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Accumulation of neoplastic traits prior to spontaneous *in vitro* transformation of rat cholangiocytes determines susceptibility to activated ErbB-2/Neu

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Abstract

Cholangiocarcinoma, a severe form of biliary cancer, has a high mortality rate resulting partially from the advanced stage of disease at earliest diagnosis. A better understanding of the progressive molecular and cellular changes occurring during spontaneous cholangiocarcinogenesis is needed to identify potential biomarkers for diagnosis/prognosis or targets for novel therapeutics. Here, we show that with continued passage (p) *in vitro*, rat bile duct epithelial cells (BDEC) accumulated neoplastic characteristics that by mid-passage (p31-85) included alterations in morphology, increased growth rate, growth factor independence, decreased cell adhesion, loss of cholangiocyte markers expressed at low passage (p<30), and onset of aneuploidy. At high passage (p>85), BDEC cultures showed increasing numbers of cells expressing activated, tyrosine phosphorylated ErbB-2/Neu, a receptor tyrosine kinase previously reported to be at elevated levels in cholangiocarcinomas. Enrichment for high passage ErbB-2/Neu-positive cells yielded several anchorage-independent sub-lines with elevated levels of activated ErbB-2/Neu and increased expression of cyclooxygenase-2 (COX-2). When injected into immunodeficient beige/nude/xid mice, these sub-lines formed poorly differentiated cystic tumors strongly positive for rat cholangiocyte markers, a finding consistent with a previous report showing the susceptibility of high passage, non-tumorigenic BDEC to transformation by activated ErbB-2/Neu. Mid passage BDEC, in contrast, were resistant to the transforming activity of activated ErbB-2/Neu and remained anchorage dependent *in vitro* and non-tumorigenic *in vivo* following stable transfection. Based on these findings, we concluded that during progression to high passage, cultured BDEC undergo preneoplastic changes that enhance their susceptibility to transformation by ErbB-2/Neu.

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The ability to generate cells at different points in the process of spontaneous neoplastic transformation offers a valuable model system for identifying molecular features that determine whether over-expression of activated ErbB-2/Neu is necessary and sufficient to induce neoplastic conversion.

Keywords

spontaneous transformation; cholangiocyte; cholangiocarcinoma; ErbB-2/Neu

Introduction

Cholangiocarcinoma is a severe form of biliary cancer arising from cholangiocytes, the epithelial cells lining the intrahepatic and extrahepatic bile ducts. These cancers are usually described as well to moderately differentiated desmoplastic ductal carcinomas. The high rate of mortality for cholangiocarcinomas stems from difficulties in making an early diagnosis and the ineffectiveness of existing therapies for advanced disease (Gores, 2003; Lai and Sirica, 1999; Sirica et al., 2002). Currently, there are no *in vitro* models that have been used to examine the progressive changes that culminate in spontaneous cholangiocarcinogenesis. This limitation has made it difficult to study the molecular events leading to this form of cancer (Gores, 2003; Sirica et al., 2002; Sirica et al., 2001).

Recent reports indicate that over-expression of the proto-oncogene ErbB-2/Neu in parallel with up-regulation of cyclooxygenase-2 (COX-2) are important and perhaps essential events during cholangiocarcinogenesis (Endo et al., 2002; Sirica et al., 2002; Sirica et al., 2001). The proto-oncogene ErbB-2/Neu, designated as Neu in rats and Her-2 in humans, is a 185 kDa transmembrane glycoprotein receptor with intrinsic kinase activities belonging to the epidermal growth factor receptor (EGFR) family (Darcy et al., 2000; Yu and Hung, 2000). ErbB-2/Neu is involved in the regulation of numerous vital cellular functions such as cell growth, differentiation, and apoptosis, and is believed to play a significant role in multiple human cancers including mammary, ovarian, liver, and prostate, through amplification or over-expression (Yu and Hung, 2000).

While the ErbB-2/Neu gene is currently one of the primary targets for cancer therapeutics, the molecular signaling events necessary for ErbB-2/Neu-mediated transformation are still not well understood (Harari and Yarden, 2000; Yu and Hung, 2000). The high level of both plasma membrane ErbB-2/Neu and cytoplasmic COX-2 in most well-differentiated cholangiocarcinomas (Endo et al., 2002; Gores, 2003; Lai et al., 2005), suggests that over-expression of these genes is a relatively early event during cholangiocarcinogenesis. However, the discovery of poorly differentiated cholangiocarcinomas that are either negative, or only weakly positive for ErbB-2/Neu and COX-2 expression raises the possibility of alternative pathways leading to malignancy (Endo et al., 2002; Zhang et al., 2004). It has also been shown that over-expression of ErbB-2/Neu is frequently associated with ligand independence, invasiveness and metastasis, later events in the carcinogenic process associated with a poor prognosis (Harari and Yarden, 2000; Yu and Hung, 2000).

While these discoveries have significantly advanced our understanding of critical events during cholangiocarcinogenesis, there is still a need for markers denoting early, preneoplastic events occurring prior to the acquisition of a tumorigenic phenotype (Endo et al., 2002; Sirica et al., 2001). In the present report, we describe a model system applicable to the analysis of molecular and cellular events leading to spontaneous *in vitro* transformation of rat cholangiocytes. Using BDE1.1 and BDE4, two long term rat bile duct epithelial cell (BDEC) lines established in our lab, we show that with continued passage, BDEC accumulate characteristics of neoplastic cells that increase their susceptibility to transformation by activated ErbB-2/Neu. Repeated selection of high passage cultures for ErbB-2/Neu-positive BDEC produced several ErbB-2/Neu positive sub-lines that displayed anchorage-independent growth *in vitro* and rapid formation *in vivo* of large hemorrhagic and cystic cholangiocarcinomas expressing elevated levels of COX-2 and activated, tyrosine phosphorylated ErbB-2/Neu. In contrast, low, mid and unselected high passage BDEC failed to form tumors when injected into immunodeficient mice.

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Materials and methods

Antibodies

Monoclonal antibodies specific for the following markers were used for phenotypic analysis: CK19, Novocastra Laboratories, United Kingdom (catalog # CD19-2); E-cadherin, Zymed Laboratories, San Francisco, CA (catalog # 33-4000); the cholangiocyte membrane markers, OC.5 and OC.10 (Hixson et al., 2000); the cholangiocyte marker, BD.1 (Yang et al., 1993a); the cholangiocyte marker, OV-6, (Dunsford et al., 1989); Hsp70, Affinity Bioreagents Inc., Golden, CO (catalog # MA3-007); Neu (F-11), Santa Cruz Biotechnology, Santa Cruz, CA (catalog # sc-7301); ErbB-2/c-Neu (Ab-4), Calbiochem, San Diego, CA (catalog # OP16); c-K-Ras, Calbiochem (catalog # OP24); phospho-Histone H3 (Ser10), Cell Signaling, Danvers, MA (catalog # 9706); and His₆, Roche Diagnostics, Indianapolis, IN (catalog # 1 922 416). Polyclonal antibodies against ErbB-2/Neu (C-18) (catalog # sc-284), COX-2 (M-19) (catalog # sc-1747) and p-Neu Y1248 (catalog # sc-12352) were from Santa Cruz Biotechnology. ErbB-2 pY1139 (catalog # ab28161) was from Ab Cam. The Alexa fluor 594-conjugated goat anti-mouse IgM (catalog # A21044) and Alexa fluor 488-conjugated goat anti-mouse IgG (catalog # A11001) secondary antibodies were obtained from Molecular Probes, Eugene, OR. The HRP-conjugated IgM and IgG were from Biosource International, Camarillo, CA.

Cell culture

BDE1.1 and BDE4, two independently derived lines of well differentiated bile duct epithelial cells described by Yang et al. were maintained as previously described in medium

supplemented with Nu Serum, growth factors and 0.1 mg/ml normocin (InvivoGen, San Diego, CA) or penicillin/streptomycin (Yang et al., 1993b). At low passage BDE1.1 cells required type 1 collagen gels but by mid passage could be maintained on plastic without undergoing senescence. BDE4 cells acquired the ability to grow on plastic at low passage and could be maintained continuously with a morphology indistinguishable from BDE1.1 cells on collagen gels. To provide a basis for discussion, low, medium and high passage cells were operationally defined as passages 1-30, 31-85 and greater than 85, respectively. For soft agar assays, each well of a six well plate was coated with 2 ml of 1% agarose in complete medium. Cells were seeded on top of the soft agar substratum at a concentration of 1×10^6 cells/well in 2 ml of complete media. Plates were incubated at 37°C and analyzed microscopically to identify anchorage-independent clones.

Growth curves were performed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) following the manufacturers protocol. For each assay, BDEC were seeded in 24-well plates at a starting concentration of 5×10^4 cells/well.

Indirect immunofluorescence

Cells were seeded in permanox chamber slides (Nalge Nunc International, Rochester, NY, USA) for indirect immunofluorescence (IIF). Slides were blocked with normal goat serum or normal donkey serum in PBS, incubated with primary antibody and treated with either Alexa fluor 594-conjugated goat anti-mouse IgM or Alexa fluor 488-conjugated goat anti-mouse IgG (Hixson et al., 1997). Quantitative analysis of ErbB-2/Neu expression was carried out using Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD) to perform microdensitometry on digital micrographs of BDEC stained by IIF for ErbB-2/Neu. Measurements were restricted to sections of plasma membrane on ErbB-2/Neu-positive cells at high passage or negative cells at low passage selected using the Image Pro Plus outlining tool. Statistical analysis was done using Graph Pad Prism Software (San Diego, CA). The total plasma membrane area analyzed for low and high passage BDEC was approximately equal (880 and 960 pixels, respectively).

FACS analysis

BDEC were plated in 24-well plates in triplicate at a starting concentration of 5×10^4 cells/ml. Cells were allowed to reach 50-60% confluence before synchronization. Cells were trypsinized and washed in Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, MO). Cells were resuspended at a concentration of 1×10^7 cells/ml and pelleted at $400 \times g$ at 4°C and fixed in ice-cold ethanol (70%) overnight at 4°C and then stored. For propidium iodide (PI) staining, cell pellets were resuspended and centrifuged for 5 min at $1,200 \times g$ and the ethanol decanted. Pellets were vortexed in residual ethanol before adding 500 µl of PI staining solution consisting of propidium iodide (0.05 mg/ml; Roche, Indianapolis, IN) and RNase A (100U/ml; Sigma) in glucose-PBS sample buffer. Cells were incubated for 30 min at room temperature with gentle rocking, and analyzed within 24 hours. ErbB-2/Neu (Neu F-11) and COX-2 expression was quantified using the Imgenex intracellular staining flow assay kit (Imgenex, San Diego, CA) following the manufacturers suggestions. Fluorescence was measured on a logarithmic scale using a band pass filter for FITC. Expression of ErbB-2/Neu and COX-2 by low, mid, and high passage BDEC was expressed as a ratio of

the geometric mean fluorescence for cells treated with anti-ErbB-2/Neu or anti-COX-2 antibody over the geometric mean fluorescence of the same cells treated with a non-specific primary antibody: anti-His₆ for ErbB-2/Neu or goat anti-mouse IgG for COX-2.

Isolation of ErbB-2-positive BDEC

High passage BDEC were sorted using the MACS MS column from Miltenyi Biotec (Auburn, CA) following the manufacturer's protocol for magnetic labeling of cells using goat anti-mouse IgG microbeads (Simper-Ronan et al., 2006). BDEC at passage 99 and 60-70% confluence were harvested with trypsin, washed with PBS and resuspended in complete BDEC medium. The cells were labeled with primary mouse IgG anti-ErbB-2/c-Neu (Ab-4) and MACS goat anti-mouse IgG microbeads. The cell suspension was applied to a MACS MS column and unbound cells were collected in the flow through. After removal of the magnetic field, the magnetically labeled cells representing the ErbB-2/Neu-positive population were flushed from the column with buffer.

Cell lysates and immunoblots

Cells (2×10^8) from low and high passage BDEC were harvested with trypsin/EGTA, and washed with HBSS. BDEC pellets were resuspended in 2 ml of STM lysis buffer containing 0.25 M sucrose, 5 mM Tris (pH 7.4), and 1 mM MgCl₂ and 40 μ l protease inhibitor cocktail (Calbiochem, San Diego, CA), vortexed, and incubated on ice. After 15 min, extracts were pelleted at $12,000 \times g$ at 4°C for 10 min to obtain the membrane fraction. The resulting supernatant was further purified by centrifugation at $145,000 \times g$ at 4°C for 90 min to obtain the soluble cytosolic fraction. Aliquots from each supernatant were mixed with four volumes of 5X non-reducing SDS sample buffer (Laemmli, 1970). Proteins separated by 7.5% SDS-PAGE were transferred onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad Laboratories) and immunoblotted as described (Yang et al., 1993a). Bound HRP was visualized by incubation with SuperSignal West Peroxide Solution (Pierce, Rockford, IL).

Aggregation assay

Low and high passage BDEC were harvested with trypsin without EDTA, pelleted and washed. The cell pellet was then treated with EDTA (0.3 mM, pH 8) in HBSS. Following centrifugation, cells were suspended in aggregation buffer consisting of BDEC complete medium supplemented with BSA (2 mg/ml), HEPES (25 mM), and DNase (20 μ g/ml), and filtered twice through a 120 micron filter (Hixson and McEntire, 1989). Cells at a concentration of 4×10^6 cells/ml were plated in a 24-well plate at 600 μ l per well and rotated at 37°C at 60 rpm. For each time point, a 20 μ l sample was removed and single cells, doublets, triplets, quadruplets, and aggregates larger than 4 cells were counted. Each experiment was done in triplicate and phase contrast micrographs were taken at 0, 45 and 90 minutes.

Tumorigenicity assay

All animal studies were performed using protocols approved by the Rhode Island Hospital Institutional Animal Care and Use Committee. Tumorigenicity was assayed in 4-6 week old female beige/nude/xid triple deficient mice. 5×10^6 cells suspended in HBSS were injected

into the interscapular fat pad and each of the two front flanks. The mice were monitored 4 times a week and kept for up to 1 month. At the time of sacrifice, tumors were measured, removed, weighed and frozen in a hexane/acetone bath and stored at -80°C . Tumors were analyzed by IIF as described above. A minimum of three animals was injected for each of the cell lines tested.

Plasmids and nucleofection

The retroviral plasmid neu*-LXSN containing the oncogenic form of neu activated by a single point mutation resulting in a Val to Glu substitution at position 664 in the transmembrane domain was a generous gift from Lisa M. Petti; Albany Medical College, Albany, NY (Petti and Ray, 2000). For transfection of low passage BDEC (p22), 2×10^6 cells at 60-70% confluence were transfected with 10 μg of plasmid DNA following the manufacturer's recommended protocol for nucleofection (Amaxa Inc., Gaithersburg, MD) of adherent cells using V solution and programs A-024 and B-016. Each nucleofection was carried out in duplicate. After incubation for 24 hours in complete medium, stable transfectants were selected by maintaining the cells in complete medium containing G418 at 600 $\mu\text{g}/\text{ml}$ with medium changes every 48 hrs. The end result was four G418 resistant sub-lines. To confirm the expression of the virally encoded ErbB-2/Neu, RT-PCR was performed with primers corresponding to a 3' ErbB-2/Neu sequence and to a plasmid sequence incorporated into the 3' end of ErbB-2/Neu transcripts encoded by the transfected retroviral expression vector. Elevated expression at the protein level was confirmed by immunoblot or IIF with antibodies specific for the activated ErbB-2.

Metaphase spreads

Ploidy was determined from metaphase spreads prepared by the genetics laboratory at Women and Infants Hospital. Briefly, exponentially growing low, mid, and high passage BDEC, between 60-85% confluence, were treated with colcemid at 0.1 $\mu\text{g}/\text{ml}$ for 30 min to disrupt the mitotic spindle. Treated cells were harvested with trypsin and resuspended in a hypotonic solution (75 mM KCl). Following a 15 min incubation at room temperature, cells were collected by centrifugation, and fixed in 3:1 (v/v) methanol: acetic acid for one hour. After two changes of fixative, cells were dropped on cold wet slides and allowed to air dry. Trypsin-Giemsa banding was performed according to the procedures of Sun et al. (Sun et al., 1974). Well-banded metaphase spreads were used to determine ploidy. A total of 20 metaphase spreads were analyzed for each passage.

Results

Low passage BDE4 cells are distinguished from BDE1.1 cells by their ability to grow on plastic

We have previously reported that BDE1.1 and BDE4 cells share a close similarity in both phenotype and morphology (Yang et al., 1993b). At low passage (<p30), both lines expressed high levels of the cholangiocyte markers OC.2, OV-6, BD.1, γ -glutamyl transpeptidase (GGT), the epithelial/cholangiocyte marker CK19, the desmosomal plaque protein, DP.I, and the cell adhesion molecule, E-cadherin (Yang et al., 1993b). We have also found that at low passage, both cell lines displayed minimal levels of ErbB-2/Neu and high

levels of the cholangiocyte markers OC.5 and OC.10 (data not shown). Morphologically, both cell lines appeared as tightly packed colonies of polygonal cells frequently displaying duct-like structures at their periphery. Both cell lines possessed near diploid karyotypes, grew at similar rates and required medium supplemented with fetal bovine serum and other growth factors (Yang et al., 1993b) for proliferation and survival. At low passage (< p30), BDE1.1 cells had to be maintained on type 1 collagen gels. When plated on plastic, they acquired a squamous morphology and underwent senescence within a few passages. However, by mid-passage, BDE1.1 cells had acquired the ability to grow on plastic without undergoing squamous degeneration. In contrast, BDE4 cells retained an epithelial morphology and could be maintained indefinitely on plastic even at low passage, a growth characteristic shared with mid and high passage BDE1.1 cells. Since collagen gels created difficulties with many of the assays of interest, the decision was made to use BDE4 cells as well as BDE1.1 for the analysis of low passage cultures, a substitution that allowed us to compare low, mid and high passage cultures and selected sub-lines attached to the same substratum. Where possible low pass BE1.1 and BDE4 cells were both analyzed to verify the equality of results with the two cell lines at low passage. In most cases low pass BDE1.1 were also analyzed Henceforth, low passage BDE4 and low, mid and high passage BDE1.1 cultures will be referred to collectively as bile duct epithelial cells (BDEC).

BDEC undergo changes in morphology with increasing passage

With continuous passage under our standard culture conditions, BDEC cells underwent changes in morphology often associated with progression towards a malignant phenotype (Kang and Krauss, 1996; Ruddon, 1995). Comparison of phase contrast micrographs of low (p21), mid (p66), and high (p135) passage BDEC revealed several morphological differences. With increasing passage, BDEC colonies became more uniform in shape and the cells were more refractive after attachment and spreading (Figs. 1A-C). High passage cells also failed to undergo morphogenic changes evidenced in low passage cultures by the formation of duct-like structures at the edges of colonies (Yang et al., 1993b). Low passage cultures formed tightly compacted colonies composed of polygonal cells that became more spindle shaped, less tightly packed and more randomly oriented with continued passage (Figs. 1A-C). At higher passage, cells began to display differences in the quantity and arrangement of cytoplasmic vesicles (Figs. 1E-F). In low passage cultures, these vesicles localized proximal to the surface of the plasma membrane in a well-ordered array (Fig. 1E). In contrast, vesicles in mid and high passage cultures were significantly decreased in quantity and were randomly distributed throughout the cytoplasm (Fig. 1F). The identity of these structures is not known at this time.

High passage BDEC proliferate more rapidly than low passage cells

When low passage cultures were maintained for 48 hrs in medium without growth factors, combined results from MTS Cell Proliferation Assays and FACS analysis (data not shown) showed that cultures rapidly underwent cell cycle arrest in G0/G1 (Fig. 1G). In contrast, high passage cells continued to proliferate in growth factor deficient medium and showed a more rapid increase in growth rate when returned to complete medium, indicating that high passage cells had become growth factor independent, a recognized hallmark of neoplastic cells (Ruddon, 1995). In addition, high passage cells exhibited a 2.5-fold higher growth rate

than low passage cells. This was especially apparent at the initiation of serum starvation when high passage cells exhibited an MTS value almost 3-fold greater than their low passage counterparts, despite seeding both low and high passage cells at identical concentrations 96 hours prior to starvation.

High passage BDEC demonstrate a decrease in E-cadherin expression and decreased capacity for aggregation relative to low passage cells

In previous studies, it has been proposed that a loss of E-cadherin triggers signaling pathways that support migration and invasion, hallmarks of malignancy (Cavallaro and Christofori, 2004; Nelson and Nusse, 2004). Analysis of BDEC by Western blot (Fig. 2A) and IIF analysis (Fig. 3) showed that E-cadherin levels decreased with increasing passage, a change consistent with neoplastic progression. This decrease was not due to a global down-regulation of protein expression since levels of the heat shock protein, Hsp70, remained constant (Fig. 2A) (Ohtsuka and Hata, 2000).

Having established that high passage BDEC exhibit a decrease in E-cadherin, it was of interest to determine if this decrease was paralleled by a reduction in intercellular adhesion. To this end, we incubated cell suspensions under conditions that were known to promote aggregation. As shown in Fig. 2B, alterations in intercellular adhesion were first evidenced by differences in resistance to dissociation into single cells. While high passage cultures readily formed single cell (75%) suspensions following trypsinization, low passage cells were more resistant to dissociation and formed suspension composed largely of doublets (13%), triplets (11%), and small aggregates (42%). With increasing time, clusters of low passage cells increased 4-5-fold in size (Fig. 2B), eventually forming aggregates containing more than 250 cells at late timepoints (not shown). In contrast, high passage cells were still present in small aggregates of less than 30 cells at the longest time point examined.

Expression of cholangiocyte markers decreases significantly with continued passage

Indirect immunofluorescent microscopy showed a marked decrease at mid to high passage in the expression of CK19, OV-6, and BD.1 (Fig. 3), three markers characteristic of mature cholangiocytes. This suggested that at high passage, BDEC were losing the well-differentiated phenotype of their low passage counterparts. Advantageously, OC.5 expression remained invariant with passage, thereby providing a marker that could be used to distinguish between mouse host and rat donor derived cells in tumorigenicity assays (described below).

High passage BDEC undergo a change in ploidy

While there is continuing controversy about the relative importance of mutation and aneuploidy as the mediators of the genetic instability that predispose a cell to malignancy, there is no question that karyotypic abnormalities are a consistent feature of neoplastic cells (Dey, 2004; Li et al., 1997; Wasch and Engelbert, 2005). When karyotypes were determined for low, mid, and high passage BDEC, chromosome counts showed a rapid escalation in the degree of aneuploidy with increasing passage (Fig. 4). Low passage BDEC at both p14 and p23 exhibited a near diploid karyotype with 100% of the metaphase spreads analyzed having chromosome counts ranging from 42-44. However, by passage 37, the percentage of near

diploid cells had dropped to 26%, a decrease paralleled by the appearance of two new cell populations with chromosome counts of 64-65 (11%) and 85 (63%). By passage 123, when BDEC acquired anchorage-independence, 100% of the cells analyzed had 65 chromosomes, a triploid karyotype with two extra chromosomes. The absence of the mid passage subpopulations with near diploid or tetraploid karyotypes, suggested that near-triploid cells, a karyotype also observed in human cholangiocarcinomas (Lai and Sirica, 1999), had a significant growth advantage over the other two subpopulations.

High Passage BDEC show an increase in ErbB-2/Neu expression

Because ErbB-2/Neu is considered an early marker of cholangiocyte transformation (Endo et al., 2002; Gores, 2003; Lai et al., 2005), it was of interest to determine if expression changed with continued passage. IIF analysis showed that the levels of this receptor were increased in high passage BDEC (Fig. 5). However expression was very heterogeneous with ErbB-2/Neu positive cells often appearing as islands surrounded by cells without detectable membrane staining.

Enrichment for high passage ErbB-2/Neu-positive cells produces an anchorage-independent population

We have previously reported that infection of high passage BDEC with a retrovirus containing activated ErbB-2/Neu leads to neoplastic transformation (Lai et al., 2005). This led us to question whether the ErbB-2/Neu-positive cells in our high passage cultures represented a subpopulation of transformed cells. Using immunomagnetic microbeads in combination with a monoclonal antibody specific for a cell surface epitope of ErbB-2/c-Neu (Ab-4), we isolated subpopulations of high passage BDEC greatly enriched for either ErbB-2/Neu positive (E+) or ErbB-2/Neu negative (E-) cells which were then tested for anchorage-independent growth and aggregation on top of a soft agar (SA) substratum, abilities previously shown to be highly correlated with soft agar colony formation and tumorigenicity *in vivo* (Kang and Krauss, 1996; Rhim, 1983; Ruddon, 1995). When plated on top of SA, most high passage E+ cells were unable to form stable aggregates and failed to proliferate. However, with continued soft agar selection we identified several large anchorage-independent cell aggregates that when transferred to plastic closely resembled high passage BDEC-E+ cells (data not shown). This was in contrast to low passage, ErbB-2/Neu negative (E-) BDEC that remained in small aggregates or single cells after 24 hrs, many of which were either distended by vacuoles or were shriveled in appearance. By 48 hours, the number of low passage E- cells had decreased significantly and those that remained were mostly present as single cells. When tested for anchorage-independence and aggregation on SA, the growth characteristics of mid passage BDEC were found to be very similar to low passage, E- cells.

High passage BDEC were tumorigenic in immunodeficient mice

The defining characteristic of neoplastic transformation is the ability to produce tumors *in vivo* in an appropriate host animal (Lai et al., 2005; Ruddon, 1995). When high passage anchorage-independent sub-lines (SA3.1, SA10.3 and SA4.1) were injected into beige/nude/xid mice, large blood-filled tumors between 7.5 – 9 mm in size were observed within 1

week with 100% incidence (Fig. 6). In contrast, low (< p30), mid- (p78) and high passage (p88) unselected BDEC cultures were nontumorigenic to weakly tumorigenic, forming small nodules that were less than 1 mm in size by 4 weeks, the longest time point examined (Fig. 6).

Histological characterization of the tumors produced from the high passage BDEC sub-lines showed them to be moderately differentiated cholangiocarcinomas composed of glandular elements and large cysts strongly positive for the rat cholangiocyte specific epitope, OC.5, (Fig. 7A) (Hixson et al., 2000). Double IIF for OC.5 and phospho-histone H3, an established marker for mitotic cells (Cheung et al., 2000; Workman and Kingston, 1998), revealed the presence of cells positive for both markers, indicating that the transplanted cells were proliferating (Fig. 7B). These ductal structures also displayed strong plasma membrane reactivity with antibodies specific for ErbB-2/Neu (C-18) and activated, phosphorylated ErbB-2/Neu (p-Neu Y1248) (Figs. 7C, E). When labeled with a COX-2 specific antibody, BDEC tumors showed strong nuclear reactivity (Fig. 7F), a localization frequently observed for this enzyme (Hashimoto et al., 2004; Parfenova et al., 2001).

Tumorigenic high passage BDEC showed changes in ErbB-2/Neu and COX-2

Quantitative analysis of ErbB-2/Neu and COX-2 expression by low and high passage BDEC was carried out by FACS analysis. Background non-specific fluorescence with anti-His6 antibodies was higher with low versus high passage BDEC, an artifact attributed to the propensity of the former cells to form aggregates (Fig. 8A). FACS analysis showed that only a small percentage of low passage BDEC (p20) expressed detectable levels of ErbB-2/Neu (13%) (Fig. 8A). However, mid-passage BDEC (p66), although not yet tumorigenic, had a three-fold increase in the percentage of ErbB-2/Neu+ cells and a 2.5-fold increase in the level of expression relative to low passage cultures (Fig. 8A). In contrast, between 80-97% of the anchorage-independent, tumorigenic cells expressed ErbB-2/Neu at levels that were 6 to 24-fold greater than their low passage counterparts. This increase in ErbB-2/Neu expression was also evident in immunoblots (Fig. 8C) that showed not only an increase in total expression but also a marked increase in the activated Y1248-phosphorylated form of ErbB-2/Neu. Consistent with FACS analysis, only low levels of total ErbB-2/Neu and essentially no phosphorylated ErbB-2/Neu were detected in immunoblots from low passage BDEC (Fig. 8C). FACS analysis for COX-2 expression (Fig. 8B) was consistent with earlier studies demonstrating a correlation between activated ErbB-2/Neu and COX-2 expression (Endo et al., 2002; Gores, 2003; Lai et al., 2005). All of the tumorigenic sub-lines showed an increase in the percentage of cells expressing COX-2 that ranged from 70-99% for tumorigenic sub-lines compared to 57% for low passage cultures. However, mid-passage, non-tumorigenic BDEC also displayed an increase in the number of cells expressing COX-2 (71%), suggesting there was not a consistent correlation between COX-2 expression and tumorigenicity in BDEC (Fig. 8B).

Expression of activated ErbB-2/Neu does not lead to neoplastic conversion of low or mid passage BDEC

In a previous report, it was shown that over-expression of activated ErbB-2/Neu by retroviral infection was sufficient to transform high passage BDEC (Lai et al., 2005). To

address the question of whether the accumulation of activated ErbB-2/Neu in BDEC was truly sufficient to induce neoplastic transformation, we nucleofected a plasmid carrying both neo and the constitutively activated transmembrane domain mutant of ErbB-2/Neu (ErbB-2/Neu-M) into low and mid passage BDEC and selected for stable transfectants by maintaining cultures in G418. RT-PCR analysis with primers specific for transcripts encoded by the transfected but not the endogenous gene confirmed transcription of ErbB-2/Neu-M in low passage transfectants. When analyzed by RT-PCR and IIF, low and mid pass ErbB-2/Neu-M transfectants showed elevated levels of activated ErbB-2/Neu relative to low pass non-transfected BDEC (Fig. 9). Morphologically, ErbB-2/Neu-M positive sub-lines closely resembled non-transfected controls but differed significantly from their high passage counterparts (compare Figs. 1 A and D). Neither of the low or mid passage BDEC sub-lines stably transfected with ErbB-2/Neu-M showed a notable change in their rate of proliferation, growth under anchorage-independent conditions or tumorigenicity in immunodeficient mice (data not shown), suggesting that expression of activated ErbB-2/Neu was not sufficient to induce transformation of low or mid passage BDEC.

Discussion

In the present report, we have chronicled neoplastic traits that occur *in vitro* during the progression of BDEC cholangiocytes from low to high passage. Our results show that mid-passage BDEC had acquired a number of neoplastic characteristics (Kang and Krauss, 1996; Ruddon, 1995) marked by growth factor independence, accelerated growth and increased numbers of cells with elevated ErbB-2/Neu. At high passage ($p > 85$), BDEC cultures also showed an increase in cells expressing activated, phosphorylated ErbB-2/Neu. When tested for evidence of neoplastic transformation, only a small population of high passage cells displayed anchorage-independent growth and tumorigenicity in immunodeficient mice. High passage anchorage-independent cells also showed elevated levels of both activated (phospho-Y1253) and total ErbB-2/Neu relative to their lower passage counterparts. Based on these findings, we concluded that spontaneous neoplastic transformation is the end result of a constellation of preneoplastic changes that act collectively to increase susceptibility to transformation by endogenous or exogenous ErbB-2/Neu.

In sporadic breast cancer where there is no germ line mutation, checkpoint defects can occur before or after inactivation of BRCA1 (Welsh and King, 2001). These checkpoint defects are analogous to the preneoplastic events occurring in high passage BDEC prior to altered ErbB-2/Neu, events that were not sufficient to induce transformation by themselves but were most likely necessary for increasing susceptibility to ErbB-2/Neu. It also seems likely that there are pathways leading to neoplastic transformation that do not involve ErbB-2/Neu, a possibility suggested by previous reports of ErbB-2/Neu negative human cholangiocarcinomas (Sirica, 2008) and by the insignificant difference reported by Sirica et al. in ErbB-2/Neu levels before and after spontaneous transformation of BDE1 cells (Sirica et al., 2008).

Multiple studies have previously demonstrated altered expression of cell-cell and cell-matrix adhesion molecules during tumor progression (Cavallaro and Christofori, 2004; Nelson and Nusse, 2004), the end result being diminished cell adhesion, changes in cell fate and

increased migration, alterations regarded as hallmarks of neoplastic transformation (Kang and Krauss, 1996; Nelson and Nusse, 2004). It is well documented that E-cadherin-mediated cell-cell adhesion diminishes in tandem with progression towards malignancy (Cavallaro and Christofori, 2004). During cholangiocarcinogenesis, this reduction is associated with reduced levels of E-cadherin protein and advances in tumor histologic grade (Gores, 2003). Consistent with these results, we observed a decrease in E-cadherin expression with increasing passage, a decrease closely paralleled by a reduction in cell-cell adhesion (Fig. 2).

While there is ongoing debate as to whether aneuploidy is a cause or an effect of the transformation process, it is generally accepted that abnormal chromosomal counts are one of the most consistent characteristics of both preneoplastic cells and malignant tumors (Dey, 2004; Li et al., 1997; Wasch and Engelbert, 2005). Current evidence suggests that the regrouping of chromosomes during aneuploidy creates an imbalance that leads to alterations in multiple genetic programs including cell growth, differentiation, morphology, and invasiveness (Li et al., 1997). Immortality of malignant cells has also been marked by an increase in chromosome number with continual passage, suggesting an ongoing selection during the transformation process for cells with an increase in growth-promoting genes (Ruddon, 1995). Based on these considerations, the changes in the ploidy of BDEC with increasing passage were not unexpected. As shown in Fig. 4, BDEC displayed a near diploid chromosome number at low passage, became karyotypically heterogeneous at mid-passage, and acquired a stable near triploid karyotype with a chromosome number of 65 at high passage. These findings are in close agreement with previous studies showing human cholangiocarcinomas to be highly aneuploid with chromosome counts between 60-70, a near triploid karyotype similar to what was observed in high passage BDEC. Thus, there appears to be a significant overlap in the genetic events involved in spontaneous cholangiocarcinogenesis *in vitro* and those directing the progression to cholangiocarcinoma *in vivo* (Lai and Sirica, 1999; Ruddon, 1995).

Multiple studies have shown that over-expression of ErbB-2/Neu in human tumors is associated with poor prognosis and invasiveness (Benlimame et al., 2005; Yu and Hung, 2000). Over-expression of ErbB-2/Neu also enhances metastasis-related aggressiveness, as well as resistance to treatment (Darcy et al., 2000; Yu and Hung, 2000) and is associated with ligand independence (Darcy et al., 2000). Together, these studies point to a prominent role for ErbB-2/Neu during progression towards a more aggressive, invasive and metastatic mode of growth. This is in contrast to the relatively benign changes in growth induced by preneoplastic events essential for the manifestation of a tumorigenic phenotype when there is overexpression of ErbB-2/Neu. In agreement with this viewpoint are previous studies showing that the transforming potential of ErbB-2/Neu is manifested only in the presence of other activated oncogenes. Over-expression of both ErbB-2/Neu or the EGF receptor, for example, was sufficient to induce transformation of fibroblasts but overexpression of either gene alone was ineffective (Ruddon, 1995), suggesting that complete transformation of fibroblasts by ErbB-2/Neu requires the activation or over-expression of another receptor tyrosine kinase (Ruddon, 1995). This need for synergy with other genes also appears to hold for BDEC as was demonstrated by the inability of activated ErbB-2/Neu alone in low/mid passage or the up-regulation of ErbB-2/Neu in mid-passage to transform BDEC. The

previously reported ability of activated ErbB-2/Neu to transform high passage BDEC further implies that additional changes during the transition from low to high passage must be required to render these cells susceptible to the effects of activated ErbB-2/Neu (Lai et al., 2005). Likely candidates include COX-2 which is elevated in high passage BDEC, and activated (phosphorylated) Akt, a kinase with a central role in cell survival signaling pathways that is elevated in ErbB-2/Neu transformed high passage BDEC cells (Lai et al., 2005) and Sirica et al. (Sirica et al., 2008).

In a recent report, Lai et al (Lai et al., 2005) derived a spontaneously transformed, anchorage-independent cell line from BDEC1.1 cells (Yang et al., 1993a) using the selection scheme of Hooth et al (Hooth et al., 1998). Designated BDEsp (Lai et al., 2005), this subline expressed levels of total and phosphorylated ErbB-2/Neu similar to those displayed by the high passage parental BDEC1.1 line from which it was derived, a finding consistent with the increase in ErbB-2/Neu observed at high passage in the present report. However, BDEsp were also reported to form tumors *in vivo*, an ability not displayed in the present study by either high passage BDEC or by an ErbB-2/Neu positive subline selected from high passage cultures. This disparity could reflect the vastly different animal models used to assess tumorigenicity, namely subcutaneous injection into immunodeficient mice and infusion into rats via the extrahepatic duct. Alternatively, the culture scheme used to derive BDEsp may select for cells susceptible to transformation at endogenous levels of ErbB-2/Neu.

Although cholangiocarcinoma is a relatively rare form of cancer incidence is on the rise for reasons that remain unclear. Since the prognosis is very poor by the time of detection, there is a need to define biomarkers that signal early preneoplastic events. We believe that the collection of low, mid and high passage BDEC lines described in this report, together with their spontaneously transformed counterparts, constitutes a valuable *in vitro* model system for identifying the molecular correlates of preneoplastic events occurring during continued passage *in vitro*. The close concordance between the phenotypic, morphological, and molecular alterations that characterize primary cholangiocarcinomas and those that accumulate during spontaneous neoplastic transformation *in vitro* highlight the potential of this model for delineating molecular events required for malignant transformation of BDEC.

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Fig. 1.

BDEC undergo changes in morphology and growth with increasing passage. (A-C) Phase contrast micrographs show morphological changes in BDE cultures at low (A; p21), mid (B; p66) and high (C; p135) passage. Images were taken when cultures had reached 50-60% confluence. Low passage cultures formed tightly compacted colonies containing polygonal shaped cells (A). In contrast, high passage cells became more spindle shaped and less tightly packed with increased passage (C). (E,F) The number and distribution of cytoplasmic vesicles decreased with increasing passage number. In low passage BDEC (p21) cytoplasmic vesicles were arranged in a well-ordered array proximal to the cell surface membrane (E). In contrast, cytoplasmic vesicles in mid and high passage cells were decreased in number and less organized as compared to low passage cells (F). (D) Phase contrast shows there are no significant morphological differences between low passage BDEC (A) and low passage BDEC nucleofected with the constitutively activated transmembrane domain mutant of ErbB-2/Neu (ErbB-2/Neu-M). (G) Growth curves show that high (p99) passage BDEC display a 2.5-fold increase in growth rate relative to low (p29) passage cultures. High passage cells also showed resistance to the effects of serum starvation. Low passage cells underwent growth arrest under similar conditions. Scale bar represents 50 μ m.

Fig. 2.

Decreased E-cadherin expression in high passage cells correlates with decreased intercellular adhesion. (A) Immunoblot analysis of cell lysates from low (p29) and high (p99) passage BDEC showed reduced expression of E-cadherin in high passage cells. The anti-Hsp70 antibody was used to demonstrate equal loading of cell lysates. (B) Aggregation of single cell suspensions of low (p26) and high (p99) passage BDEC was determined at time points ranging from 0 to 90 minutes. Reduced expression of E-cadherin by high passage cells (A) closely correlated with decreased intercellular adhesion as evidenced by the formation of large cell aggregates in low but not high passage cultures.

Fig. 3.

Indirect immunofluorescence shows loss of ductal markers with increasing passage. Comparison of low (p18) and high (p88) passage BDEC showed decreased expression of ductal markers OV-6, CK19 and BD.1. E-cadherin expression was also reduced in high passage cells as shown also in Fig. 2 by immunoblot analysis. Expression of the ductal marker OC.5 remained constant with increasing passage. Scale bar represents 50 μ m.

Fig. 4.

Karyotypic analysis of BDEC showed rapid escalation in the degree of aneuploidy with increasing passage. Low passage BDEC (BDE4 (p14) and BDE1.1 (p23)) showed a near normal diploid karyotype with chromosome counts ranging from 42-44.

Chromosomal instability was evident by mid passage (p37) as 26% of the spreads were diploid, 63% of spreads had 85 chromosomes and 11% of the spreads had 64-65 chromosomes. In contrast, high passage anchorage-independent clones had acquired a triploid karyotype with 100% of metaphase spreads showing 64-65 chromosomes. A total of 20 metaphase spreads were prepared for each passage.

Fig. 5.

ErbB-2/Neu expression is increased in high passage BDEC. (A) Indirect immunofluorescence demonstrated that ErbB-2/Neu expression in high passage BDEC (p99) was upregulated relative to low passage (p19) cells. (B) Quantitative analysis of ErbB-2/Neu (Ab-4) membrane staining using the two-tailed Student's T test with 99% confidence intervals demonstrated that the mean fluorescence of low and high passage cells was significantly different ($p = 0.0001$). The mean values were: BDEC p99, 29.85 ± 0.926 , BDEC p19, 20.03 ± 0.208 . Scale bar represents 25 μm .

Fig. 6.

High passage anchorage-independent BDEC were tumorigenic when injected into immunodeficient beige/nude/xid mice. (A, B) Tumors produced in beige/nude/xid mice from two soft agar (SA) anchorage-independent sublines, SA 3.1 (p130) and SA 10.3 (p123), are shown as representative examples of tumorigenicity observed at one week following injection. (C) When mid-passage BDEC (p78) were injected into immunodeficient mice, only small nodules were detected after four weeks.

Fig. 7.

Cloned high passage anchorage-independent BDEC sub-lines form desmoplastic ductal cholangiocarcinomas when injected into immunodeficient mice.

(A) Histological characterization by hematoxylin and eosin staining demonstrates clearly defined ducts throughout the tissue, marked by arrows. (B-F) Phenotypic characterization of tumors by indirect immunofluorescence. (B) Double-labeling for the ductal marker OC.5 (green) and phosphorylated histone 3 (red) demonstrated actively proliferating ducts containing BDEC. (C-F) Ductal cells in tumors were strongly positive for plasma membrane ErbB-2, (C; Neu C-18), phosphorylated ErbB-2 (E; p-neu Y1248) and nuclear COX-2 (F). The inset in panel F shows a magnification of the region outlined in the rectangle. (D) The DAPI image that corresponds to Panel C defined a distinct morphology of ductal cells relative to surrounding epithelial tissue. Scale bars represent 50 μ m.

Fig. 8.

High passage anchorage-independent BDEC express activated ErbB-2/Neu and COX-2 proteins. (A) FACS analysis for ErbB-2 (Neu F-11) expression in low (p20), mid (p66), and anchorage-independent BDEC sub-lines. 13% of low passage BDEC (p20) expressed detectable levels of ErbB-2/Neu protein. In contrast, 80-97% of high passage, tumorigenic sub-lines expressed ErbB-2/Neu showing that these tumorigenic sub-lines expressed a 6-24-fold increase in ErbB-2/Neu expression relative to low passage cultures. Mid passage cells (p66) had a 3-fold increase in both the percentage of cells expressing ErbB-2 and fluorescence intensity as compared to low passage BDEC. (B) FACS analysis for COX-2 expression in low, mid, and high passage BDEC. While 57% of low passage BDEC (p20) expressed COX-2, the percentage of cell expressing COX-2 in tumorigenic sub-lines was between 70-99%, although this increase in positive cells did not always correlate with an increase in expression levels. Mid-passage BDEC also displayed an increase in the number of cells positive for COX-2. (C) Cell lysates from low passage BDEC (p35) and anchorage-independent sub-lines were analyzed by immunoblot for ErbB-2/Neu (C-18) and phosphorylated ErbB-2/Neu (p-Neu Tyr 1248). Activated, phosphorylated ErbB-2/Neu expression was elevated in tumorigenic sublines (lanes 2-6) as compared to low passage BDEC (lane 1) and unselected, nontumorigenic high passage anchorage-independent BDEC (SA.NT; lane 7). Hsp70 expression demonstrates equal loading between lanes.

Fig. 9.

Expression of activated ErbB-2 is not sufficient to induce transformation of low or mid pass BDEC. (A) RT-PCR shows that transfected low passage BDEC (p22) express activated ErbB-2/Neu transcript (lane 4). ErbB-2/Neu expression was not detected in non-nucleofected low pass BDEC (lane 1), high pass BDEC (lane 2) or cultured hepatocytes (lane 3). Indirect immunofluorescence confirmed protein expression of activated ErbB-2/Neu (Neu C-18-G) in nucleofected low pass BDEC (C). ErbB-2/Neu expression was not detected in control non-nucleofected low passage BDEC (B). OC.5 expression, shown as a control, was not affected by nucleofection (D, E). Scale bar represents 50 μ m.