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ESTABLISHMENT AND CHARACTERIZATION OF THREE NEW BREAST-CANCER CELL LINES

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We have established and characterized 3 new breast-cancer cell lines from pleural effusions of patients with advanced breast cancer. All 3 cell lines, designated IBEP-1, IBEP-2 and IBEP-3, showed typical ultrastructural characteristics of epithelial mammary tumor cells. Electron microscopy showed, among other characteristics, the presence of numerous microvilli, desmosomal junctions, intracytoplasmic duct-like vacuoles, well-developed endoplasmic reticulum and large nuclei. Immunohistochemical and biochemical studies revealed that the 3 cell lines expressed cytokeratin, epithelial membrane antigen, CEA and CA 15-3, but all showed negative immunoreaction for vimentin. On the other hand, other antigens (LEU-M1, GCDFP 15, c-erbB-2) were expressed by some of the cell lines, but in a variable manner. Ploidy studies confirmed the neoplastic origin of the cell lines. The doubling times were 68 hr for IBEP-1, 29 hr for IBEP-2 and 39 hr for IBEP-3. Only IBEP-2 cells expressed estrogen receptors (ER^+), which were down-regulated after pre-incubation with E_2, but they did not express progesterone receptors (PgR^-). IBEP-1 and IBEP-3 cells were ER^- but expressed PgR (PgR^+). In these 2 cell lines, PgR were down-regulated after pre-incubation of the cells with progesterone (10^{-8} M) for 24 hr. Estradiol (E_2) increased the proliferation rate of IBEP-2 cells and progesterone increased the proliferation of IBEP-1 and -3 cell lines. S.C. injection of the 3 IBEP cell lines into nude mice resulted in the growth of solid tumors between 11 and 16 weeks after inoculation. These cell lines could thus be new models for studying various aspects of the biology and the tumorigenicity of breast-cancer cells. A major interest of these new cell lines is that 2 of them were ER^- and PgR^+, which is an exceptional phenotypic feature. These 2 cell lines could be interesting models for studying the regulation of PgR and the effects of progestins and antiprogestins independently of the presence of ER. *Int. J. Cancer* 76:677–683, 1998.
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The difficulty of obtaining permanent cell lines from primary breast-cancer tissue is well known (Amadori *et al.,* 1993). It is thus no surprise that the majority of breast-cancer cell lines have been established from malignant effusions of patients with advanced breast cancer (Cailleau *et al.,* 1974). Such effusions are considered as an adequate source of viable tumor cells, with little contamination by stromal cells. Moreover, most *in vitro* studies using breast-cancer cells are based on a few well-characterized cell lines, such as MCF-7, T-47D and MDA-MB-231 which have been established in culture for up to 20 years (Engel and Young, 1978). However, the phenotype of cancer cells can change in long-term cultures and, due to extensive sub-cloning, many of these established cell lines occasionally show phenotypic and genotypic instability, with eventual loss of the characteristics of the tumors from which they originated (Engel and Young, 1978).

Measurement of estrogen-receptor (ER) and progesterone-receptor (PgR) levels has been shown to be of predictive value both for overall survival and for response to endocrine therapy in patients with breast carcinoma (Fuqua and Wolf, 1995). Most ER^+ cells are also positive for PgR, and in breast-cancer cell lines, expression of PgR is up-regulated by estradiol, whose effect is mediated by ER (Nardulli and Katzenellenbogen, 1988). The presence of PgR in a ER^- tumor is a factor of good prognosis, as the disease-free survival (DFS) and overall survival (OS) of PgR^+ patients is significantly longer than for patients with PgR^- tumors. Moreover, to study the actions of progesterone and synthetic progestins and antiprogestins in human cells, it is important to understand the basic mechanisms responsible for both the inhibitory as well as the stimulatory activity of these hormones. The ZR-75-31 cell line (Engel and Young, 1978) remains the only available ER^- PgR^+ line, but it resulted from sub-cloning. Lasfargues *et al.* (1978) reported that BT-474 and BT-483 cell line were ER^- and PgR^+, but other investigators reported those cells to be ER^+ (Van Slooten *et al.,* 1995). Moreover, to the best of our knowledge, none of the cell lines described by Healicon *et al.* (1993) is ER^- and PgR^+. Most of the studies on the effects of progestational agents have thus been performed with T-47D cells, which express high levels of PgR but are also ER^+ (Kalkhoven *et al.,* 1994), or with MCF-7 cells transfected with a PgR expression vector to increase the number of PgR sites (Salvodi *et al.,* 1996).

We established and characterized 3 new breast-cancer cell lines from pleural effusions of breast-cancer patients. One was ER^+ and PgR-inducible, while the other 2 were ER^- but constitutively expressed PgR receptors, which were down-regulated after incubation by progesterone. These cell lines could thus be novel and useful models to study PgR independently of the presence of ER in breast-cancer cells. Moreover, s.c. injection of these 3 cell lines to nude mice resulted in the growth of solid tumors (between 11 weeks and 16 weeks after inoculation). These cell lines could thus be new *in vivo* models for studying the biology of breast cancer. Lastly, 2 of the cell lines came from patients who presented bone metastasis; the skeleton is the most common metastatic site in breast cancer, and our cell lines could thus be useful for studying the pathogenesis of breast-cancer-induced osteolysis (Body *et al.,* 1996).

MATERIAL AND METHODS

Origin of cells

In 1993 and 1994, we successfully established 3 cell lines from 15 malignant effusions cultured in the laboratory for that purpose.

The first cell line, IBEP-1, was derived from the pleural effusion (obtained at the end of 1993) of a 58-year-old woman mastectomized for a primary breast cancer in 1984. The histology of the tumor showed an invasive well-differentiated carcinoma with involvement of 1/13 axillary lymph nodes. The cells of the primary tumor (3 cm) were ER^+ (43 fmoles/mg prot) and PgR^+ (89

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fmoles/mg prot). The patient was treated with hormone therapy (3 lines; Nolvadex, Orimeten and Provera) and chemotherapy (2 lines; FEC and Taxotere); she presented bone metastases in 1990 and liver metastases in 1993.

The second patient (74 years old) was mastectomized for an invasive well-differentiated ductal carcinoma of the breast (2×1 cm) in 1985. The primary breast tumor was ER^+, and tamoxifen treatment was started. In 1992, she presented skeletal and lymph-node (3/13) metastases. She was treated with various chemotherapeutic agents (5 lines; CMF, taxotere, mitoxantrone, mitomycin C and 5FU). She developed malignant pleural effusion as well as liver and lung metastases in 1993. Pleural tumor cells obtained at recurrence in 1994 were used to establish the second cell line (IBEP-2).

The third cell line, IBEP-3, was obtained in 1994 from the pleural effusion of a 56-year-old patient who had presented breast cancer in 1977. She had an invasive ductal carcinoma (1.8 cm) with one lymph node infiltrated. The recurrent tumor in 1992 was ER^- and PgR^-. She was treated with 3 subsequent lines of chemotherapy (doxorubicine, taxol, 5FU). The patient developed skin metastases and a malignant pleural effusion in 1993, and brain and bronchial metastases in 1994, but she never presented bone metastases.

Cell culture

Cells were sedimented by low-speed centrifugation. The supernatant was removed and the cell pellet was re-suspended in Dulbecco's modified essential medium (DMEM; GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 2% penicillin-streptomycin (10,000 U/ml) and 10% heat-inactivated FCS (Gibco). The cells were plated in Petri dishes and cultured in an atmosphere of 5% CO_2 at 37°C. After 48 hr of incubation, the supernatant, containing the tumor cells which did not initially attach to the plate, was transferred to new Petri dishes in the same medium. Fresh cultured medium was supplied every 2 days and, when confluent, the cells were dissociated with trypsin (0.5%) and plated in T25 flasks (Nunc, Roskilde, Denmark). The cells were routinely cultured in RPMI medium supplemented with 10% FCS.

Light and electron microscopy

Breast-cancer cells at various passages were directly examined with an inverted-phase microscope (Olympus, Tokyo, Japan). Cells cultured on coverslips were fixed in Bouin, stained with hematoxylin-eosin, and examined with a light microscope. For electron microscopy, cells were removed from tissue-culture flasks and pelleted by centrifugation. The pellet was fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer and post-fixed with OsO_4 (1%) in the same buffer. After dehydration in alcohol, the samples were embedded in an Epon mixture. The blocks were ultra-sectioned on a LKB (Bromma, Sweden) microtome. Thin silver-gray sections were stained with uranyl acetate and Reynolds' lead citrate and examined on a Zeiss EM9A electron microscope.

Immunohistochemistry

Cells were plated on coverslips (VEL, Leuven, Belgium) and cultured in RPMI medium supplemented with 10% of FCS. At confluence, the cells were washed in PBS and fixed in Bouin or acetone (1 min), after which Immunocytochemical tests were performed. We used, as first antibodies, anti-low-molecular-weight cytokeratins (CAM 5.2, Becton Dickinson, Erembodegem, Belgium), anti-epithelial membrane antigen (anti-EMA; Dako, Glostrup, Denmark), anti-vimentin (Dako), anti-human-breast-gross-cystic-disease-fluid protein (anti-GCDFP 15; BRST2, Signet, Hamburg, Germany), anti-Leu M1 (CD15, Immunotech, Marseille, France), anti-serum against c-erbB2 oncoprotein (Biogenex, San Ramon, CA), anti-ER (ERID5, Immunotech) and anti-PgR (PR10A9, Immunotech). Control experiments were performed by substituting non-immune serum for the serum containing the first antibodies. Following incubation, cells were washed extensively with PBS, then incubated with biotinated sheep anti-mouse antibody and peroxydase-conjugated streptavidin (for CAM 5.2, EMA, VIM and GCDFP 15, Boehringer, Mannheim, Germany) or with supersensitive multilink and label (for CD15, c-erbB-2, ER and PgR, Biogenex kit). Diaminobenzidine (DAB) was used as chromogen. The cells were also counterstained with hematoxylin.

Calculation of growth rate

Cells (10^5 cells per flask) from each of the 3 lines were plated in 25-cm^2 flasks in RPMI supplemented with 10% of FCS. The culture medium was changed every 2 days. Triplicate flasks were trypsinized at various times (see ''Results'') and the cells were counted. To determine the response of IBEP cells to estradiol (E_2) and progesterone (ORG 2058, Organon, Oss, The Netherlands), cells were incubated in the same medium containing E_2 or ORG 2058 (10^{-8} M) and counted in the same conditions. The doubling time of the 3 cell lines, pre-incubated or not with E_2 or ORG 2058, was calculated in the exponential-growth phase.

Presence of steroid receptors (Scatchard and mRNA analysis)

The quantification of ER and PgR was determined by Scatchard analysis in cytosol and by computer-assisted image analysis with the Cell Analysis System 200 (CAS 200) machine after immunohistochemical techniques.

Cells were removed from the T-175 flasks with 1 mM EDTA in PBS and centrifuged 10 min at 300 *g*. Cells were re-suspended in 10 mM phosphate buffer, pH 7.5, containing 1.5 mM EDTA, 1 mM monothioglycerol and 10% glycerol. For PgR determination, BSA 1 mg/ml was added to the phosphate buffer. Cells were homogenized with a Teflon-glass homogenizer and ultracentrifuged for 30 min at 100,000 *g*. The cytosolic ER and PgR levels were determined by multipoint dextran-coated charcoal (DCC) assays according to EORTC (1980) recommendations using [^{3}H]-E_2 and [^{3}H]-ORG 2058 as labeled ligands. Cells with more than 10 fmol ER or PgR/mg protein were considered as positive by Scatchard analysis. To determine the effects of estrogens and progesterone on receptor numbers (ER and PgR), the cells were pre-incubated with 10^{-8} M E_2 or ORG 2058 for 24 hr before Scatchard analysis. Similar results were obtained when using usual medium or phenol red-free medium with charcoal-stripped serum.

For CAS-200 analysis, the cell pellets were fixed in formol for 30 min and incorporated in agar before immunolabelings with anti-ER and anti-PgR. The stained slides were analyzed and a quantitative immunocytochemical score (QIC score) was generated by use of the following equation: QIC score = positive percentage $\times$ positive stain intensity/10. A QIC score below 18 was considered positive (Bacus *et al.*, 1988).

For mRNA analysis, total RNA was extracted from confluent cells with RNAzol (Cinna/Biotecx, Houston, TX). After thawing and de-naturation, RNA was submitted to electrophoresis through a 1% agarose formaldehyde gel, capillary transferred to a Hybond-N membrane (Amersham, Aylesbury, UK) and treated according to the manufacturer's instructions. Blots were hybridized sequentially with a ^{32}P-labeled ER cDNA probe (10^9 cpm/μg cDNA, produced by random priming; Boehringer) and a control 28S RNA oligonucleotide probe (Clontech, Palo Alto, CA); pre-hybridization and hybridization were performed in classical buffers for 4 and 18 hr respectively. The membrane was then washed with citrate-sodium solutions (SSC) of increasing stringency, the last wash being performed in 0.3 $\times$ SSC containing 0.1% SDS. Blots were visualized by exposure of the membrane to a Kodak XAR-5 film in an autoradiography cassette with intensifying screen.

Measurement of tumor-associated antigens

The presence of tumor markers CEA and CA15-3 was quantified in culture medium and in cytosols with an IRMA (Tandem-R CEA kit, Hybritech Europe, Liège, Belgium and BYK-Gulden, DF3 and 115D8 antibodies). HBL-100 cells (Polanowski *et al.*, 1976) were used as control cells; they were grown and treated in the same

conditions. The concentration in culture medium alone (controls) was 1.5 ng/ml for CEA and 1.1 U/ml for CA 15-3.

Ploidy study and cytogenetic analysis

The cells were fixed in formol for 30 min, and after Feulgen coloration, they were analyzed for DNA content by computer-assisted image analysis (CAS 200, $0.9 < \text{DNA index} < 1.1$ for diploidy).

For cytogenetic analyses, semi-confluent cells were exposed to 1 µg/ml colcemid for 2 hr at 37°C, then detached with trypsin. Hypotonic treatment was performed in 0.075 M potassium chloride for 10 min and the cells were fixed with 3:1 methanol-glacial-acetic-acid. The slides were stained conventionally with 3% Giemsa for morphological examination and counting of the chromosomes. G-banding of the chromosomes was obtained by heating at 60°C overnight and staining with Wright stain freshly diluted 1:3 in phosphate buffer, pH 6.8.

Tumorigenicity

Breast-cancer cells (5×10^6 cells/0.1 ml of PBS) were injected s.c. into 5 female nude mice (*nu/nu*, 4 weeks old, Iffa Credo, Font Saint Landry, Belgium). The mice were examined every week for the development of palpable tumors. At the end of the experiment, the animals were killed and the tumors were excised.

RESULTS

Morphology

The 3 breast-cancer cell lines were developed in culture as monolayers of epithelial cells.

IBEP-1 cells appeared as small clusters of spherical or fibroblastic poorly differentiated cells (Fig. 1*a*). Electron-microscopy analysis indicated the presence of large nuclei, of large number of intracytoplasmic inclusions and of duct-like vacuoles, but the cells contained few desmosomes (Fig. 2*a*).

IBEP-2 cells appeared as a layer of uniformly sized, large polyclonal cells, possessing a large nucleus with either a single nucleolus or numerous lobulated nucleoli. These cells had a well-developed endoplasmic reticulum (Fig. 1*b*). Under electron-microscopic examination, most of the cells showed several characteristics typical of epithelial mammary tumor cells (*e.g.*, numerous microvilli on the surface, Fig. 2*b*).

IBEP-3 cells demonstrated variable degrees of attachment to the plastic (Fig. 1*c*). The well-attached cells were polyhedral in shape, with large nuclei and 1 or 2 prominent nucleoli. The loosely attached cells were spherical, and clusters of these cells frequently overlaid the well-attached cells. Electron-microscopy analysis showed epithelial characteristics, such as high numbers of desmosomes and tight junctions (Fig. 2*c*).

The cell morphology of the 3 cell lines was quite stable for over 20 passages. The ultrastructural characteristics (large nuclei, intracytoplasmic duct-like vacuoles, abundant microvilli on the cell border, desmosomes) of the 3 cell lines suggested their epithelial nature and their neoplastic origin (Fig. 2).

Immunohistochemistry

Immunohistochemical data are summarized in Table I. Each of the cell lines showed positive immunoreaction for EMA and low-molecular-weight cytokeratins (CAM 5.2), which are markers of epithelial differentiation. IBEP-1 cells, however, showed only weak positivity for the cytokeratins. The immunoreaction was negative for vimentin in all 3 cell lines. IBEP-1 and IBEP-3 cells expressed carcino-embryonic antigen (CEA) and c-erbB-2 protein, and showed weak staining for GCDFP 15, which is a human breast-gross-cystic-disease-fluid protein. IBEP-2 and, to a lesser extent, IBEP-3 cells showed specific immunocytochemical reactions with antibodies for LEU-M1 (CD-15), which is expressed in epithelial cells but not in mesothelial cells.

Presence of steroid receptors

The presence of estrogen and progesterone receptors in the 3 cell lines was detected by Scatchard analysis in the cytosol. IBEP-1 and IBEP-3 cells were ER-negative and PgR-positive. IBEP-2 cells were ER-positive and PgR-negative, but PgR was inducible by E_2.

The binding capacity of ER in IBEP-2 cells was 205 fmol/mg of protein with a dissociation constant of 1.06×10^{-10} M. ER concentrations in IBEP-2 cells were down-regulated after pre-incubation with E_2 (10^{-8} M for 24 hr; Fig. 3*a*). Similarly, the mRNA for estrogen receptor was detected in IBEP-2 cells but not in IBEP-1 and IBEP-3 cells (Fig. 4).

The binding capacities of PgR in IBEP-1 and IBEP-3 cells (Fig. 3*b*) were 1139 fmol/mg of protein ($K_D = 8.3 \times 10^{-10}$ M) and 475 fmol/mg protein ($K_D = 1.6 \times 10^{-10}$ M), respectively. Moreover, PgR levels in these 2 cell lines were completely down-regulated after pre-incubation with ORG 2058 (10^{-8} M for 24 hr). On the other hand, neither induction nor inhibition of binding capacities of PgR were observed after E_2 treatment in these two cell lines. On the contrary, in IBEP-2 cells, no PgR was detected but, after pre-incubation with E_2, the binding capacity of PgR reached 318 fmol/mg protein ($K_D = 4.8 \times 10^{-10}$ M; Fig. 3*c*). Similar results were obtained in 3 separate experiments.

These data were confirmed by immunohistochemical analysis and quantification by CAS 200. The QIC score for PgR in IBEP-1 and IBEP-3 was 736 and 370 respectively, and in IBEP-2 cells, the QIC score was 63 for ER and 14 for PgR, which is considered to be negative (see ''Material and Methods'').

Biochemical analysis

The presence of the tumor-associated antigens CEA and CA 15-3 was determined in the culture medium and in the cytosol of the 3 cell lines. The concentrations of tumor markers in cytosols of IBEP-1, IBEP-2 and IBEP-3 cells were 5.2, <1.5 and 2.3 ng/ml, respectively, for CEA and 30, 12 and 5 U/ml, respectively, for CA 15-3. In culture medium, however, the tumor markers were all undetectable, except for CA 15-3 with IBEP-1 cells (8.4 U/ml). Neither CEA nor CA 15-3 were detectable in cytosol or culture medium of the control HBL-100 cells.

Proliferation

Growth curves were established for the 3 cell lines (Fig. 5), and the doubling population times for IBEP-1, IBEP-2 and IBEP-3 cells were 68, 29 and 39 hr respectively. E_2 increased the proliferation rate of IBEP-2 cells by 38%, but E_2 did not exert any detectable effect on the growth of IBEP-1 and IBEP-3 cells. Progesterone (ORG 2058) had no effect on the growth of IBEP-2 cells, but increased the proliferation rate of IBEP-1 and -3 cells by 67 and 33% respectively.

Ploidy study and karyotype analysis

The 3 cell lines were aneuploid with a DNA index of 1.25 for IBEP-1, 1.75 for IBEP-2 and 1.31 for IBEP-3. A karyotype analysis was carried out to gain some information on the modal number of chromosomes, the presence of specific markers and chromosome abnormalities. The modal number of chromosomes was determined after conventional Giemsa staining. Chromosome numbers in IBEP-1, IBEP-2 and IBEP-3 cells ranged between 47 to 67, 72 to 75 and 55 to 60 with median values of 52, 74 and 57 respectively. For IBEP-1 cells, the karyotype was mostly diploid but several chromosome abnormalities were observed. A few normal human chromosomes could be identified, including chromosomes 7, 10 and 11. For IBEP-2 cells, the karyotype was mostly triploid, presenting extensive rearrangements leading to many unidentified chromosomes. Chromosomes 7, 8, 11, 15, 17 and 21 appeared disomic and chromosomes 9 and 10 were not identified. For IBEP-3 cells, normal human chromosomes were most abundant (6, 7, 9, 10, 14, 15 and X). However, in all 3 cell lines, chromosomes of unidentified origin were present.

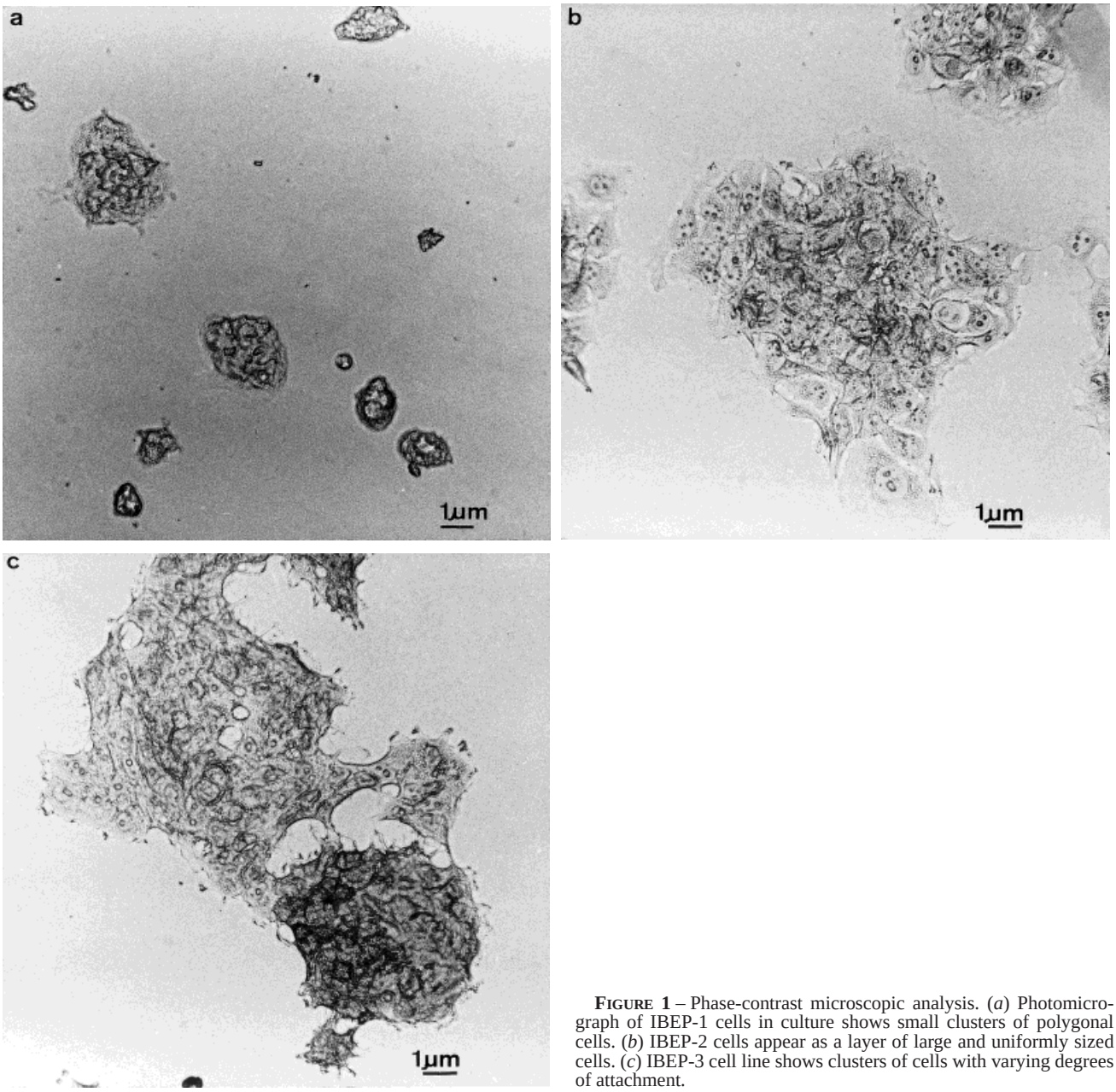


FIGURE 1 – Phase-contrast microscopic analysis. (*a*) Photomicrograph of IBEP-1 cells in culture shows small clusters of polygonal cells. (*b*) IBEP-2 cells appear as a layer of large and uniformly sized cells. (*c*) IBEP-3 cell line shows clusters of cells with varying degrees of attachment.

Tumorigenicity

Nude mice (3 groups of 5 each) were injected s.c. with 5×10^6 IBEP-1, IBEP-2 and IBEP-3 cells. Local tumors were observed in 5/5, 5/5 and 4/5 mice respectively. The latency was 11, 14 and 16 weeks for IBEP-1, -2 and -3 cells, respectively. Cells appeared to be poorly differentiated.

DISCUSSION

We established 3 new malignant breast-cancer cell lines, designated IBEP-1, IBEP-2 and IBEP-3, from pleural effusions of breast-cancer patients. Interestingly, 2 of these cell lines (IBEP-1 and IBEP-3) were PgR-positive and ER-negative, and one (IBEP-2) was more classically ER-positive and PgR-inducible. To the best of our knowledge, the ER^- PgR^+ phenotype of IBEP-1 and IBEP-3 is unique. Moreover, the PgR concentrations of these 2 cell lines were down-regulated after progesterone pre-incubation. IBEP-1 and IBEP-3 cells grew more slowly than IBEP-2 cells, in agreement with the finding that cells expressing ER are associated with a higher proliferation rate than ER^- cells (Fanelli *et al.*, 1996). The growth curves showed different confluent densities. Only IBEP-2 cells arrived at confluence after about 10 days. IBEP-1 cells never arrived at confluence and remained as small clusters before cell detachment. IBEP-3 cells showed variable degrees of attachment and had a morphology different from that of IBEP-2 cells. Moreover, IBEP-3 cells overlaid well-attached cells, inducing their death. These variable growth patterns could explain the final differences in cell density. E_2 increased the proliferation rate of IBEP-2 cells and progesterone stimulated the proliferation rate of IBEP-1 and IBEP-3 cells.

The 3 cell lines can be classified as epithelial because of their morphologic appearance in monolayer cultures (fibroblastic, spherical or epithelioid) and because of their unique ultrastructure. The ultrastructural features generally accepted as specific traits of

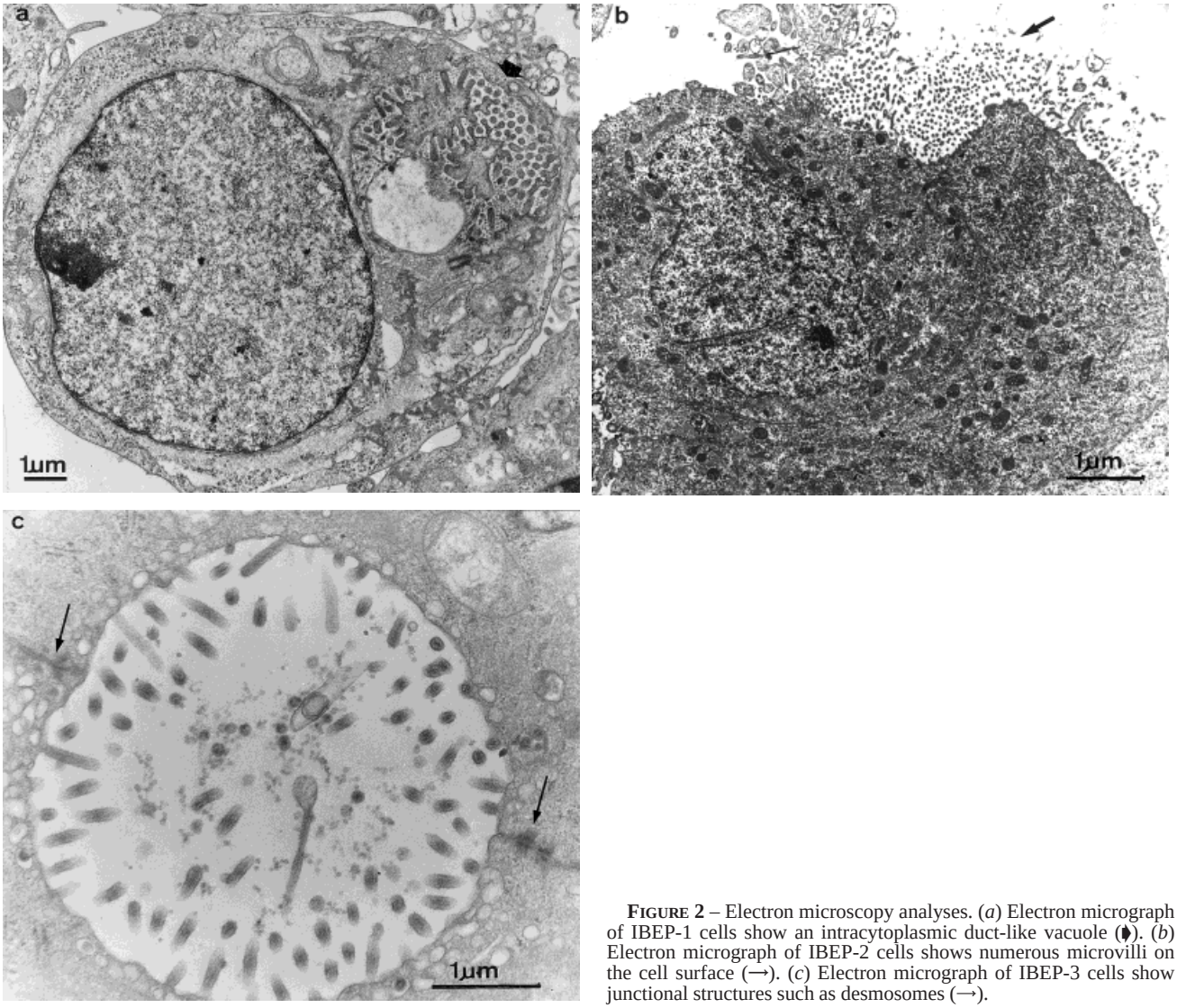


FIGURE 2 – Electron microscopy analyses. (*a*) Electron micrograph of IBEP-1 cells show an intracytoplasmic duct-like vacuole (⧫). (*b*) Electron micrograph of IBEP-2 cells shows numerous microvilli on the cell surface (⟶). (*c*) Electron micrograph of IBEP-3 cells show junctional structures such as desmosomes (⟶).

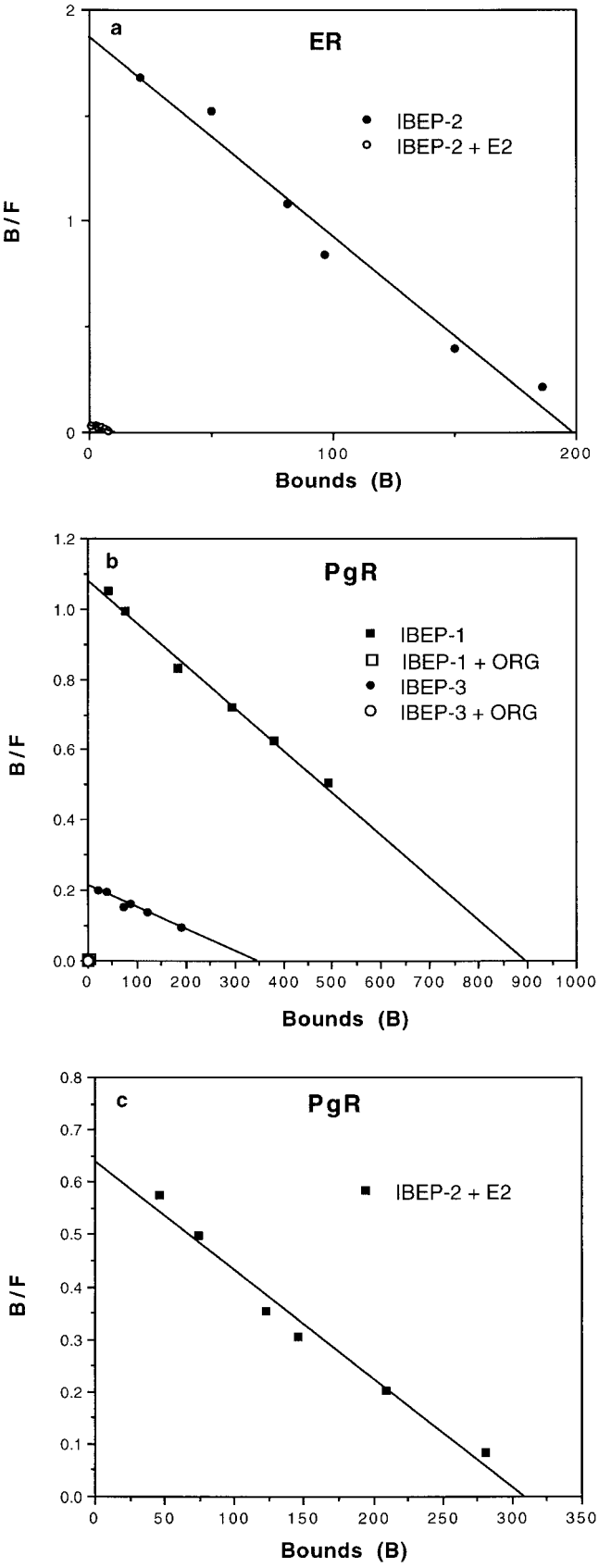
TABLE I – IMMUNOHISTOCHEMICAL CHARACTERISTICS OF THE 3 CELL LINES

	IBEP-1	IBEP-2	IBEP-3
EMA	+	+	+
Cytokeratin	+/−	+	+
Vimentin	−	−	−
GCDFP 15	+/−	−	+/−
LEU-M1	−	+	+/−
c-erbB-2	+	−	+
ER	−	+	−
PgR	+	−	+
CEA	+	−	+

epithelial cells (Buehring and Hackett, 1974) were found in all 3 cell lines at variable levels. There were tight junctions, desmosomes and abundant microvilli on the cell border. Moreover, intracytoplasmic duct-like vacuoles with protruding microvilli are also described as a specific feature of breast-tumor cells. Immunohistochemical analysis (EMA and low-molecular-weight cytokeratin) also confirmed their epithelial origin.

The 3 cell lines displayed aneuploid karyotypes, with a median of 52, 74 and 57 chromosomes, respectively. Moreover, all 3 cell types had an increased number of chromosomes with structural abnormalities and many unidentified chromosomes. In fact, aneuploidy is frequently observed in breast tumors, but not in normal or reactive mesothelial cells, and has been reported to be a marker of poor prognosis (Toikkanen *et al.*, 1989). The neoplastic nature of our cell lines is thus well demonstrated by their ploidy status, the high modal number of chromosomes, the frequency of multinucleated cells and the presence of tumor markers such CA 15-3, which is a classical marker of breast cancer, in the cytosol of the 3 cell lines. Definite proof was given by the development of tumors in *nu/nu* mice after s.c. injection of the cell lines. Our observations indicate that the 3 cell lines are highly malignant and that they maintain the ability to generate tumors.

These cell lines could be new *in vivo* models for studying the biology of breast cancer. It is well known that breast-cancer cell lines tend to be less tumorigenic and less metastatic than other cell lines derived from lung, renal and colon carcinoma when cells are injected s.c. in nude mice (Price *et al.*, 1990). Our cell lines therefore appear particularly interesting. Moreover, while most breast-cancer cell lines are predominantly ER^+, and must be injected with E_2 and/or matrigel to develop local tumors (Bao *et al.*, 1994; Van Slooten *et al.*, 1995), it is noteworthy that our IBEP-2 cells could grow independently of E_2 supplementation in female nude mice. Possibly a low level of endogenous E_2 may be sufficient for IBEP-2 cells to grow *in vivo*.



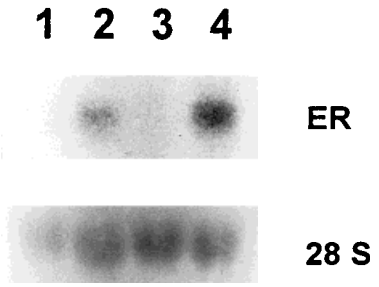


FIGURE 4 – Expression of mRNA for ER in IBEP-1 (1), IBEP-2 (2), IBEP-3 (3) and T-47D (4) cell lines.

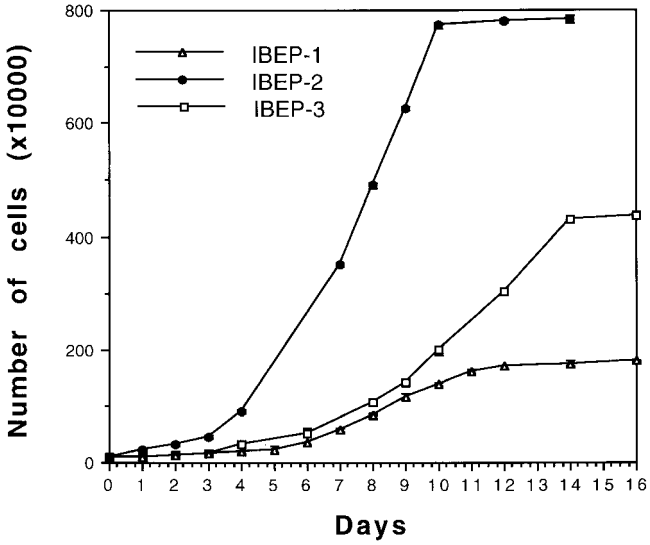


FIGURE 5 – Growth curves of IBEP-1, IBEP-2 and IBEP-3 cell lines. The doubling times were 68, 29 and 39 hr respectively.

Expression of ER is one of the most characteristic features of breast-cancer cells. The mRNA for ER was detected only in IBEP-2 cells. Both the primary tumor cells and IBEP-2 cells showed the presence of ER and inducible PgR, whereas IBEP-3 cells did not express ER, whether in the primary tumor or in culture; and IBEP-1 cells did not express ER although it was present in the primary tumor of the patient. This could be explained by anti-neoplastic treatment with tamoxifen. The expression level of ER in IBEP-2 cells (205 fmol/mg of protein) was comparable with or higher than in the majority of ER^+ breast-cancer cell lines but lower than in MCF-7 cells (±700 fmol/mg of protein). PgR was constitutively expressed by IBEP-1 and IBEP-3 cells and down-regulated by progesterone pre-incubation. These receptors were measured by a semi-quantitative score after immunocytochemical staining (QIC score) and by Scatchard analyses. The role of progesterone in cell proliferation remains controversial: this hormone may be growth-stimulatory or growth-inhibitory for breast-cancer cells, but the stimulatory effects of progestins could be masked in the presence of estrogen (Clarke and Sutherland, 1990). The effects of progester-

FIGURE 3 – Er and PgR determination in cytosol of IBEP cells by the dextran-coated charcoal method (Scatchard analysis, fmol/mg protein). (*a*) ER concentrations in IBEP-2 cells (●); ER was down-regulated after pre-incubation with E_2 10^{-8} M for 24 hr (○); (*b*) PgR in IBEP-1 and IBEP-3 cells; PgR were completely down-regulated after pre-incubation with ORG 2058 10^{-8} M for 24 hr; (*c*) induction of PgR in IBEP-2 cells after pre-incubation with E_2 10^{-8} M for 24 hr.

one are also mediated through its receptor and binding to hormone-responsive elements (HREs) that modulate gene expression (Truss and Beato, 1993). As mentioned above, none of the established breast-cancer cell lines is ER^- and PgR^+. The constitutively high level of PgR in T-47D cells made them the essential model for studying the actions of progesterone in breast cancer, but this cell line is also ER^+, and PgR is an estrogen-regulated protein. Our 2 new cell lines, IBEP-1 and IBEP-3 could thus be useful in studying the regulation of PgR and the effects of progestins and antiprogestins on the growth rate of breast-cancer cells.

Since IBEP-1 and IBEP-2 cells were isolated from patients with breast cancer and bone metastases but IBEP-3 cells from a patient without skeletal involvement, such cell lines could also provide new models for studying the pathogenesis of tumor-induced osteolysis. We have shown that established breast-cancer cell lines release factors that modulate osteoblast properties (Siwek *et al.*, 1997) and preliminary findings obtained with the 3 new cell lines go in the same direction.

In conclusion, we have established and characterized 3 new breast-cancer cell lines with distinct ultrastructural, immunological and genetic characteristics that are typical of breast-cancer cells. These cell lines should provide new models for studying the regulation of estrogen and progesterone receptors independently, since 2 of our cell lines were ER^- and PgR^+. Moreover, all 3 cell lines appear to be particularly tumorigenic after s.c. injection in nude mice, which is classically the case only for ER^+ cells (Van Slooten *et al.*, 1995). In addition, our cell lines might help to clarify the pathogenesis of breast-cancer-induced osteolysis, especially since bone involvement is observed more frequently in patients with $ER^- PgR^+$ tumors than in those with $ER^+ PgR^+$ tumors (Body *et al.*, 1996).

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