Essential role of biliary glycoprotein (CD66a) in morphogenesis of the human mammary epithelial cell line MCF10F

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SUMMARY

Normal mammary epithelial cells express the cell surface protein biliary glycoprotein (BGP or CD66a) in a polarized manner, suggesting that this protein may play a role in the formation of mammary acini. In order to test this hypothesis, we interrupted the expression of BGP in the mammary epithelial line MCF10F when cultured in or on Matrigel, a source of extracellular matrix (ECM). When analyzed by immunofluorescence confocal microscopy, the BGP staining is confined to the luminal surface and colocalizes with actin.

Sequential scanning electron microscopy demonstrates that the MCF10F cells migrate to form clusters, followed by apoptotic cell death within the center, resulting in lumen formation. Transmission electron micrographs reveal the presence of tight junctions and desmosomes between the cells, microvilli along the luminal surface, and typical apoptotic bodies within the lumen. When the MCF10F cells are transfected with the BGP antisense gene and grown in Matrigel, they exhibit reduced acini formation (12% and 20%) compared to untransfected cells (52%) or to cells transfected with vector only (62%). Acini formation is also significantly reduced when MCF10F cells grown in Matrigel are treated with anti-BGP antibody (18% at 100 µg/ml), or recombinant soluble BGP (18% at 0.4 µM). In contrast, the BGP-negative MCF7 breast tumor cell line, which does not form acini when grown in matrigel, exhibits >60% cell death with the occasional formation of acini, when transfected with the BGP sense gene and grown in Matrigel. These results support the hypothesis that BGP plays a role in the normal differentiation program of mammary epithelial cells, indicating that its expression is essential to the formation of the lumen. Furthermore, and as shown by others, the differentiation program depends on the presence of ECM. The lack of expression of BGP in the MCF7 breast cancer cell line suggests that the downregulation of BGP expression confers a growth advantage to these cells in ECM. In addition, we found that the MCF10F cells could be separated into a BGP-positive epithelial fraction (MCF10F-e), and a BGP-negative myoepithelial fraction (MCF10F-m). When the myoepithelial cell-enriched fraction is grown on Matrigel, web-like structures are formed. These cells have a typical spindle shape cell morphology and express keratin, α-smooth muscle actin and vimentin, markers of the myoepithelial cell phenotype. When MCF10F-m cells are treated with IFNγ, they express CEA (carcinoembryonic antigen) but not BGP. Since breast carcinomas, especially in situ carcinomas, express CEA, this finding may suggest a heretofore unappreciated relationship between myoepithelial cells and breast cancer.

Key words: Biliary glycoprotein, CD66a, Mammary epithelial cell, Myoepithelial cell, Matrigel, Extracellular matrix, Morphogenesis

INTRODUCTION

Biliary glycoprotein (BGP), also known as CD66a due to its expression in granulocytes, is a cell surface glycoprotein mainly expressed on the luminal surface of epithelial cells. BGP is a member of the CEA (carcinoembryonic antigen) gene family, which in turn is a member of the immunoglobulin superfamily (Williams and Barclay, 1988). As part of our ongoing studies on the biology of BGP, we have investigated its expression in the normal and malignant breast (Huang et al., 1998). These studies were prompted by the observation that BGP is expressed in a polarized, luminal orientation in the liver (Wagener et al., 1983b), colon (Frangsmyr et al., 1995) and breast (Riethdorf et al., 1997), and is downregulated in premalignant adenomas (Nollau et al., 1997) and colon tumors (Neumaier et al., 1993). Although we found that BGP is downregulated in only 30% of breast cancers (Huang et al., 1998) compared to >90% in colon cancers (Neumaier et al., 1993), it is likely that BGP delivers a strong growth inhibitory signal upon its expression in fully differentiated epithelial cells,
and that many cancers of epithelial origin overcome this growth inhibition by downregulation of the BGP gene. This property of BGP is further indicated by its tumor growth inhibitory activity when the BGP gene is transfected and expressed in prostate (Kleinerman et al., 1995), bladder (Kleinerman et al., 1996) or breast cancer cells (Luo et al., 1997). The fact that breast tumors show a lower incidence of downregulation of the BGP gene may relate to the changing role of BGP during morphogenesis. In this respect, we hypothesize that BGP may play a role in the formation of the lumen. In order to test this hypothesis, we investigated BGP expression in a mammary epithelial cell model of morphogenesis.

Normal mammary glands have a polarized orientation of epithelial cells, with their secretory surfaces facing a central lumen and the basal surfaces surrounded by a layer of myoepithelial cells. When these cells are separately isolated and cultured together in Matrigel, a source of extracellular matrix (ECM), they form a reconstituted alveolar structure (Gomm et al., 1997). When the epithelial cells are cultured separately in Matrigel or collagen gel, respectively, they may form either spheroids (Gomm et al., 1997) or branching structures (Foster et al., 1983).

In vitro models have shown that mammary epithelial cells undergo differentiation to acini and tubule structures when grown on Matrigel (Barcellos-Hoff et al., 1989; Bergstraesser and Weitzman, 1993; Gomm et al., 1997; Petersen et al., 1992). The spontaneously immortalized human breast cell line MCF10 (Soule et al., 1990) mimics the behaviour of primary mammary epithelial cells when grown on Matrigel and has been used as a model for the study of alveolar (Howlett et al., 1995; Petersen et al., 1992) and branching morphogenesis (Stahl et al., 1997). This cell line has been further divided into two lines designated MCF10A for attached and MCF10F for floating (Soule et al., 1990). In the original report (Soule et al., 1990) both cell lines exhibited the polarized phenotype of epithelial cells while neither cell line was observed to contain myoepithelial cells. In subsequent studies, MCF10A cells were shown to favor acini structures when mixed with liquid Matrigel and grown in thick Matrigel culture (Howlett et al., 1995), but predominantly formed tubules when seeded on the top of thin Matrigel cultures at a concentration of 2.5×10⁴ cells/cm² (Stahl et al., 1997). Tubule formation was found to depend on the seeding density, with the cells forming acini at 1.25×10⁴ cells/cm² or below. Tubule formation (branching morphogenesis) was found to depend on the expression of α6β4 integrin on these cells and the presence of laminin-5 in Matrigel (Stahl et al., 1997). Treatment of these cells with antibodies to laminin or α6β4 integrin blocked the formation of tubules on thin Matrigel. Similarly, treatment of the HMT-3522 breast epithelial cell line with blocking antibodies to α6β4 integrin prevented acini formation in thick Matrigel (Weaver et al., 1997).

In other studies, a variety of growth factors, including hepatocyte growth factor (Brinkmann et al., 1995; Soriano et al., 1995), epidermal growth factor (Gomm et al., 1997), neuregulin/hereregulin (Yang et al., 1995), keratinocyte growth factor (Hirai et al., 1998) and fibroblast growth factor (Li and Shipley, 1991) were found to promote branching morphogenesis. In addition, cell surface glycoproteins on stromal cells or the epithelial cells such as epimorphin (Hirai et al., 1998) play a role in morphogenesis of the mammary gland.

In this study, we have chosen the MCF10F cell line rather than MCF10A because MCF10F cells produce a higher level of acini when grown in Matrigel (approx. 50%) compared to MCF10A cells (<20%). We show here that MCF10F cells can be divided into epithelial and myoepithelial sublines, and that when the epithelial cells are cultured on Matrigel they form acinar structures. Furthermore, interruption of BGP expression with anti-BGP antibody or a BGP antisense gene or treatment with recombinant soluble BGP significantly reduces the formation of acini.

**MATERIALS AND METHODS**

**Cell culture and treatments**

MCF10F, MCF10A and MCF7 cell lines were obtained from ATCC. MCF10F and MCF10A cells were grown in mammary epithelial cell growth medium (MEGM) (Clonetics) with 10 ng/ml hEGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B and 52 μg/ml bovine pituitary extract. MCF10F cells were separated into two sublines, MCF10F-e (strongly adherent) and MCF10F-m (weakly adherent). MCF10F-e cells were grown in mammary epithelial cell growth medium, and MCF10F-m cells were grown in DMEM-F12 (Gibco-BRL) with 5 μg/ml insulin, 5 μg/ml transferrin, 20 ng/ml epidermal growth factor, 5×10⁻⁸ M dexamethasone and 5% FBS. MCF7 cells were maintained in MEM with Earle’s salts and 10% FBS (Irvine Scientific). Matrigel culture: 2×10⁶ cells were plated either on a thin layer (0.5 mm) or in a thick layer (1 mm) of Matrigel (Collaborative Biomedical Products) in 2-well chamber slides. To harvest cells, the gels were incubated with dispase (0.2 ml of 50 U/ml per cm²; Collaborative Biomedical Products) at 37°C for 2 hours. Dispersed were inactivated by dilution with medium. Cytokine treatment: cells cultured as above were treated with either 500 U/ml of IFNγ or 50 ng/ml of TNF-α for the times indicated.

MCF10F-e cells (2.5×10⁵) were treated with anti-BGP mAb T84.1 or control mAb T84.66 (anti-CEA) at concentrations of 10, 50 and 100 μg/ml at room temperature for 15 minutes prior to plating in Matrigel. The same concentrations of antibodies were maintained in the culture medium over the length of the experiment. A synthetic peptide (BGP-N1B) containing residues 5-18 of the N-domain of BGP (Barnett et al., 1989) was mixed with MCF10F-e cells (2.5×10⁵) at 1 and 10 μM and cultured as above. A cDNA fragment containing the extracellular domains of BGP (N, A1, B1 and A2 domains) was cloned into a glutamine synthetase expression vector (details to be published elsewhere) and the construct was expressed in NS0 cells. Recombinant soluble BGP was purified from culture medium supernatant using T84.1 mAb affinity chromatography and used at 0.4 μM to treat MCF10F cells (2.5×10⁵) as described above.

**Cell transfections**

A 1.6 kb full-length cDNA of BGP (BGPa) was cloned into the SalI and HindIII restriction sites of an expression vector pHβ-actin (Gunning et al., 1987) in either the sense or antisense orientation, designated as BGPa/pHβ and BGPAS/pHβ, respectively. The pHβ vector contains a neomycin-resistant gene and a β-actin promoter.

**MCF10F cells**

8×10⁵ cells were plated in each well of 6-well plates and transfected with 1.5 μg of plasmid DNA constructs BGPa/pHβ, BGPAS/pHβ or pHβ-actin vector using Lipofectin (Life Technologies, Ltd), according to the manufacturer’s instructions. Cells were grown in DMEM-F12 (Gibco-BRL) with 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 20 ng/ml epidermal growth factor (Sigma), 5×10⁻⁸ M...
dexamethasone (Sigma) and selected with 0.4-0.5 mg/ml of genetin (Life Technologies) for 4 weeks. The G418-resistant colonies were expanded and analyzed by FACS. Two of the stably transfected lines (AS6 and AS9) were grown in Matrigel and analyzed by immunohistochemistry. Colonies were scored for the presence (acinus formation) or absence of lumen and compared by the chi-squared test.

**MCF7 cells**

The cells were grown in 100 mm dishes until they reached 80% confluence, then the cells were transfected with 4 μg of plasmid DNA construct BGPα/pHβ or pHβ-actin vector, respectively, using the lipofectin method. G418-resistant cells were grown in Matrigel and scored for acinus formation as described above.

**Immunohistochemistry**

In monolayer culture, cells were fixed with 10% formalin for 10 minutes, followed by cold methanol for 5 minutes, and chilled acetone at −20°C for 1 minute. In Matrigel culture, cells were first fixed with 10% formalin for 10 minutes, embedded in 3% agarose, then treated with 10% formalin overnight, followed by 70% ethanol. Paraffin-embedded sections were prepared and used for Hematoxylin and Eosin staining and immunohistochemistry. Monoclonal antibodies against α-smooth muscle actin (1:10,000; Sigma), vimentin (1:200; Dako), pankeratin (1:100; Ventana) and cytokeratin-14 (1:20; Neomark) were used. Monoclonal antibody 4DIC (1 μg/ml) was used to detect BGP (Drzeneik et al., 1991). Immunohistochemical staining was performed using the standard avidin-biotin-peroxidase technique. In brief, sections were blocked in 5% normal horse serum for 20 minutes, incubated with primary antibodies at room temperature for overnight, and further incubated with biotinylated horse anti-mouse secondary antibody (1:400; Vector) for 1 hour. After washing with PBS, the sections were incubated with avidin-biotin-peroxidase complex (1:200; Vector) for 1 hour and with 0.05% 3,3′-diaminobenzidine/0.03% H₂O₂ for 7 minutes, washed and counterstained with Hematoxylin.

**Electron microscopy**

For scanning electron microscopy, the cells were grown on thin Matrigel on coverslips for 2-4 days, fixed for 3 hours with 10% formalin, washed with 70% ethanol. Paraffin-embedded sections were prepared and used for Hematoxylin and Eosin staining and immunohistochemistry. Monoclonal antibodies against α-smooth muscle actin (1:10,000; Sigma), vimentin (1:200; Dako), pankeratin (1:100; Ventana) and cytokeratin-14 (1:20; Neomark) were used. Monoclonal antibody 4D1C2 (1 μg/ml) was used to detect BGP (Drzeneik et al., 1991). Immunohistochemical staining was performed using the standard avidin-biotin-peroxidase technique. In brief, sections were blocked in 5% normal horse serum for 20 minutes, incubated with primary antibodies at room temperature for overnight, and further incubated with biotinylated horse anti-mouse secondary antibody (1:400; Vector) for 1 hour. After washing with PBS, the sections were incubated with avidin-biotin-peroxidase complex (1:200; Vector) for 1 hour and with 0.05% 3,3′-diaminobenzidine/0.03% H₂O₂ for 7 minutes, washed and counterstained with Hematoxylin.

**RT-PCR**

Total RNA was extracted from tissue culture cells using ToTALLY RNA Kit (Ambion). 1 μg of total RNA was used for synthesis of first-strand cDNA. Reverse transcription reaction was carried out at 42°C for 30 minutes as described previously (Huang et al., 1998), using 1 μM BGP antisense primer (5′-GGTGCAACTCCAGCATTAACTTGA-3′; antisense primer: 5′-GGTGCAACTCCAGCATTAACTTGA-3′) was used. PCR was carried out with denaturation at 94°C for 2 minutes followed by 72 cycles at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes. RNA from HT-29 cells was used as a positive control (Huang et al., 1998). PCR products were separated by electrophoresis on a 2% agarose gel and transferred to a nylon membrane (Qiagen). Following prehybridization with 100 μg/ml salmon sperm DNA in 5X SSC, 20 mM sodium phosphate, pH 7.0, 7% SDS, 10 μg/ml Denhardt’s solution, 10% dextran sulfate for 5 hours at 50°C, the blot was hybridized with [α-32P]UTP labeled BGP probe (5′-GAAATGGCCCTCTACCTGGA-3′) at 50°C, overnight. Autoradiography was performed using BioMax MR films.

**Ribonuclease protection assay**

A 190-bp fragment of the BGP cDNA (1435-1625) was subcloned into Bluescript at the HindIII/BamHI sites. BGP antisense riboprobe was synthesized and labeled with 50 μCi of [α-32P]UTP using MAXIscript T7/T3 Kit (Ambion). Samples (10 μg) of total RNA were coprecipitated with the BGP probe and a 316-bp [α-35S]UTP-labeled GAPDH probe (as internal control), and hybridized in hybridization solution containing 80% formamide at 42°C, overnight. After digestion with RNases A and T1, the protected RNA hybrids were precipitated, dissolved in denaturing loading buffer, and separated by electrophoresis on 7 M urea, 5% polyacrylamide gels. Autoradiography was performed at −70°C using BioMax MS films with intensifying screen. The intensities of bands were quantitated using a Bio-Rad scanning densitometer.

**FACS analysis**

For the detection of cell surface expression of BGP and cell sorting, MCF10F cells were first incubated with mAb T84.1 (1 μg/ml) at 4°C for 1 hour, washed with PBS, and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-mouse IgG, 1:250; Boehringer Mannheim) at 4°C for 1 hour. Cells were sorted on the MoFlo Optical Bench (Cytomation). After sorting, T84.1 positive cells and T84.1 negative cells were cultured separately, both on plastic and in Matrigel, as described above.

**Confocal microscopy**

The cells were grown on Matrigel, fixed and permeabilized with 10% formaldehyde/0.1% Triton X-100 at room temperature for 10 minutes and stained with both FITC-conjugated phallolidin (200 ng/ml; Sigma) and mAb T84.1 (1 μg/ml) for 1 hour followed by Texas Red-conjugated goat anti-mouse IgG (1:100; Molecular Probes) for 1 hour. A Zeiss Model 310 confocal microscope was used to acquire data.

**RESULTS**

**Characterization of MCF10F cell phenotypes**

In order to study BGP expression in an in vitro model system, we chose MCF10F, a mammary epithelial cell line, which in preliminary experiments produced high yields of acini when cultured in Matrigel. However, we also noted that this cell line contained at least two phenotypes, prompting the following phenotypic characterization. When MCF10F cells were grown in serum-free medium on plastic, the majority of cells exhibited...
a cobblestone morphology with occasional spindle-shaped cells. When the loosely attached cells were repetitively passaged on plastic with removal of tightly adhering cells, cultures containing >95% spindle-shaped cells were obtained. In contrast, repetitive passaging of the tightly attached cells on plastic and removal of the loosely attached cells led to >95% epithelial cells with a typical cobblestone morphology. Immunostaining of the spindle-shaped cells (MCF10F-m) was positive for keratin, α-smooth muscle actin and vimentin (Fig. 1A–C), confirming the myoepithelial phenotype of these cells. Immunostaining of the cobblestone-like cells (MCF10F-e) was positive for keratin only (Fig. 1D–F), confirming their epithelial phenotype. When stained for BGP using a BGP-specific antibody, the MCF10F-e cells were weakly positive and the myoepithelial cells were negative (data not shown). When the MCF10F-e cells were grown in thick Matrigel they formed acini (approx. 50% of the colonies formed acini after 14 days) but no tubule structures, staining positively for BGP on the luminal side (Fig. 2A,B). When grown on thin Matrigel, the MCF10F-m cells formed thin web-like structures (Fig. 2C). In contrast, when grown on thin Matrigel the MCF10F-e cells formed donut-shaped acini structures. When the acini structures were examined by confocal microscopy and stained for BGP and actin, they showed colocalization of BGP and actin on the luminal surface (Fig. 2D).

**Electron microscopy**

The formation of acini by MCF10F-e cells grown on thin Matrigel was further studied by electron microscopy. This approach permitted the direct examination of morphological changes that the cells undergo while developing acini. Scanning electron micrographs of the MCF10F-e cells grown on thin Matrigel revealed small colonies (<8 cells per colony) with few isolated cells after 1-2 days of culture (Fig. 3A). After 2-4 days of culture, the colonies increased in size (>12 cells) and began to form central lumens (Fig. 3B,C). Close inspection of the developing lumens revealed bright, condensed bodies with numerous blebs. The consistent finding of bright, condensed bodies on the tops of the colonies and within the developing lumen suggested that the lumen was formed by cell death within the central region of the colony. In addition, the mature acini exhibited cell flattening with a polarized orientation around the lumen. Cells at the base of the colonies were strongly attached to the ECM and showed either evidence of pulling matrix fibrils up along the sides of the colonies (Fig. 3B,C) or the extension of cellular processes to the ECM. Based on a close examination of the fibrils (under TEM), we conclude that the fibrils originate from the ECM and not from the cell. A similar conclusion was derived by Barcellos-Hoff et al. (1989), who performed EM studies on acini formation by freshly isolated murine mammary epithelial cells grown on Matrigel.

To further confirm the cellular morphology, representative colonies were embedded, sectioned and analyzed by transmission electron microscopy (Fig. 4). The cell surfaces facing the lumen contained microvilli, while the intercellular boundaries exhibited tight junctions and desmosomes, a characteristic finding for mammary epithelial cells grown on Matrigel (Barcellos-Hoff et al., 1989). The lumen contained numerous small vesicles (20-50 nm), which were not present elsewhere and probably represent vesicle formation by the differentiated cells. Close examination of the lumen revealed cellular bodies with well defined membranes, compacted chromatin, and condensed cytoplasm with well preserved organelles (vacuoles, Golgi and mitochondria). In particular, a section through the nucleus of one of the bodies (Fig. 4B) reveals several areas of condensed chromatin and an irregular nuclear boundary. These cellular bodies correspond to apoptotic bodies described in detail by other investigators using

**Fig. 1.** Characterization of MCF10F cell phenotypes. MCF10F-m cells (A–C) or MCF10F-e cells (D–F) were grown on plastic and immunostained with anti-pan-keratin (A,D), anti-α-smooth muscle actin (B,E) or anti-vimentin (C,F) antibodies. The spindle-shaped morphology of the MCF10F-m cells and their strong staining with all three antibodies confirms their myoepithelial cell phenotype, while the MCF10F-e cells have an epithelial cell phenotype.
Upregulation of BGP mRNA and protein expression

Once stable phenotypes were established for MCF10F-m and MCF10F-e cells, it was necessary to determine the effect of Matrigel on BGP expression at the mRNA and protein levels. When mRNA levels were analyzed by RNase protection assays, BGP mRNA increased by over five- to tenfold when cultured for 6 days on Matrigel (Fig. 5A). However, when the protein levels of BGP were determined by western blot analysis (Fig. 6A), only a modest increase (20-50%) in the level was observed before and after culture of MCF10F cells on Matrigel. While the mechanism of BGP mRNA induction by Matrigel is unknown, previous studies on colonic epithelial cells have shown that BGP mRNA is strongly induced by TNFα and IFNγ (Chen et al., 1996; Takahashi et al., 1993). Therefore, we tested the effect of these cytokines on MCF10F cells. When the cells grown on plastic were treated with 50 ng/ml of TNFα or 500 U/ml of IFNγ, BGP expression increased over twofold when analyzed by RNase protection (Fig. 5B) or western blots (Fig. 6B). When the cells were grown in Matrigel with the addition of 500 U/ml of IFNγ or 50 ng/ml of TNFα, no change in rate of growth or morphology was detected (data not shown), and a less dramatic increase in BGP protein expression was observed (Fig. 6A). For purposes of comparison with the non-tumorigenic MCF10F cell line, the breast carcinoma cell line MCF7 was also analyzed for BGP expression under the same conditions. MCF7 cells were found not to express BGP at either the mRNA (Fig. 5A) or the protein level (Fig. 6D). However, these cells do express CEA, which was detected as a 180 kDa band on western blots with the T84.1 antibody (Fig. 6D), and confirmed by reaction with CEA specific antibody T84.66 (data not shown). Similarly, MCF10F-m cells did not produce detectable amounts of BGP, but after treatment with IFNγ, CEA expression was induced (Fig. 6C). The CEA production of cytokine-treated MCF10F-m cells was confirmed by RT-PCR (data not shown).
Since BGP mRNA can exist in at least four isoforms, it was also necessary to perform an isoform-specific RT-PCR analysis (Takahashi et al., 1993) on mRNA from MCF10F-e cells before and after growth in Matrigel. The analysis is based on two primers common to the four major isoforms of BGP (Fig. 5C). This analysis (Fig. 5D) revealed that only three of the four BGP isoforms are produced, mimicking the situation in normal breast glands (Huang et al., 1998). No change in isoform expression was found upon growth in Matrigel.

Morphology of BGP sorted cells

Since it was possible that the expression levels of BGP played a role in lumen formation, we decided to sort MCF10F cells into BGP high and low populations in the expectation that their initial BGP levels would influence their morphologies when cultured in Matrigel. Cell sorting analysis of MCF10F cells revealed that 60-70% of the cells were BGP positive. When the cells were sorted into BGP high (>90% positive) and BGP low (<10% positive) populations, passaged on plastic for 2 weeks, and reanalyzed, the BGP low population was 30% positive (Fig. 7A) and the BGP high population was 80% positive (Fig. 7D). When grown on thin Matrigel, the BGP low population gave rise to either acini (Fig. 7B) or tubule structures (Fig. 7C), some with a surrounding layer of myoepithelial cells. In contrast, the BGP high population gave rise to exclusively acini-forming colonies (Fig. 7E). When the BGP high cells were grown in thick Matrigel and stained for BGP, intense staining was observed at the lumenal surfaces of the acini (Fig. 7F). When the BGP low cells were grown on thin Matrigel and stained for BGP, intense staining was observed at the lumenal surfaces of the acinar and tubular structures (data not shown). Thus, we found that BGP low sorted cells still retain the ability to express BGP, and when grown in or on Matrigel, can form glandular structures, often including myoepithelial cells. These results can be explained by assuming that while the BGP high cells contained few or no myoepithelial cells, the BGP low cells contained significant numbers of myoepithelial cells. We also conclude that either the presence of myoepithelial cells in the mixed cell cultures or the lower amounts of BGP induced tubule formation, a result usually seen with MCF10A cells.

**Table 1. Acinus formation for MCF10F cells transfected with the BGP antisense and sense gene**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Acini/colonies</th>
<th>% acini</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected</td>
<td>52/100</td>
<td>52</td>
<td>-</td>
</tr>
<tr>
<td>Vector</td>
<td>62/99</td>
<td>62</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Antisense AS6</td>
<td>31/156</td>
<td>20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antisense AS9</td>
<td>14/117</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sense S3</td>
<td>113/198</td>
<td>57</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

P values were calculated by the \( \chi^2 \) method.

**Fig. 4.** Transmission electron micrographs of MCF10F-e cells grown on thin Matrigel for 4 days. (A) A section of a polarized surface showing lumen (lu), microvilli (mi), vesicles (ve), desmosomes (de) and an apoptotic body (a). (B) Section of an apoptotic body (a) within the lumen exhibiting dark bands of condensed chromatin, convoluted nucleus, and condensed cytoplasm with retention of the cell membrane and organelles.
positive cells (Fig. 8A) compared to 60% for untransfected controls (not shown). The slight increase in BGP-positive cells was not significant. Two lines were established from the BGP antisense transfected cells, AS9 and AS6, showing 8% and 30% positive staining for BGP, respectively (Fig. 8B, and not shown). A line established from the BGP sense transfected cells showed 90% positive staining for BGP (Fig. 8C). When these lines were grown in thick Matrigel and scored for acini formation by immunohistochemical staining, the antisense transfected lines showed a significant reduction in acini formation compared to controls (Fig. 8D).
formation compared to the untransfected, vector transfected, and BGP sense transfected lines (Table 1). In the case of the untransfected control, 52% of the colonies exhibited acini formation compared to 62% for the vector transfected line ($P>0.05$). In the case of the antisense transfected lines AS6 and AS9, 20% and 12% positive acini formation were scored, respectively ($P<0.001$). As a further control, the sense transfected line S3 scored 57% positive for acini ($P>0.05$). Thus, the inhibition of acini formation correlated with the lower expression levels of BGP in the antisense transfected lines.

Immunostaining for BGP showed high levels of BGP expressed in a polarized manner for the cells transfected with vector only (Fig. 9A), while the two lines obtained from transfection with the antisense BGP gene showed reduced staining for BGP and reduced acini formation (Fig. 9B,C). These results further confirm that transfection of MCF10F cells with the BGP antisense gene has lowered the expression levels of BGP in the antisense transfected lines.

Treatment of MCF10F-e cells grown in thick Matrigel with increasing concentrations of either anti-BGP antibody T84.1 or with a single concentration of recombinant soluble BGP also resulted in a significant reduction of acini formation (Table 2). In this series of experiments, each treatment group included a separate untreated control group. In the case of the anti-BGP antibody (T84.1)-treated cells, an antibody control (T84.66) was also included. While the control antibody T84.66 (an anti-CEA antibody) had no effect at three dose levels (10, 50 and 100 μg/ml), the T84.1 anti-BGP antibody was able to reduce

![Fig. 7. FACS analysis and morphology of BGP sorted cells.](image)

![Fig. 8. FACS analysis of MCF10F (A-C) and MCF7 (D-F) cells transfected with BGP genes.](image)
acini formation from 43% (control) to 18% ($P<0.001$) at the highest concentration (100 μg/ml). The percentage reduction was also significant at the two lower dose levels with the lowest dose level bordering on significance ($P<0.05$). Thus, antibody inhibited acini formation in a BGP-specific and dose-dependent manner.

It is important to note here that antibody T84.1 recognizes an epitope on the N-terminal domain of BGP, and that this domain has been previously shown to mediate BGP’s cell-cell adhesion effects (Cheung et al., 1993; Obrink, 1997). With this in mind, we decided to test if either a synthetic peptide or a recombinant soluble form of BGP could inhibit acini formation of MCF10F cells grown in Matrigel. When the synthetic peptide N1B was tested at two levels (1 μM and 10 μM) a slight, but statistically borderline, reduction in acini formation was observed (Table 2). When recombinant soluble BGP was tested at 0.4 μM, a more potent effect was observed (18% positive acini formation versus 43% for the control, $P<0.001$). Although insufficient quantities of recombinant soluble BGP were available for a dose-response study, we can conclude that interruption of BGP interactions at the cell surface by either anti-BGP antibody or a soluble form of BGP resulted in reduced acini formation.

Transfection of MCF7 cells with the BGP gene

Since these studies provided strong evidence that expression of BGP is essential to lumen formation in the BGP-positive MCF10F model system, and breast tumor lines lack BGP expression, we attempted to restore a more normal phenotype to MCF7 cells by transfection with the BGP gene. MCF7 cells express the oncofetal protein CEA, but not BGP (Figs 5, 6) and thus react with mAb T84.1 (70% positive, Fig. 8D). A line established from MCF7 cells transfected with vector only showed reduced positivity for CEA (50% positive, Fig. 8E). While the reduction in CEA positivity borders on being significant, the mechanism of reduction is unknown, and may be due to the G418 selection conditions. A line (S1) established from MCF7 cells transfected with vector only showed reduced positivity for CEA (50% positive, Fig. 8E). While the reduction in CEA positivity borders on being significant, the mechanism of reduction is unknown, and may be due to the G418 selection conditions. A line (S1) established from MCF7 cells transfected with the sense BGP gene showed 95% positivity for T84.1 (Fig. 8F) suggesting that BGP was expressed in addition to CEA. BGP expression was confirmed by western blot (data not shown). When two lines (S1 and S6) were grown in Matrigel and the colonies analyzed by immunohistochemistry using the BGP-specific mAb 4D1C2, they showed significant BGP expression and cell death, and occasional acini formation, compared to the untransfected and vector transfected lines which showed no BGP expression, no cell death and no acini formation (Table 3). The untransfected cells (not shown) or vector transfected controls formed large spherical colonies with no central lumen (Fig. 9D). In contrast, the BGP-transfected line exhibited significant cell death (approx. 60% of the colonies, Fig. 9E) with an occasional surviving colony exhibiting lumen formation (Fig. 9F). The central dead cells showed intense staining for BGP (Fig. 9F) compared to a normal mouse IgG control (Fig. 9G). Cells within the surviving colonies exhibited a polarized expression of BGP around the central lumen with dead cells within the lumen staining intensely for BGP. These results demonstrate that while BGP expression in MCF7/BGP cells grown on plastic is not growth inhibitory, it is growth inhibitory for MCF/BGP cells grown in Matrigel.

Fig. 9. Immunohistochemical analysis of MCF10F (A-C) and MCF7 (D-G) BGP transfected cells grown in thick Matrigel. The colonies were stained with 4D1C2. MCF10F: (A) vector only, (B) antisense line AS6, (C) antisense line AS9. MCF7: (D) vector only, (E) colonies undergoing cell death, (F) example of a surviving colony, (G) example of surviving colony stained with normal mouse serum (control).
Table 2. Acinus formation of MCF10F cells treated with antibodies, peptide or soluble BGP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Acini/colonies</th>
<th>% acini</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-a</td>
<td>–</td>
<td>109/208</td>
<td>52.4</td>
<td>–</td>
</tr>
<tr>
<td>Control-b</td>
<td>–</td>
<td>61/124</td>
<td>49.2</td>
<td>–</td>
</tr>
<tr>
<td>Control-c</td>
<td>–</td>
<td>55/127</td>
<td>43.3</td>
<td>–</td>
</tr>
<tr>
<td>mAb T84.66, a</td>
<td>10 μg/ml</td>
<td>74/136</td>
<td>54.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>mAb T84.66, b</td>
<td>50 μg/ml</td>
<td>102/203</td>
<td>50.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>mAb T84.66, c</td>
<td>100 μg/ml</td>
<td>80/194</td>
<td>41.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>mAb T84.1, a</td>
<td>10 μg/ml</td>
<td>65/209</td>
<td>31.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mAb T84.1, b</td>
<td>50 μg/ml</td>
<td>18/100</td>
<td>18.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mAb T84.1, c</td>
<td>100 μg/ml</td>
<td>88/215</td>
<td>40.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Peptide N1B, a</td>
<td>1 μM</td>
<td>88/204</td>
<td>43.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Peptide N1B, b</td>
<td>10 μM</td>
<td>30/161</td>
<td>18.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sol-BGP, c</td>
<td>0.4 μM</td>
<td>55/127</td>
<td>43.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>61/124</td>
<td>49.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>109/208</td>
<td>52.4</td>
<td></td>
</tr>
</tbody>
</table>

Cells were analyzed as described in Table 1 in the presence of increasing concentrations of antibody (T84.66 or T84.1), peptide N1B, or a single concentration of recombinant soluble BGP. Three sets of experiments were performed, each with its own control group, designated a, b and c, and indicated with the appropriate letter.

DISCUSSION

MCF10F cell phenotypes
In order to test our hypothesis that the cell-cell adhesion molecule BGP played a role in lumen formation, we selected MCF10F cells grown in or on Matrigel as a model system. In contrast to the first report describing the isolation and characterization of MCF10A and MCF10F cell lines (Soule et al., 1990), we found that at least one of these lines (MCF10F), contained myoepithelial cells (Fig. 1). It is not clear from our studies whether these cells were always present as contaminating cells, or whether they arose spontaneously during routine cell culture. Due to their differential attachment to plastic, it was possible to greatly enrich cultures in cells belonging to either the epithelial (MCF10F-e) or myoepithelial (MCF10F-m) phenotype. When cultured on Matrigel, the MCF10F-e cells produced acini structures while the MCF10F-m cells produced thin web-like structures. In order to maintain and expand the MCF10F-m population, it was necessary to grow these cells in serum-containing medium. In spite of their exposure to serum, there was no effect on their morphology when grown on Matrigel. We are not aware of any other publications describing the presence of myoepithelial cells in MCF10 cell lines; however, most groups use the MCF10A line when grown in or on Matrigel as a model system. In contrast to the first report describing the isolation and characterization of MCF10A and MCF10F cell lines (Soule et al., 1990), we found that at least one of these lines (MCF10F), contained myoepithelial cells (Fig. 1). It is not clear from our studies whether these cells were always present as contaminating cells, or whether they arose spontaneously during routine cell culture. Due to their differential attachment to plastic, it was possible to greatly enrich cultures in cells belonging to either the epithelial (MCF10F-e) or myoepithelial (MCF10F-m) phenotype. When cultured on Matrigel, the MCF10F-e cells produced acini structures while the MCF10F-m cells produced thin web-like structures. In order to maintain and expand the MCF10F-m population, it was necessary to grow these cells in serum-containing medium. In spite of their exposure to serum, there was no effect on their morphology when grown on Matrigel. We are not aware of any other publications describing the presence of myoepithelial cells in MCF10 cell lines; however, most groups use the MCF10A line when grown in or on Matrigel, as described later, the cells within the lumen have not been subjected to stress (lack of nutrients or oxygen) and thus appeared to have been programmed to die, rather than undergoing necrosis. Finally, we note that while necrotic cells are easily recognized by TEM, exhibiting dissolved nuclear and plasma membranes, and disintegrating cytoplasmic organelles (Wyllie et al., 1980), none were observed in our electron micrographs. These results support the conclusion that the MCF10F-e cells have a differentiated phenotype typical of polarized mammary epithelial cells and that the central lumen is formed by apoptosis of the cells in the center of the colony. Since BGP expression was limited to the luminal surface in...
the mature acini and the lumen formed by death of the cells within the center of the colony, it was tempting to speculate that BGP was involved in the delivery of the death signal. Since we know that BGP expression by itself is not fatal, its action on adjacent cells in the as-yet unformed lumen must require an additional signal(s). The additional signal may depend on the loss of contact of the central cells to extracellular matrix (ECM), leading to a phenomenon referred to as anoikis (Frisch and Ruoslahti, 1997). Anoikis is a popular theory used to distinguish tumorigenic from non-tumorigenic epithelial cells. It is postulated that normal epithelial cells die when they lose contact with ECM while tumor cells (due to some gene expression change) continue to live. In our model system, MCF10F-e cells require both loss of contact with ECM and expression of BGP to apoptose (form a central lumen), because the cells transfected with the BGP antisense gene form colonies with no central lumen. Thus, the cells in the center of these colonies do not die even though they have lost contact with the ECM. This fact demonstrates that central cells do not die due to lack of nutrients or oxygen, but rather due to an inherent differentiation program. In support of this mechanism, the MCF7/BGP cells exhibit pronounced cell death during colony formation in Matrigel, but grow well on plastic. In this case, the apoptotic signal is stronger for MCF7/BGP cells than for MCF10F-e cells, perhaps due to lower expression of cell surface integrins (which interact with ECM) on MCF7 cells (Noel et al., 1993). Based on our studies, we propose that anoikis may be a more complicated phenomenon than just the loss of cell contact with ECM.

The lack of expression of BGP in all breast tumor cell lines that we have examined suggested that breast tumor cells may escape apoptosis by downregulation of the BGP gene, a situation already found for colon cancer (Neumaier et al., 1993). However, we have found that BGP was only downregulated in 30% of breast cancers (Huang et al., 1998). This finding can be reconciled by postulating that the apoptotic signal transduction mechanism was obviated in the other 70% of breast tumors. Nonetheless, it is intriguing that BGP gene regulation is fundamentally different between colon and breast cancers.

**Role of BGP in cell-cell adhesion**

Members of the CEA gene family, including BGP, have been postulated to mediate homotypic cell adhesion (Benchimol et al., 1989). In particular rat BGP was originally cloned as an ecto-ATPase by Lin and coworkers (Lin and Guidotti, 1989; Lin et al., 1991) and identified as the cell adhesion molecule Cell-Cell Adhesion Molecule-1 (CCAM1 or CCAM105) by Obrink and coworkers (Aurivillius et al., 1990; Edlund et al., 1993; Hansson et al., 1989; Ocklind and Obrink, 1982; Odin et al., 1988). Transfection of the BGP gene into a variety of non-adhesive cell lines results in transient cell-cell adhesion, supporting this view (Oikawa et al., 1992; Rojas et al., 1990; Turbide et al., 1991). However, immunohistochemical staining of BGP on colon (Frangsmyr et al., 1995), prostate (Hsieh and Lin, 1994), breast (Huang et al., 1998; Riethdorf et al., 1997) and bile canalicular ducts (Svenberg et al., 1979) reveals a distinct polarized orientation of BGP, while Hansson et al. (1989) have observed BGP expression at both the luminal and basolateral surface of intestinal epithelial cells. To rationalize the apparent discrepancy between the postulated role of BGP in cell-cell adhesion versus its observed luminal location, we propose here that BGP-BGP intercellular interactions may be a transient phenomenon, not leading to permanent cell-cell contacts, but rather guiding the cells towards formation of acini or tubules with a central lumen. Thus, when cells sense BGP-BGP intercellular contacts, they know that they have not yet achieved the formation of a polarized surface or lumen and continue the polarization program, but once the cells no longer sense BGP-BGP intercellular contacts and BGP has migrated to an exclusively luminal orientation they know that they have achieved their polarized state and cease the polarization program. In this scheme, BGP would be a dynamic cell-cell adhesion molecule, in which its function as a cell adhesion molecule is temporal and changes during the differentiation program.

A mechanism for how BGP may mediate transient cell adhesion has been proposed by Obrink (1997). Since BGP molecules may form inter- or intra-cellular dimers, they may switch between the two states to cause first adhesion (trans dimers) and then disaggregation (cis dimers). If the BGP cytoplasmic domain confers the signal to switch between the two states, then its deletion would not be expected to prevent cell adhesion. However, its deletion may affect the ability of the cell to continue the polarization program. Interactions of the BGP cytoplasmic domain with calmodulin (Edlund et al., 1996; Edlund and Obrink, 1993; Edlund et al., 1998) and the cytoskeleton (see Fig. 2D) could be key determinants of this program. The expression of different isoforms (long and short cytoplasmic forms) of BGP would give the cells the potential to modulate the polarization program, with only the long form capable of delivering a negative growth signal via its ITIM (Beauchemin et al., 1997; Huber et al., 1999). We predict that the ITIM of BGP must be fully phosphorylated (inhibitory) only in the completely polarized cells where further cell division would be unnecessary or even disrupt the acini structure.

**Effect of cytokines on BGP expression**

As we have previously described for colon carcinoma cell lines (Chen et al., 1996; Takahashi et al., 1993), BGP is strongly induced by inflammatory cytokines such as IFN-γ and TNF-α. In this study we found that both cytokines were capable of inducing BGP in MCF10F-e, but not in BGP negative MCF10F-m cells. However, CEA was induced by IFN-γ in the MCF10F-m cells. This intriguing finding may provide a clue to the high incidence of CEA production in breast cancers, especially in situ ductal carcinomas. While very few breast carcinomas are believed to arise from myoepithelial cells, we note here that we found both epithelial and myoepithelial cells in the MCF10F cell line, suggesting that the two types of cells coexist and may under some circumstances interconvert. It is also intriguing that the MCF7 breast carcinoma cell line produces CEA, but not BGP, even when treated with IFN-γ and TNF-α. Thus, the downregulation of BGP in breast cancer epithelial cells is a permanent event and cannot be reversed by cytokines, an otherwise strong inducer of BGP. In this respect the regulatory phenotype of MCF7 cells resembles a myoepithelial rather than an epithelial one. Therefore, further studies aimed at deciphering the regulatory changes in the BGP gene during tumorigenesis are warranted.
Conclusion
We conclude that the MCF10F cell line is a valuable source of both mammary epithelial and myoepithelial cells, and in the case of the MCF10F-e cells, is an excellent model for the study of acini formation when grown in or on Matrigel. In addition, and under appropriate conditions, the two types of cells can collaborate to form gland-like structures resembling myoepithelial-surrounded acini and tubules in the normal mammary gland. BGP is an excellent marker for cell polarization in this system and is specific for epithelial cell expression. BGP appears to follow a programmed reorganization of the cell surface proteins during lumen formation. We hypothesize here that the transient cell-cell adhesion properties of BGP may assist in this process by sensing the position of the cell surface relative to other cells during the reorganization process. Finally, as demonstrated by interruption of BGP expression by a BGP antisense gene, anti-BGP antibody, or recombinant soluble BGP, we showed that BGP plays an essential role in lumen formation, most likely by promoting apoptosis of the cells in the center of the colony that lack contact with ECM.

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