Isolation of Two Distinct Epithelial Cell Lines from a Single Feline Mammary Carcinoma with Different Tumorigenic Potential in Nude Mice and Expressing Different Levels of Epidermal Growth Factor Receptors

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ABSTRACT

From a single spontaneous feline mammary carcinoma, two subpopulations of epithelial tumor cells have been isolated. The variant cells were established as cell lines designated K248C and K248P. DNA ploidy analysis showed that the two cell lines represented cell populations already present in the original tumor. Chromosome analysis confirmed the feline origin of K248C and K248P and demonstrated that in addition to unique marker chromosomes characteristic for each cell line, both cell lines had several marker chromosomes in common. These data suggest that the two cell populations arose from a hypothetical single ancestor which diverged during tumor progression. The K248C and K248P cell lines differed from one another with respect to their tumorigenicity in athymic mice and epidermal growth factor (EGF) receptor content. The K248C cells were highly tumorigenic as indicated by a short latency period and high take rate. The K248P cells were poorly tumorigenic. Southern blot analysis revealed that the K248C cells contained an amplified EGF receptor gene that was accompanied by elevated levels of EGF receptor RNA and protein. The K248C cells were growth inhibited in vitro at EGF concentrations that stimulated growth of K248P cells. The amplification of the EGF receptor gene could be detected only in DNA derived from K248C cells at high passage numbers and not in DNA derived from the original tumor and K248C cells at low passage numbers. These data suggest that amplification of the EGF receptor gene occurred during establishment of the K248C cell line.

INTRODUCTION

Studies on tumors in domestic animals can contribute to a better understanding of similar diseases in humans. Several reports (1-4) have focused on feline mammary carcinoma as a potential model for human breast cancer. Comparative studies have shown that feline mammary carcinoma shares important features with human breast cancer, including similar biological behavior and histological appearance (1, 2). The tumors show local infiltrative-destructive growth and metastasize to regional lymph nodes, lungs, pleura, and kidney. The tumor was classified according to the WHO classification for tumors in domestic animals (8). The classification was extended by the introduction of a distinct cribriform tumor type. Histologically, the tumor was classified as a compound adenocarcinoma with areas of papillary, cribriform, and solid tumor growth.

Establishment of the K248 Cell Lines. After autopsy the pulmonary metastasis of cat 248 was placed in ice-cold tissue culture medium and within 1 h was processed for tissue culture. Part of the tumor was removed for histopathological diagnosis, and the remainder of the tumor was debrided of necrosis and minced into 1- to 2-mm3 pieces. The minced tumor was disaggregated overnight in 0.9% collagenase (CLS III; Cooper Biochemicals) in DMEM (Gibco, Grand Island, NY) at room temperature with constant stirring. The resulting cell suspension was cultured in DMEM supplemented with 10% FCS (Gibco), 10 μg/ml insulin (Sigma, St. Louis, MO), 100 IU/ml penicillin, and 1 mg/ml streptomycin.

MATERIALS AND METHODS

Clinical Data on the K248 Donor. The tumor cells were collected at autopsy from a pulmonary metastasis of a mammary carcinoma in a 14-year-old spayed Siamese cat (K248), which had undergone radical mastectomy 3 months earlier for infiltrating adenocarcinoma of the mammary gland. No chemo- or radiotherapy had been given. Autopsy disclosed a widespread disease, with metastases to the regional lymph nodes, presternal lymph node, lungs, pleura, and kidney. The tumor was classified according to the WHO classification for tumors in domestic animals (8). The classification was extended by the introduction of a distinct cribriform tumor type. Histologically, the tumor was classified as a compound adenocarcinoma with areas of papillary, cribriform, and solid tumor growth.

Received 11/19/90; accepted 5/24/91.

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1 E. S. was supported by Dutch Cancer Society Grant NK87-12.

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The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; DF medium, DMEM/Ham F-12 medium; MAb, monoclonal antibody; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (NaCl 150 mM-Na3 citrate-2H2O,15 mM, pH 7.0); CDNA, complementary DNA; DI, DNA index; UM, unidentified marker; HSR, homogeneously staining region; FelV-gp70, M, 70,000 envelope glycoprotein of feline leukemia virus.
ml streptomycin (both from Seromed), and after washing in PBS it was xenografted s.c. between the scapulae in two 8-12-week-old athymic male B6Kh nude mice (0.15 ml centrifuged packed cell clumps in PBS). Confluent monolayers were dissociated with 0.25% trypsin (Difco) and 0.02% EDTA in Ca2+- and Mg2+-free PBS.

In the primary culture of tumor K248 two different cell types were identified. These variants were isolated by repeated differential trypsinization and two successive rounds of single cell cloning using 6.5-mm cloning cylinders as described previously (9). All cells were screened monthly for Mycoplasma contamination by Hoechst staining and were always found to be negative.

Cloning Efficiency in Soft Agar. Dispersed cells were resuspended in 0.25% agar (Difco) in culture medium at concentrations of 10³ and 5 x 10³ cells/ml and layered on top of 0.5% agar in culture medium. For anchorage-independent growth assay in the presence of EGF cells were plated identically, except that the medium added the next day and once every week contained 50, 0.5, or 0.01 ng/ml EGF (culture grade; Collaborative Research). Four weeks after initiating cultures, colonies were counted.

Saturation Densities and Doubling Times. Logarithmically growing cell cultures were trypsinized, and 5 x 10⁶ cells were plated in triplicate in 12-well plates (Costar) and cultured in DMEM supplemented with 10% FCS. Cells were counted every second day by hemacytometer. The doubling time and saturation densities were calculated from a plot of cell numbers against time.

Growth in Serum-free Medium. Tryptinized cells were washed once in a 1:1 medium mixture of DF (Gibco) with 10% FCS to inactivate trypsin. Subsequently, the cells were washed three times with DF medium, seeded in the same medium without FCS, and supplemented with 10 µg/ml insulin and 10 µg/ml transferrin (Sigma). When subcultured, 0.1% (w/v) soybean trypsin inhibitor (Sigma) in DF medium instead of 10% FCS-supplemented medium was used to inactivate trypsin.

Effect of EGF on Cell Proliferation. K248C, K248P, and A431 cells were seeded on 35-mm plates (Falcon) at a density of 10⁶ cells/plates in DF medium containing 10% FCS. After attachment overnight, cultures were washed three times with PBS, and the medium was changed to DF medium containing 10 µg/ml insulin and 10 µg/ml transferrin. After overnight incubation, the growth assay was performed in the presence of various concentrations of EGF (0.01-50 ng/ml) and in the absence of EGF. Cells were incubated for 10 days (K248C and K248P) or 6 days (A431), with medium changes every 48 h. The cells were then trypsinized and counted by hemocytometer. Data shown are the average of determinations from triplicate plates. Results were expressed as the percentage change in cell number for cells grown in EGF-supplemented media compared with cells grown in control media.

Other Cell Lines and Monoclonal Antibodies. A431, a human squamous carcinoma cell line with an amplified and rearranged EGF receptor gene (10), was obtained from the American Type Culture Collection. FEA, a feline fibroblast line, was the gift of Dr. O. Jarre (11). Both cell lines were grown in DMEM plus 10% FCS. The feline lymphoblast cell lines, F422 and FL74, were the gift of Dr. K. Weijer and were grown in RPMI 1640 (Gibco) supplemented with 10% FCS. Mouse MAb 425 was the generous gift of Dr. U. Rodeck (Wistar Institute, Philadelphia) and was directed against the protein backbone of the extracellular domain of the human EGF receptor (12). Mouse MAb 139H2 was raised against human epilaspin (13). Mouse MAb E.C4 reacts with keratin 8 and 18 in both humans and cats. Mouse MAb 3.17 was raised against FeLV-gp70 (14).

Electron Microscopy. Cells were grown in culture flasks and fixed in situ with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h and postfixed with 1% osmium tetroxide in the same buffer for 2 h. After fixation the cells were scraped off the flasks using a rubber policeman and centrifuged. The pellet was dehydrated and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead hydroxide. The preparations were examined with a Philips CM10 electron microscope.

Karyotype Analysis. Colchicine (1 µg/ml) was added to logarithmically growing cell cultures, followed by an incubation for 20 min at 37°C. The cells were harvested with trypsin and incubated for 12 min at 37°C in a hypotonic solution containing 0.075 M KCl. After centrifugation, the pellet was fixed in three changes of fresh methanol/glacial acetic acid (3:1, v/v). The fixed cells were placed on 6 x HCl-treated slides and air dried.

Chromosome spreads were “aged” at 60°C for 16 h and subsequently either conventionally stained in Giemsa for chromosome counts or G-banded. For each culture a minimum of 100 spreads was evaluated for modal chromosome number, and at least 7 G-banded karyotypes were analyzed for the detection of chromosomal aberrations. Karyotypes of each cell line were arranged according to the recommendations of the first international conference on standardization of banded karyotypes of domestic animals (15).

Ploidy Analysis. Multiple samples derived from frozen and 40-µm paraffin-embedded sections of the original K248 pulmonary metastasis were used for flow cytometric DNA analysis according to the method previously described (16). Briefly, frozen sections were thawed at room temperature, cut into small pieces, treated with detergent-trypsin, and stained with propidium iodide (Sigma). Nuclear suspensions were analyzed with chicken RBC and normal feline WBC as internal standards. From deparaffinized sections, cell suspensions were prepared by treatment with a solution of pepsin (no. P7000; Sigma) and stained with 4',6-diamidino-2-phenylindole dihydrochloride (Boehringer, Mannheim, Germany). Stained samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, MA).

Suspensions of cultured cells in 40 mM citrate buffer, pH 7.6, containing 250 mM sucrose and 5% (v/v) dimethyl sulfoxide, were frozen in liquid nitrogen and analyzed as described for nuclei derived from frozen sections.

Tumorigenicity. Eight- to 12-week-old male B6Kh nu/nu mice were used as recipients for tumor cell injections. Cell cultures at passage 60 were trypsinized and washed 3 times in PBS, and 10⁶ cells suspended in 0.1 ml PBS were injected s.c. between the shoulder blades. For i.v. injections, mice were anesthetized with ether, and 8 x 10⁶ cells in 0.1 ml PBS were injected in the tail vein with a 25-gauge needle. All animals were sacrificed by cervical dislocation when the tumors reached a diameter of 2 cm or after 4 months in the absence of palpable tumors. Tumors and selected organs (axillary lymph nodes, lungs, liver, and kidneys) were removed, fixed in phosphate-buffered 10% formalin, and processed for histopathological examination.

Immunofluorescence. For detection of keratins, cells were grown on glass coverslips and fixed in cold acetone for 4 min. For FeLV-gp70 analysis, a suspension of 10⁶ cells/ml was incubated on multitest slides (Flow, Irving, Scotland) for 1 h at 37°C. The reaction of monoclonal antibodies in the appropriate dilutions with the two cell lines was tested in an indirect immunofluorescence assay using fluorescein-conjugated goat anti-mouse immunoglobulin as the second antibody (Nordic Diagnostik, Tilburg, The Netherlands). Normal mouse serum (diluted 1:100) served as a negative control. The cells were examined under a fluorescence microscope.

DNA Isolation and Blotting. High-molecular-weight genomic DNA was isolated by SDS-proteinase K lysis, phenol/chloroform extractions, and ethanol precipitation as described (17). Fifteen µg DNA were digested with restriction enzymes (EcoRI, BamHI, HindIII, and XbaI) according to the manufacturer's recommendations (Boehringer), fractionated on 0.8% agarose gels in buffer (0.04 M Tris-acetate, pH 8.2-0.02 M sodium acetate-0.018 M NaCl-0.002 M EDTA), transferred onto nitrocellulose filters (Schleicher and Schuell) according to the method of Southern (18), and fixed at 80°C for 2 h.

RNA Isolation and Blotting. Total RNA was isolated in 3 M lithium chloride/6 M urea as described previously (19). Twenty µg of total RNA were electrophoresed on 1% agarose gels, transferred with 20 x SSC to nitrocellulose filters, and fixed by heating at 80°C for 4 h.

Hybridization and Probes. Nitrocellulose filters, containing DNA or RNA, were prehybridized for 2 h in 10% dextran sulfate, 5 x Denhardt's solution (1 x = 0.02% polyvinylpyrrolidone-0.02% Ficol-0.02% bovine serum albumin), and 50 µg/ml salmon sperm DNA at 65°C. 32P-Labeled

4 D. Ivanyi, personal communication.
probes were added and incubated for 16 h at 65°C. The filters were washed once with 3 x SSC, 0.1% SDS, once with 1 x SSC, 0.1% SDS, and twice with 0.1 x SSC, 0.1% SDS, for 30 min at 65°C, depending on the homology of the probes. The filters were dried and exposed to Kodak XAR-5 films with Dupont Lightning-Plus intensifying screens.

Whole plasmids or purified DNA fragments were labeled by random priming using [32P]dCTP (specific activity 10^9 cpm/µg DNA; Amer sham). The following probes were used in this study: a human HER 64.1 cDNA clone (20); a BamHI fragment of rat neu (21); a 1.3-kilobase BamHI genomic feline sis fragment; a 2.3-kilobase BamHI genomic feline myc fragment (both obtained from Dr. W. van de Ven); a 1.8-kilobase HindIII/PstI env fragment of feline RD 114 (provided by Dr. P. Roy-Burman); and a 1.3-kilobase PstI fragment of rat glyceraldehyde triphosphate dehydrogenase cDNA (22).

Immunoblotting. Membrane preparations (40 µg) were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions and blotted onto nitrocellulose according to the method described by Towbin et al. (23). To prevent nonspecific binding the blot was blocked with 0.5% gelatin in PBS for 60 min at room temperature. After 3 washes in PBS, the blot was incubated for 60 min at room temperature with protein A-purified MAb 425 and MAb 139H2 (13) as a negative control labeled with 125I (0.2 x 10^6 cpm/ml). After 5 washes in PBS the blot was dried and exposed to a Kodak XAR-5 film at -70°C.

Immune Complex Kinase Assay. Trypsinized cells (2 x 10^6) were washed 3 times in PBS and solubilized in lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 10% glycerol, 100 units/ml of trysylol, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation at 12,000 x g for 15 min at 4°C. The cell lysates were precleared for 15 min at room temperature in the presence or absence of EGF (400 ng/ml) as described earlier (24). Immunoprecipitation of the EGF receptor was accomplished with MAb 425 (4.65 mg/ml). The cell lysates were incubated for 1 h at 4°C with 1 µl of MAb 425 or with 1 µl normal mouse serum/ml cell lysate. The immune complexes were precipitated for 30 min at 4°C with 25 µl donkey anti-mouse immunoglobulin-coated beads (IDS). The immune precipitates were washed three times in lysis buffer and resuspended in 25 µl kinase buffer (50 mM Tris-HCl, pH 7.5-8.0, 50 mM NaCl, 0.1% Triton X-100-10% glycerol-100 µM Na3VO4, 10 mM MnCl2, 1 mM phenylmethylsulfonyl fluoride). The kinase reaction was initiated by adding 10 µCl γ-ATP followed by a 10-min incubation on ice. The reaction was terminated by boiling with 1.5 x SDS sample buffer (0.1 M Tris/HCl, pH 6.8-2% SDS-6 M urea-7% mercaptoethanol-10% glycerol-0.15% bromphenol blue). Proteins were analyzed on 7.5% SDS-polyacrylamide gels under reducing conditions. Dried gels were exposed to Kodak XAR-5 films at -70°C.

Receptor Analysis. The receptors for estrogen, progesterone, and EGF were determined according to methods previously described (25, 26). Briefly, competition assays were performed on intact cells for the detection of EGF receptors and on cell lysates for the detection of estrogen receptor and progesterone receptor, using 125I-labeled ligand and multiple concentrations of cold ligand. The EGF receptor data were analyzed according to the method of Scatchard (27); regression curves were calculated with the Ligand computer program (26). The estrogen receptor and progesterone receptor binding data were analyzed according to the method of Scatchard. Values of >10 and >20 fmol/mg cytosolic protein were taken as positive for estrogen and progesterone receptor, respectively.

RESULTS

Establishment of Cell Lines. The K248 primary culture contained at least two morphologically different cell types. One cell type, called K248C, was isolated by repeated differential trypsinization from the second cell type adhering strongly to the culture flask, referred to as K248P. At passage 6, both cultures entered a "crisis," during which most cells died and detached from the culture vessel. Some of the K248C and K248P cells survived the crisis and were cloned for two successive rounds of single cell cloning. Only cell beyond passage 60 were characterized unless otherwise stated.

Characteristics of the K248 Cell Lines. The K248C cells grew in tightly packed colonies, which detached readily after trypsinization. At saturation density, the cells were heterogeneous in size and focally formed ridges (Fig. 1A). Dome formation was present only after several days of confluency. The K248P culture consisted of more open colonies. At high cell densities the K248P cells formed a uniform layer of dark, "droplet" cells. Abundant dome formation occurred spontaneously shortly after the cultures reached confluency (Fig. 1B). The number of domes in the K248P cell cultures was much higher compared to the number of domes in the K248 cultures. The morphology remained unchanged during 2.5 years of continuous culture (over 200 passages).

The main growth characteristics (saturation density, doubling time, soft agar growth, and serum-free growth) of both cell lines are depicted in Table 1. Doubling times for the two variants were not significantly different. K248C reached a higher saturation density. Both cell lines were serum dependent for growth and did not form colonies in soft agar.

Ultrastructural analysis (Table 2) showed that both cell lines contained desmosomes, a typical feature of epithelial cells. The epithelial phenotype of K248C and K248P cells was further confirmed by the detection of keratin 8 and 18 using MAb E,C4. The presence of FeLV in both cell lines was investigated immunologically and electron microscopically. No FeLV-gp70

Fig. 1. Phase contrast micrographs of K248 established cell lines. A, K248C cells at saturation density. × 240. B, K248P cells at saturation density with dome. × 384.
could be detected in either cell line in an indirect immunofluorescence assay using MAb 3-17. No virus-like particles could be detected by electron microscopy. The presence of RD-114 provirus, an endogenous virus of felines, was demonstrated by hybridization studies with a RD-114 envelope-specific probe on DNA derived from both cell lines. No estrogen receptors and low amounts of progesterone receptor were detected in K248C DNA derived from both cell lines. No virus-like particles could be detected in either cell line in an indirect immunofluorescence assay using MAb 3-17, directed against feline leukemia virus gp-70.

DNA Ploidy. Table 3 presents the DNA indices of the original tumor and the cell lines at various passage numbers. The pulmonary metastasis from which the cell lines were derived was multiploid and contained three cell populations corresponding to DI of 1.09, 1.15, and 1.23. Flow cytometric analysis of the mixed primary culture (passage 2) showed that two cell populations (DI 1.09 and DI 1.25) were represented in the primary culture. Repeated differential trypsinization separated the two cell populations. At passage 4, the K248C cells had a DI of 1.27, whereas the K248P culture still contained two cell populations, one with a DI of 1.09 and a minor cell population with a DI of 1.24. During crisis a new cell population emerged in the K248C culture with a DI of 1.17; the K248P cells remained in the near-diploid range (DI 1.06). Therefore the ploidy of K248C and K248P remained constant for more than 160 passages (2.5 years of culture).

Tumorigenicity. The xenografted K248 pulmonary metastasis produced a tumor 2 weeks after s.c. inoculation in nude mice and could be maintained as a serially transplantable tumor line. The K248C and K248P cell lines showed variation with respect to their tumorigenic abilities when injected into athymic nude mice (Table 4). K248C cells (passage 60) formed tumors at both high and low cell concentrations in all of the s.c. inoculated male nude mice with a latency period of 2–3 weeks. The tumors reached a diameter of 1–2 cm after 3 months. Histologically, the tumors were classified as cribriform mammary carcinoma (Fig. 3B) resembling the cribriform growth pattern seen in part of the original tumor (Fig. 3A). In 1 of 13 tumors vascular invasion was observed. Experimental metastasis was tested by injection of 8 x 10^5 K248C cells i.v., resulting in lung colonization in 3 of 4 recipient animals within 5 months after inoculation. K248P cells (passage 60) were poorly tumorigenic at both high (3 of 14 take) and low (1 of 9 take) cell concentration upon s.c. inoculation into male nude mice within an observation period of 4 months. The latency time was variable, ranging from 4 to 16 weeks. The 4 tumors that arose proliferated very slowly. All K248P tumors were classified as adenocarcinomas (Fig. 3C). Extensive necrosis was characteristic of all tumors. The presence of vascular invasion in 3 of 4 tumors was remarkable. Injection of 8 x 10^5 K248P cells i.v. did not result in lung colonization as verified at autopsy 3.5 months later. Tumorigenicity experiments with K248C and K248P cells were repeated at passage 112 in female mice; similar results were obtained.
In conclusion, K248C is a highly tumorigenic cell line, while K248P is poorly tumorigenic.

Oncogene Activation in the K248 Cell Lines: EGF Receptor Gene. To investigate whether oncogenes, known to be involved in human breast cancer development, were activated in the original K248 tumor and K248 cell lines, DNA was isolated from the original tumor and from the K248C and K248P cells at low passage (<7 and 22) and high passage (>60) number and screened for amplification and rearrangements of the myc and neu oncogenes and the EGF receptor gene.

Hybridization using the 64.1 EGF receptor cDNA probe showed that the K248C cells at high passage number contained an amplified (4-6 times) EGF receptor gene (Fig. 4, Lane 5). The EGF receptor gene in K248P cells at high passage number was not amplified (Fig. 4, Lane 6). DNA isolated from normal feline placenta was used as a control for normal EGF receptor gene copy number (Fig. 4, Lane 4). Hybridization of the same filters to a rat neu (Fig. 4, Lanes 4-6), a feline sis (Fig. 4, Lanes 4-6), and a feline myc probe (not shown) indicated that equal amounts of DNA were loaded on the gel. The original K248 tumor (not shown), the K248C cells (Fig. 4, Lane 2), and K248P cells (Fig. 4, Lane 3) at low passage number (<7 and 22) were all negative for EGF receptor gene amplification (DNA from passage 22 is shown). DNA isolated from normal feline liver served as a control for normal EGF receptor gene copy number (Fig. 4, Lane 1). Hybridization of the same filter...
Table 3 Comparison of the DNA indices of the original K248 tumor with the DNA ploidy of the K248 cell lines at different passage numbers

<table>
<thead>
<tr>
<th>Tumor/cell culture</th>
<th>DNA index</th>
</tr>
</thead>
<tbody>
<tr>
<td>K248 original tumor</td>
<td>1.09; 1.15; 1.23</td>
</tr>
<tr>
<td>Primary culturea</td>
<td>1.08; 1.25</td>
</tr>
<tr>
<td>K248C at passage 4</td>
<td>1.27</td>
</tr>
<tr>
<td>12</td>
<td>1.17</td>
</tr>
<tr>
<td>86</td>
<td>1.18</td>
</tr>
<tr>
<td>K248P at passage 4</td>
<td>1.09; 1.24a</td>
</tr>
<tr>
<td>26</td>
<td>1.06</td>
</tr>
<tr>
<td>85</td>
<td>1.11</td>
</tr>
</tbody>
</table>

*a Analyzed at passage 2.
*a Minor cell population.

Table 4 Tumorigenicity of K248C and K248P in male B6Kh-nu/nu mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inoculum (x10⁶)</th>
<th>Site of injection</th>
<th>Latent period (wk)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>K248C</td>
<td>0.8</td>
<td>3/4</td>
<td>2-3</td>
<td>Cribriform</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9/9a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4/4a</td>
<td>2-3</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>K248P</td>
<td>0.8</td>
<td>0/3</td>
<td>15</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1/9a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3/14a</td>
<td>4, 8, 15</td>
<td></td>
</tr>
</tbody>
</table>

*a Weeks after s.c. inoculation to development of palpable tumors.
*a Differences in tumorigenic potential between K248C (13 of 13 take) and K248P (4 of 23 take) are statistically significant (P < 0.0001; Fisher exact test).

...to a rat neu probe (Fig. 4, Lanes 1–3) showed that equal amounts of DNA were loaded on the gel.

The EcoRI (Fig. 4, Lanes 5 and 6), HindIII (Fig. 4, Lanes 5 and 6), BamHI, and XbaI (both not shown) restriction patterns did not show any evidence of rearrangements of the EGF receptor gene, sis gene, and neu gene in the K248C and K248P cells at high passage number. No EGF receptor gene or neu rearrangements were found in the EcoRI digest of DNA isolated from the original K248 tumor (not shown) and the K248C (Fig. 4, Lane 2) and K248P (Fig. 4, Lane 3) cells at low passage number. Hybridization using the 64.1 probe revealed an amplified and rearranged EGF receptor gene in the control A431 cell line (not shown).

To determine whether amplification of the EGF receptor gene in the K248C cells could lead to increased expression of the EGF receptor, total RNA was isolated from the K248 tumor, the K248 cell lines at high (passage 96) and low (passages 22 and 23) passage number, and the A431 cell line; blotted to nitrocellulose; and analyzed for levels of EGF receptor transcripts. The filter was hybridized to the 64.1 EGF receptor cDNA probe (Fig. 5A) and to a rat glyceraldehyde triphosphate dehydrogenase probe (Fig. 5B) to check for the amount of RNA loaded on the gel. After correction for the amount of RNA loaded on the gel, we concluded that the K248C cells at high passage number contained increased levels of normal-sized EGF receptor transcripts (Fig. 5A). The size of normal EGF receptor transcripts was determined using normal feline salivary gland RNA (not shown). The EGF receptor transcripts were present in lower quantities in the original K248 tumor (not shown), in the K248P cells at high passage number (Fig. 5A), and in the K248C and K248P cells at low passage number (Fig. 5A). In addition, no expression of the EGF receptor gene was found in the FL74 and F422 feline lymphoblast cell lines (not shown). No truncated EGF receptor transcripts were detected...
HETEROGENEITY OF CELLS FROM A FELINE MAMMARY CARCINOMA

Eco RI

1 2 3

Eco RI Hind III

4 5 6 4 5 6

EGFR

23.0 - 9.4 - 6.6 - 4.4 -

425 and tested for autophosphorylation activity. The A431 cells were used as a control for autophosphorylation activity. Fig. 7 shows that both the K248C and the K248P cells contained a basal level of autophosphorylation activity. The basal levels of autophosphorylation activity in K248C and K248P cannot be compared with each other since the membrane preparations loaded onto the gel were not corrected for the amount of receptors present. Both K248C and K248P cells responded to EGF stimulation by an increased autophosphorylation of the EGF receptor. These results suggest that in both K248C and K248P a functional EGF receptor was present. Only a slight increase in phosphorylation activity of the EGF receptor was observed in the A431 cell line after addition of EGF (Fig. 7).

Estimation of EGF Binding Sites. Scatchard plot analysis of the EGF binding data (Fig. 8) revealed two receptor classes for each cell type with dissociation constants in the range of $10^{-8}$ to $10^{-10}$ M. The total amount of EGF receptors in K248C was showed that amplification of the EGF receptor gene in K248C cells at high passage number correlated with overexpression of the EGF receptor gene, without rearranging the gene.

Further characterization of the EGF receptor on the protein level was performed with K248C and K248P cells beyond passage 60.

Analysis of EGF Receptor Levels in Cell Lines. In order to determine whether increased EGF receptor gene expression correlated with elevated levels of EGF receptor protein, we analyzed the amount of EGF receptor in K248C and K248P by Western immunoblotting using purified 125I-labeled monoclonal antibody 425. As a negative control MAb 139H2 was used. Equal amounts of membrane proteins, as determined with the BioRad protein assay, were loaded on the gel. The results (Fig. 6) revealed that K248C contained more $M_r 170,000$ EGF receptors than did K248P. In conclusion, the difference in EGF receptor RNA levels between the K248C and K248P cell lines was reflected at the protein level.

EGF Receptor Kinase Activity. Upon ligand binding to the EGF receptor a tyrosine-specific kinase becomes activated which results in autophosphorylation of the EGF receptor. To determine whether the EGF receptors of both cell lines were functionally active, EGF receptors from both K248C and K248P were immune precipitated with monoclonal antibody SIS

Fig. 4. Southern blot analysis of DNA from the K248C and K248P cell lines at high and low passage number, normal feline liver and normal feline placenta. DNA was digested with EcoRI or HindIII restriction endonuclease, blotted to nitrocellulose and hybridized to the 64.1 EGF receptor cDNA probe (EGFR) to a rat neu(r-Neu) and a feline sis (SIS) probe. Lane 1, normal feline liver; Lane 2, K248C at passage 22; Lane 3, K248P at passage 22; Lane 4, normal feline placenta; Lane 5, K248C at high passage number (>60); Lane 6, K248P at high passage number (>60). Molecular markers (in kilobases) are derived from electrophoresis of λDNA digested with HindIII.

Fig. 5. Northern blot analysis of total RNA from K248C and K248P at low passage (23 and 22, respectively) and high passage (96) number and from A431. The same nitrocellulose filter was hybridized to the 64.1 EGF receptor cDNA probe (A) and the glyceraldehyde triphosphate dehydrogenase probe (B). Kb, kilobases.
HETEROGENEITY OF CELLS FROM A FELINE MAMMARY CARCINOMA

In Vitro Response of Cell Lines to EGF. The effect of increasing concentrations of EGF on K248C, K248P, and A431 cell proliferation is shown in Fig. 9. Under the serum-free conditions used, the proliferative response of K248C cells to the addition of EGF was biphasic with stimulation of growth at 0.01 and 0.1 ng/ml EGF and inhibition at EGF concentrations exceeding 1 ng/ml. The K248C cell line was capable of growth in serum-free medium in the presence of 0.01 ng/ml EGF for an observation period as long as 3 months.

In contrast, EGF at doses ranging from 0.01 to 50 ng/ml stimulated the growth of K248P cells under the serum-free conditions used. K248P cells were capable of growth in serum-free medium in the presence of EGF at concentrations ranging from 0.01 to 50 ng/ml and have been carried as long as 3 months in this manner. The observed effect of EGF on proliferation of A431 cells is in accordance with literature data (28). EGF did not induce growth of K248C and K248P cells in soft agar.

DISCUSSION

Thus far, the establishment of feline mammary carcinoma cell lines has met with low success rates. Recently, a cell line was isolated from 1 of 30 mammary carcinomas (7). In another study, no feline mammary carcinoma cell lines were established from 14 mammary carcinomas (29). We succeeded in establishing 4 cell lines of 135 feline mammary carcinomas; 3 of the 4

![Fig. 6. Western immunoblot analysis of the EGF receptor from A431, and from K248C and K248P at high passage number. Equal amounts of membrane protein were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions and blotted onto nitrocellulose filters. The EGF receptor was detected by protein A-purified MAb 425 coupled to 125I. The specificity of the reaction was determined by incubating the filter with protein A-purified MAb 139H2 (negative control) coupled to 125I. Size markers are in thousands.](image1)

5.6 times the number calculated for K248P (Table 5). When a distinction was made between the number of high- and low-affinity receptors in both lines it was observed that K248C contained 5 times more low-affinity receptors and 10 times more high-affinity receptors than K248P (Table 5).

**In Vitro** Response of Cell Lines to EGF. The effect of increasing concentrations of EGF on K248C, K248P, and A431 cell proliferation is shown in Fig. 9. Under the serum-free conditions used, the proliferative response of K248C cells to the addition of EGF was biphasic with stimulation of growth at 0.01 and 0.1 ng/ml EGF and inhibition at EGF concentrations exceeding 1 ng/ml. The K248C cell line was capable of growth in serum-free medium in the presence of 0.01 ng/ml EGF for an observation period as long as 3 months.

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![Fig. 7. Analysis of protein kinase activity of the EGF receptor from A431 cells and from K248C and K248P at high passage number. Cell lysates from 2 × 10^6 K248C, K248P, and A431 cells were incubated for 15 min at 20°C in the presence or absence of EGF (400 ng/ml) and immunoprecipitated with either MAb 425 or normal mouse serum (NMS). Immunoprecipitates were incubated for 10 min at 4°C with 10 μCi [γ-32P]ATP. The reaction was terminated by addition of sample buffer, and the proteins were analyzed by gel electrophoresis under reducing conditions and autoradiography. Size markers are in thousands.](image2)

![Fig. 8. Scatchard analysis of EGF binding to K248C and K248P cells. Cells were incubated at 4°C for 3 h with a constant amount of 125I-labeled EGF together with increasing amounts of unlabeled EGF. Cell-associated radioactivity was measured and Scatchard analysis was performed as described in "Materials and Methods." Nonspecific binding was determined using a 1000-fold excess of unlabeled EGF.](image3)
cell lines were isolated from pulmonary metastases. Here, we (*, P < 0.01; •, P < 0.005; **, P < 0.001: Student's t test).

of the control without EGF; bars, SD; *, significant differences from the control.

present at low passage number, suggesting that the two cell

lines, both cell lines had several marker chromosomes.

that in addition to unique marker chromosomes characteristic

of the original tumor. Chromosome analysis showed

K248P cells suggested that the two cell types were already

ison of DNA indices of the original tumor and the K248C and

from a single multiploid feline mammary carcinoma. Compar

report the properties of two distinct epithelial cell lines derived

made at day 10 for K248C and K248P and at day 6 for A431. Points, percentage

to DMEM/F-12 with insulin and transferrin and increasing concentrations of

medium containing 10% FCS. After attachment overnight, medium was changed

overexpression of the EGF receptor gene was accompanied by

shown that in the K248C cell line at high passage number,

several human mammary carcinoma cell lines (37), we consider

receptor content in the K248P cell line was comparable with

other tumor systems in different species (9, 31-36).

discussed elsewhere (30). Tumor heterogeneity and the isolation

this observation on tumor therapy is obvious and has been

related but distinct tumor cell subpopulations. The impact of

in the histológica! appearance of individual feline mammary

carcinoma cells in culture or that overexpression of the EGF

sion of the EGF receptor gene is restricted to feline mammary

presented in this study emphasize the risk of extrapolating

establishment as a cell line. A similar observation has been

made in human squamous cell carcinomas, which showed a low

incidence of amplification in primary tumors but a high inci
dence in cell lines established from these tumors (41). The data

presented in this study emphasize the risk of extrapolating results obtained in vitro to the in vivo situation.

A more fundamental question is whether abnormal expres

sion of the EGF receptor gene is restricted to feline mammary

carcinoma cells in culture or that overexpression of the EGF

receptor gene is also causally related to the development of

feline mammary carcinomas. To address these issues a system

atic study on EGF receptor alterations in a large series of feline

primary and metastatic mammary carcinomas is now in progres

at our institute. Studies on human breast (6, 40), bladder

(42), and squamous cell carcinomas (43) suggest a causal rela

tionship between EGF receptor overexpression and tumorigenesis.

The tyrosine kinase activity of the EGF receptor, functioning

as a second messenger, is essential for many biological re

ponses, including DNA synthesis (44). The EGF receptor

proteins identified by Mab 425 showed functional tyrosine kinase activity as detected by receptor autophosphorylation.

The EGF receptor in K248C and K248P cells was biologically

competent since both cell lines were EGF dependent for growth

under serum-free conditions. However, the effect of EGF on

the growth of the two cell lines was different. A correlation was

found between the number of EGF receptors and the proliferative

response of K248C and K248P cells to the addition of

EGF. These data are consistent with the threshold model pro

duced by Kawamoto et al. (28), indicating an optimum ratio

between EGF and EGF receptors.

The observed relationship between the high number of EGF

receptors in K248C and its tumorigenic potential in nude mice has also been described for the human A431 (45) and MDA-

468 (46) cell lines in which the extent of amplification of the

EGF receptor gene in the parental and isolated variant lines correlated with the rate of tumor growth and length of the

latency period in athymic mice.

The K248 cell lines established in this work could be useful

for extending insight into the biology and heterogeneity of

feline mammary carcinomas. Since these lines can be grown in

cats (47) they may provide useful models for in vitro and in vivo

targeting studies with monoclonal antibodies.

ACKNOWLEDGMENTS

We gratefully acknowledge the gifts of monoclonal antibodies from

Dr. U. Rodeck (425), Dr. K. Weijer (3.17), and Dr. D. Ivanyi (E3C4)

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Our thanks are due to Dr. A. Sonnenberg, Dr. G. Verheijden, Dr. A. Bosma, and Dr. A. Jongsmma for useful discussions and to Dr. W. Mooi for critical reading of the manuscript.

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Isolation of Two Distinct Epithelial Cell Lines from a Single Feline Mammary Carcinoma with Different Tumorigenic Potential in Nude Mice and Expressing Different Levels of Epidermal Growth Factor Receptors

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