCF-10A cells, which are dependent on EGF for growth, were used as indicator cells to assay for secreted EGF-like ligands in conditioned medium from SUM-102PT cells. MCF-10A cells were seeded at 5 X 10^5 cells/well in 35-mm wells and grown with or without 10 ng/ml EGF or with 48 h conditioned media (50% v/v) from SUM-102PT cells grown in the absence of exogenous EGF. Media were changed every 2 days. Values are the mean number of cells per well for triplicate wells after 7 days; bars, SD.

Fig. 8. HB-EGF expression in SUM-102PT cells. A, HB-EGF Northern Blot of SUM-102PT. Total RNA was isolated from SUM-102PT cells grown in the three media or MCF10A cells grown in SF media with insulin, hydrocortisone, and EGF. Twenty μg of RNA were electrophoresed through 1.5% agarose, transferred, and probed with a 1.1-kb HB-EGF probe. Lane 1, MCF-10A. Lane 2, SUM-102PT cultured in SF-IH medium. Lane 3, SUM-102PT cultured in SF-IHP medium. Lane 4, SUM-102PT cultured in SF-IHP medium. 36B4 was used to standardize relative amounts of RNA (lower band). Note that HB-EGF message is expressed in the SUM-102PT cells, which showed high levels of activated EGFR. B, HB-EGF immunochemistry of SUM-102PT Cells. Photomicrographs show SUM-102PT cells immunostained using antibody to HB-EGF (A) or nonimmune IgG control (B).
SUM-102PT cells express high levels of EGFR, both at the message and protein levels, without amplification of the EGFR gene. Thus, these cells appear to be representative of a large subset of human breast cancer cells that overexpress EGFR in the absence of gene amplification. Overexpression of EGFR without gene amplification occurs in approximately 30% of cases and has been observed repeatedly to be associated with poor outcome (16–23). This is potentially of mechanistic significance because overexpression of a growth factor receptor may be a central factor in transforming an autocrine or paracrine loop from a physiological to a pathological process.

HB-EGF appears to be the ligand that drives constitutive tyrosine phosphorylation of EGFR and proliferation of SUM-102PT cells cultured continuously in SF-IHP medium. SUM-102PT cells express HB-EGF mRNA and protein detectable at the cell surface. EGFR-like mitogenic activity was also observed in conditioned medium obtained from these cells, and this activity was retained on a heparin-agarose affinity column. The 1.0 M salt eluate from this column contained the EGFR-like mitogenic activity. DT effectively killed SUM-102PT cells, indicating the presence of HG-EGF on the cell surface. However, antibodies to HB-EGF, or a nontoxic analogue of DT, were only marginally effective at blocking the growth of these cells. The relatively poor ability of these reagents to block growth of these cells may be a reflection of true cellular autonomy of HB-EGF or may reflect the inability of this particular antibody to block the interaction of HB-EGF with the EGFR, especially when the growth factor itself is located predominantly on the cell surface. In this regard, its worth noting that these same antibodies were only poorly effective at blocking the growth of epidermal keratinocytes, which have also been shown to synthesize and secrete HB-EGF (42).

Heparin-binding epidermal like growth factor was originally isolated from macrophage-like U937 cells as a M subunit 22,000 heparin-binding factor that is mitogenic for smooth muscle cells (43). HB-EGF was found to have 40–50% homology to other known EGF-like...
factors, having the conserved six-cysteine domain characteristic of EGF family members, and is a ligand for the EGFR (44). HB-EGF was subsequently discovered to function as a membrane-bound precursor as well as a secreted molecule (45, 46). In its membrane-bound form, proHB-EGF functions as the DT receptor. DT binds proHB-EGF in the EGF-like region, which allows internalization of DT, with resultant toxicity, and inhibits binding of HB-EGF to EGFR (31, 32, 47, 48). HB-EGF expression has been shown in many tissues, particularly lung, skeletal muscle, brain, and heart (45). In keratinocytes, HB-EGF has been implicated in autocrine/paracrine interactions; normal human keratinocytes have EGFRs, express HB-EGF mRNA, and respond to exogenous HB-EGF by proliferation (42). HB-EGF has also been demonstrated in mammary carcinoma cell lines (46), but its functional role in these lines is unknown. To our knowledge, the results reported here represent the first report of HB-EGF autocrine/paracrine activity in a human breast carcinoma cell line.

It is interesting that the HB-EGF autocrine/paracrine loop appeared to function most efficiently in cells grown in progesterone-containing medium. These cells had a faster doubling time and higher levels of EGFR activation than cells isolated and grown without progesterone. Progesterone has been reported to increase HB-EGF mRNA levels in paracrine activity in a human breast carcinoma cell line.

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We also found that SUM-102PT cells express a constitutively active STAT-3 complex that is not detectable in MCF-10A human mammary epithelial cells cultured under the same conditions. The STAT family of proteins was first identified by their involvement in the IFN response (38, 51, 52), which leads to STAT complex formation, tyrosine phosphorylation, and translocation to the nucleus, where the complex binds to enhancer elements of genes activated by the IFNs (53, 54). Tyrosine kinase-activated receptors, such as the EGFR, have also been found to use the STAT pathway. EGFR-activated STAT complexes typically consist of homo- and heterodimers of the STAT-1 and STAT-3 proteins. These activated STAT complexes ultimately bind the SIE of the c-fos promoter, thus contributing to immediate-early gene expression (55, 56). We found that SUM-102PT cells contain constitutively activated STAT-3 homodimeric complexes that bind the c-fos SIE with high affinity. In previous reports, STAT-3 complex formation has been demonstrated in EGF-stimulated hepatocytes (35) in cells with very high levels of EGFR, such as A431 cells, (52, 57), or in EGFR transfectants (58). Although it is possible that the level of EGFR activation in SUM-102PT cells is itself sufficient for STAT-3 complex formation, an alternative explanation is that STAT-3 is being activated by other kinases. Experiments underway are aimed at determining the tyrosine kinase directly responsible for STAT-3 activation in SUM-102PT cells and other EGFR-positive HBC cells.

The significance of STAT activation in human breast cancer is unknown. Previous studies demonstrated that STAT-3 is constitutively activated by the src oncprotein, providing the first link between a specific oncprotein and activation of STAT signaling pathways (24). STAT-DNA binding activity has been demonstrated in breast cancer nuclear extracts but not in normal breast tissue histology samples (59). Our finding that SUM-102PT cells grown in EGHD had constitutive STAT activation, whereas MCF10A cells that were grown under the same conditions did not express activated STAT-3, suggests that STAT activation is part of an abnormal signal transduction process in some malignant breast cells. Consistent with this hypothesis was our observation that MDA-468 human breast cancer cells, which have an amplification of the EGFR gene and which also exhibit constitutive EGFR activity, also express high levels of the activated STAT-3 complex.

In our laboratory, we have recently developed 12 new human breast cancer cell lines. These lines have been examined for STAT-3 activation, and the results indicate that STAT-3 activation is associated with EGFR expression in these human breast cancer cells. Thus, constitutive STAT-3 activation appears to be a property of breast cancer cells with constitutive EGFR activation.

In summary, the SUM-102PT human breast cancer cell line represents a novel cell line that can be cultured under defined conditions in vitro. These cells may be representative of that subset of human breast cancer characterized by estrogen receptor negativity and overexpression of EGFR in the absence of gene amplification. The data presented here indicate that the overexpression of the EGFR is of direct importance to the altered growth potential of these cells.

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