Cell–cell adhesion molecule CEACAM1 is expressed in normal breast and milk and associates with β1 integrin in a 3D model of morphogenesis

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Summary

CEA cell adhesion molecule-1 (CEACAM1) is a cell–cell adhesion molecule that, paradoxically, is expressed in an apical location in normal breast epithelium. Strong luminal membrane staining is observed in 100% of normal glands (11/11), low in atypical hyperplasia (2/6), high in cribiform ductal carcinoma in situ (DCIS) (8/8), but low in other types of DCIS (2/15). Although most invasive ductal carcinomas express CEACAM1 (21/26), the staining pattern tends to be weak and cytoplasmic in tumours with minimal lumena formation (grades 2–3), while there is membrane staining in well-differentiated tumours (grade 1). The ‘normal’ breast epithelial line MCF10F forms acini with lumena in Matrigel with apical membrane expression of CEACAM1. MCF7 cells that do not express CEACAM1 and fail to form lumena in Matrigel, revert to a lumen forming phenotype when transfected with the CEACAM1-4S but not the -4L isoform. CEACAM1 directly associates with and down-regulates the expression of β1-integrin. Immuno-electron microscopy reveals numerous vesicles coated with CEACAM1 within the lumena, and as predicted by this finding, CEACAM1 is found in the lipid fraction of breast milk. Thus, CEACAM1 is a critical molecule in mammary morphogenesis and may play a role in the absorption of the lipid vesicles of milk in the infant intestinal tract.

Introduction

CEA cell adhesion molecule-1 (CEACAM1) is a member of the carcinoembryonic antigen (CEA) gene family that was originally described in the bile ducts of liver as biliary glycoprotein (BGP) and shown to cross-react with anti-CEA antibodies (Svenberg 1976). Later, it was shown to be apically expressed in intestinal epithelia (Svenberg et al. 1979). Studies in rat hepatocytes demonstrated that it was a cell–cell adhesion molecule and it was given the name Cell-CAM-105 (Ocklind & Obrink 1982). The human gene was cloned by Hinoda et al. (1988) and Barnett et al. (1989) who showed that it was alternatively spliced into at least eight isoforms (Barnett et al. 1993). The rat gene was originally cloned as a Ca2+/Mg2+-ecto-ATPase (Lin & Guidotti 1989), based on its ability to confer this activity to transfected COS cells, and it has been cloned as a bile transporter gene (Sippel et al. 1993) and for opa+ Nisseriae strains (Virji et al. 1996, Gray-Owen et al. 1997, Bos et al. 1998) and H. influenzae (Virji et al. 2000) in man. CEACAM1 associates with the insulin receptor in rat liver (Najjar et al. 1993), is rapidly phosphorylated upon binding of insulin to its receptor (Najjar et al. 1995), and upon mutation of Ser-503 in the cytoplasmic domain of CEACAM1, confers insulin resistance in transgenic mice (Poy et al. 2002a,b). CEACAM1 is down-regulated early in >90% of colon cancers (Neumaier et al. 1993), in prostate cancer (Kleinerman et al. 1995a), and to a lesser extent, in breast cancer (Riethdorf et al. 1997, Huang et al. 1998). Transfection of the rat gene into prostate (Hsieh et al. 1995), bladder (Kleinerman et al. 1996), or breast cancer (Luo et al. 1997) cells renders the tumours less tumorigenic in nude mice.

The morphology of normal mammary gland is characterized by an inner layer of polarized epithelial cells with secretory surfaces facing a central lumen and basal surfaces surrounded by an outer layer of myoepithelial cells. When these cells are cultured ex vivo in Matrigel, a source of extracellular matrix (ECM), they form alveolar structures resembling their in vivo morphology (Gomm et al. 1997). Other in vitro models that utilize the same Matrigel culture system have shown that normal mammary epithelial cells differentiate into acini or tubular structures (Barcellos-Hoff et al. 1989, Petersen et al. 1992, Bergstraesser & Weitzman 1993, Gomm et al. 1997). Upon contact with ECM proteins such as laminin and collagen IV, normal mammary epithelial cells become polarized, form spherical acini or branched tubules, and in the presence of prolactin initiate β-casein synthesis.
secretion (Muschler et al. 1999). In contrast to normal cells, tumour mammary epithelial cells form large, non-polarized, undifferentiated colonies without lumena when grown in Matrigel (Petersen et al. 1992).

A variety of growth factors such as hepatocyte growth factor (Brinkmann et al. 1995, Soriano et al. 1995), epidermal growth factor (Gomm et al. 1997), keratinocyte growth factor (Hirai et al. 1998), and fibroblast growth factor (Li & Shipley 1991) promote branching morphogenesis of cells grown on ECM. In addition to growth factors, integrins play an important role in establishing alveolar differentiation while aberrant integrin expression confers a tumorigenic phenotype. Integrins \( \alpha_1 \) and \( \alpha_2 \) confer invasive behaviour by regulating stromelysin-1 (matrix metalloproteinase) expression, and \( \alpha_6 \)-integrin has been shown to regulate cell motility (Lochter et al. 1999). Integrins \( \alpha_6 \) and \( \beta_1 \) cause metastatic invasion in mouse mammary carcinoma cells and are required for cell movement in 2-dimensional (2D) cell assays on reconstituted basement membranes. In agreement with these findings, antibodies to \( \alpha_1 \), \( \alpha_2 \), \( \alpha_6 \), and \( \beta_1 \)-integrins were able to block cell motility (Lochter et al. 1999). Moreover, blocking antibodies which inhibit expression of \( \beta_1 \)-integrin or EGFR in tumour cells grown in Matrigel have been shown to induce down-regulation of \( \beta_1 \) and EGFR, and cause growth arrest and reversion to normal morphogenesis as indicated by the formation of acinus-like structures (Li et al. 1997, Weaver et al. 1999).

Assembled lipid droplets in the mammary gland are secreted from the cytoplasm and enveloped by cellular membranes. Milk lipids originate as small droplets of triacyl-glycerol, synthesized in or on the surface of rough endoplasmic reticulum membrane and are released into the cytoplasm with a surface coat of protein and polar lipid. Droplets of varying size are transported to the apical cytoplasm, and when secreted from the cell, are coated with an outer membrane bilayer. Thus, the cream fraction of milk comprises lipid droplets coated with cellular membranes. Two possible mechanisms for lipid secretion have been proposed: an apical mechanism, in which lipid droplets are enveloped with apical plasma membrane, and a secretory-vesicle mechanism, in which fat droplets are surrounded by secretory vesicles in the cytoplasm and are released from the surface by exocytosis from intracytoplasmic vacuoles (Mather & Keenan 1998, Mather et al. 2001, Oshima et al. 2002). A number of proteins are found on the surface of secreted milk-fat globules including mucin 1 (Mather et al. 2001) and milk fat globule-EGF factor 8, a peripheral membrane glycoprotein abundantly expressed in lactating mammary glands and thought to play a role in vesicle secretion (Oshima et al. 2002). The presence of sialyl-Lewis ligands on milk oligosaccharides and their known role as selectin ligands suggests that they could either play a role in inflammatory processes (Rudloff et al. 2002) or milk uptake in the infant intestinal tract. Interestingly, mastitis milk has the same anti-inflammatory components and characteristics of normal milk, with elevations in selected components/activities that may help protect the nursing infant from developing infections due to feeding on mastitis milk (Buescher 2001). High levels of cell adhesion molecules ICAM-1 and VCAM-1 are also found in milk, which could further augment the immune system of the neonate (Xyni et al. 2000). Moreover, multimeric \( \alpha \)-lactalbumin, a potent \( \text{Ca}^{2+} \)-elevating and apoptosis-inducing agent with broad, yet selective, cytotoxic activity was found in milk. Multimeric \( \alpha \)-lactalbumin killed all transformed, embryonic, and lymphoid cells tested, but spared mature epithelial elements. These findings raise the possibility that milk contributes to mucosal immunity not only by furnishing antimicrobial molecules but also by activating lymphocytes and epithelium (Hakansson et al. 1995).

We are currently studying the role of CEACAM1 in normal and malignant breast. Previously, we demonstrated that human CEACAM1 is expressed in the normal breast cell line MCF10 and is essential for lumen formation when the cells are cultured in Matrigel (Huang et al. 1999). Blocking CEACAM1 expression either with an anti-sense gene or anti-CEACAM1 antibodies reverts the cells to a more malignant phenotype. Conversely, MCF7 breast cancer cells fail to express CEACAM1 or produce lumena when grown in Matrigel. When MCF7 breast cancer cells were transfected with CEACAM1-4S, the short cytoplasmic domain isoform that predominates in normal breast (Huang et al. 1998), reversion to a more normal phenotype was evidenced by lumen formation when the cells were grown in Matrigel (Kirshner et al. 2003). In the study presented here, we examine the expression of CEACAM1 in a wide variety of cancer cell lines grown in Matrigel, in normal, pre-malignant, and malignant breast tissue, and for the first time, show that CEACAM1 is secreted in breast milk on the membrane of lipid vesicles and in alveolar cells.

Materials and methods

Tissues and cells

With institutional review board approval, tissues were obtained from the anatomic pathology file at the City of Hope National Medical Center. Normal mammary epithelial cells (HMEC, MCF10A, MCF10F), mammary carcinoma cells (MCF7, MB-MDA-231, MB-MDA-468, ZR-75, T47D, SKBR3, BT474), cervical carcinoma (HeLa), and colon carcinoma (HT29) cells were obtained from ATCC. MCF7-CEACAM1-4S-ectoGFPP cells were generously provided by Dr. Charrg-Jui Chen and were made as described previously (Kirshner et al. 2003).

Culture conditions

MCF10Fe cells were grown in mammary epithelial cell growth medium (MEGM) supplemented with SingleQuotes (10 ng/ml hEGF, 5 \( \mu \)g/ml insulin, 0.5 \( \mu \)g/ml hydrocortisone, 50 \( \mu \)g/ml gentamicin, 50 ng/ml amphotericin B, and 52 \( \mu \)g/ml bovine pituitary extract) (Clonetics). MCF10Fm cells were grown in DMEM-F12 (Gibco-BRL) with 5 \( \mu \)g/ml insulin, 5 \( \mu \)g/ml transferrin, 20 ng/ml hEGF, 5 \( \times \)10\(^{-8}\) M
dexamethasone and 5% FBS (Huang et al. 1999). MCF7 and HeLa cells were grown in MEM with Earle’s salts and 10% FBS (Irvine Scientific). MCF7, HeLa, HT29 cells were grown in RPMI 1640 (Irvine Scientific) and supplemented with 10% FBS (Irvine Scientific), l-glutamine (GibcoBRL), and penicillin/streptomycin (GibcoBRL). MB-MDA-231, MB-MDA-468, ZR-75, T47D, SKBR3, HBL-100, and BT474 were grown per ATCC instructions.

Matrigel culture

Cells (2.5 × 10⁵) were plated in a thick layer (1 mm) of Matrigel (Collaborative Biomedical Products) in two well chamber slides (Nunc). Solidified Matrigel was overlaid with MEGM. To harvest cells, the gels were incubated in MatriSperse solution (Collaborative Biomedical Products) for 1 h at 4 °C. Cells grown in Matrigel were scored for the presence (acinus formation) or absence of lumena and compared by Student’s t-test or the chi-squared test.

Gene array

GEArray Q Series Human Apoptosis and Human ECM and Adhesion Molecules gene arrays were purchased from SuperArray. Hybridization with the RNA from MCF/vector and MCF7/CEACAM1-4S cells grown on tissue culture plastic or in Matrigel for 4 days was performed according to the manufacturer’s instructions. For cDNA positions and identification lists, see http://www.superarray.com.

FACS analysis

For the detection of cell surface integrins 1 × 10⁶ cells were incubated with 1 µg/ml of anti-integrin mAbs (Chemicon) for 1 h, washed with phosphate buffered saline (PBS), and then incubated with Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes) for 1 h followed by washes with PBS. Cells were analysed on a MoFlo (Cytomation) FACS.

Immunohistochemistry

Matrigel cultures were fixed with 10% neutral buffered formalin (NBF) (10% formalin in PBS) for 10 min, embedded in 3% agar, and treated with 10% NBF overnight, followed by 70% ethanol until processed. Paraffin-embedded sections were prepared and used for haematoxylin and eosin staining and immunohistochemistry. Monoclonal antibody 4D1C2 (1 µg/ml) (Drzeniek et al. 1991) was used to detect CEACAM1. Immunohistochemical staining was performed as described by Huang et al. (1999).

Confocal microscopy

Cells were grown in Matrigel and stained with MitoSensor JC-1 cationic dye (Clontech), annexin V-FITC, or anti-integrin mAbs (Chemicon) according to manufacturer’s instructions. Confocal microscopy was performed on a Zeiss Model 310 confocal microscope.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed according to (Huang et al. 1999). In short, MCF10F, MCF7/pHβ, and MCF7/CEACAM1-4S cells were grown in Matrigel for 5–12 days or isolated from human breast milk by centrifugation at 2000 rpm for 10 min. Acini were isolated from Matrigel as previously described, fixed for 3 h with 1% glutaraldehyde in 0.2 M cacodylate buffer, washed 3× in PBS, and post-fixed with 1% osmium tetroxide for 1 h at 4 °C. Following three PBS washes, the cells were placed in microcentrifuge tubes for graded ethanol rinses. The samples were treated with propylene oxide, embedded in Eponate with araldite, and polymerized for 72 h at 70 °C. Sections (50 nm-thick) were cut on an LKB Ultrotome III, placed on 300 mesh, uncoated copper grids, and stained with 5% uranyl acetate for 10 min, followed by 1 min in Sato’s lead solution. TEM was performed on a Philips CM 10 TEM.

Immuno-electron microscopy

MCF10F and MCF7/CEACAM1-4S cells were grown in Matrigel for 5–12 days, resulting acini were isolated with MatriSperse, fixed for 2 h with 4% paraformaldehyde–0.01% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, and washed with cacodylate buffer. Following cryoprotection with 2.3 M sucrose overnight, the specimen was frozen by immersion into liquefied nitrogen and sectioned at 70 nm on a Leica Ultracat UCT equipped with EMFCS cryoattachment unit at −100 °C. The sections were picked up and transferred to formvar coated, carbon coated, glow discharged 300 mesh nickel grids. The grids were placed on drops of sucrose, washed with 3× on 50 µl drops of PBS–6% BSA for 5 min each and incubated for 45 min at room temperature on 30 µl drops of PBS–6% BSA (negative control) or mAb T84.1 (1 µg/ml) diluted with PBS–6% BSA. Subsequently, the grids were washed 3× on 50 µl drops of PBS–6% BSA for 2 min each and then incubated for 1 h on 50 µl drops of 1 : 100 Protein G gold (20 nm) diluted with PBS–6% BSA. After washing with PBS–6% BSA 5× for 2 min each, grids were incubated on a drop of PBS–1% glutaraldehyde for 5 min, washed 5× on nano-pure water, stained on a drop of methylcellulose–uranyl acetate for 30 s, dried with filter paper, and observed and photographed with the Philips CM 10 TEM.

Immunoblotting

Fractionated or whole human milk samples were applied to reducing gradient SDS-PAGE gels (Invitrogen) in SDS loading buffer with 100 mM DTT. Following semidry electrotransfer to PVDF membranes (BioRad) and blocking with TBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) buffer containing 5% non-fat dry milk or 10% BSA (for phospho-protein blotting) overnight, the blots were incubated for 1 h with mAb T84 or pAb 22.9 at a final concentration of 1 µg/ml. Following three PBS washes, the blots were incubated with goat anti-mouse IgG-HRP or goat anti-rabbit protein blotting overnight, the blots were incubated with 1 µg/ml of anti-integrin mAbs (Chemicon) for 1 h, washed with phosphate buffered saline (PBS), and then incubated with Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes) for 1 h followed by washes with PBS. Cells were analysed on a MoFlo (Cytomation) FACS.

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IgG-HRP at a 1:3000 dilution (BioRad) for 1 h and washed again. Detection was carried out using the SuperSignal Chemiluminescent Substrate and Enhancer Kit (Pierce).

Reverse transcriptase–polymerase chain reaction

Mammary alveolar cells were isolated from milk by centrifugation and RNA was isolated using Totally RNA kit (Ambion) as per the manufacturer’s instructions. Reverse transcriptase–polymerase chain reaction (RT–PCR) was performed with sense and anti-sense primers P1 and P2 as described previously (Takahashi et al. 1993) using One Step RT–PCR kit (QIAGEN) per manufacturer’s instructions.

Results

CEACAM1 expression in breast tissues

CEACAM1 was found to be down-regulated in 30% of breast cancers (Riethdorf et al. 1997, Huang et al. 1998). Riethdorf et al. (1997) assessed the pattern of CEACAM1 expression in ductal and lobular carcinomas, and found predominantly membrane staining in the less differentiated, and occasional luminal staining in well-differentiated specimens. We have now compared normal, pre-malignant, and invasive breast cancers for their patterns of CEACAM1 expression in more detail (Table 1 and Figures 1 and 2). Strong luminal staining was observed in 11/11 normal glands examined (Figure 1A). Similarly, strong luminal staining was observed for proliferative breast disease (13/15, Figure 1B). Most atypical ductal hyperplasia specimens were negative (13/15, Figure 1C and D). All cases of ductal carcinoma in situ (DCIS) with a cribriform pattern were positive (Figure 2A), but those with a solid, comedo, and micropapillary patterns were negative or had only weak cytoplasmic staining (3/15, Figure 2B). Although most invasive ductal carcinomas showed expression of CEACAM1 (21/26) the strength and pattern varied with morphologic features of the tumours. Those tumours with well-formed tubules (Grade 1 and a few Grade 2 tumours) showed strong luminal membrane expression (Figure 2C). Tumours growing as sheets and cords with little if any tubule formation (most Grade 2 and all Grade 3 tumours) had predominately weak to moderate cytoplasmic staining (Figure 2D) with only occasional membrane positivity. A rare high-grade tumour had strong cytoplasmic expression (Figure 2E). Invasive lobular carcinomas were negative (Figure 2F) with only one of four showing weak staining of signet ring cells. These results suggest that in ductal breast cancers, the altered pattern of cellular expression of CEACAM1 reflects the tubular-differentiation state. Lower grade tumours retain the ability to localize the protein to the apical surface while in the more poorly differentiated ductal and in lobular carcinomas the protein is cytoplasmic.

Previously, we examined the pattern of CEACAM1 isoform expression in normal and malignant breast and found that patients either produced the CEACAM1-3S isoform only, or a combination of the -4L, -4S, and -3S isoforms (Huang et al. 1999). While the reason for these differences in isoform production are not clear, we noted that the -4S or -3S isoforms predominate. Later, we showed that transfection of the -4S but not the -4L isoform into MCF7 cells was sufficient to restore luminal formation in these cells when grown in Matrigel (Kirshner et al. 2003). Thus, the -S isoform appears to be the biologically relevant isoform in the human mammary gland. In the majority of breast cancer patients examined, the combined isotype expression pattern (-4L, -4S, -3S) was observed (Huang et al. 1999). It remains to be seen if the isoform expression pattern is changed during malignancy.

| Histology       | CEACAM1 expression | N | Pos | % | Notes                                                                 |
|-----------------|--------------------|----|-----|---|----------------------------------------------------------------------|
| Normal          |                    | 11 | 11  | 100 | Strong luminal membrane staining of terminal ductules and ductal epithelium. |
| PBD*            |                    | 15 | 13  | 87 | Most showed strong staining of luminal membrane but a few had cytoplasmic staining as well. |
| Fibroadenoma     |                    | 2  | 2   | 100 | Strong luminal membrane staining.                                      |
| ADH**           |                    | 6  | 2   | 33 | Most were negative but two had luminal membrane staining and some cytoplasmic staining. |
| DCIS***          |                    | 23 | 11  | 48 | All showed some luminal membrane staining with or without a cytoplasmic component. |
| Solid           |                    | 8  | 2   | 25 | Both positive cases showed only weak cytoplasmic staining.              |
| Micropapillary   |                    | 2  | 0   | 0  |                                                                       |
| Comedo          |                    | 5  | 1   | 20 |                                                                       |
| Carcinomas      |                    |    |     |    |                                                                       |
| Ductal Grade 1  |                    | 26 | 21  | 80 | Strong luminal membrane staining of tumors with prominent tubule formation. |
| Grade 2         |                    | 17 | 13  | 76 | Most had only moderate to weak cytoplasmic staining but a sub group showed strong membrane staining or luminal membrane staining of tubules. |
| Grade 3         |                    | 7  | 6   | 86 | Most showed only weak to moderate cytoplasmic staining but a few had membrane accentuation. |
| Lobular         |                    | 4  | 1   | 25 | Only one showed weak cytoplasmic staining predominately in areas of signet ring cells. |
| Papillary       |                    | 1  | 1   | 100| Strong luminal membrane staining in one papillary case.                 |

*PBD – proliferative breast disease including ductal hyperplasia and adenosis. **ADH – atypical ductal hyperplasia. ***DCIS – ductal carcinoma in situ.
CEACAM1 expression in mammary gland morphogenesis

Figure 1. Expression of CEACAM1 in normal and pre-malignant mammary tissue. Immunohistochemistry staining of tissues with CEACAM1 specific antibody 4D1C2. (A) Normal mammary gland (200×). (B) Florid ductal hyperplasia in proliferative breast disease has strong staining of luminal surfaces of epithelial cells similar to that seen in normal epithelium (100×). (C) Atypical ductal hyperplasia minimal membrane staining of the involved duct (100×). (D) Higher power of a duct with partial involvement by an atypical epithelial proliferation that demonstrates little staining of some cribriform ducts while adjacent epithelium has minimal surface immunoreactivity (400×).

CEACAM1 expression in breast cancer cell lines

We examined the pattern of CEACAM1 expression in a number of mammary epithelial cell lines grown in Matrigel to determine if CEACAM1 expression confers lumen formation across a wide range of cells. Both mammary epithelial cells obtained from breast reduction surgery (HMECs) and MCF10 cells, that are considered to mimic normal mammary epithelial cells, expressed CEACAM1 and formed acini with lumena, when grown in Matrigel (Figure 3, Table 2). The pattern of expression was identical to that of normal mammary gland, namely, strong membranous staining surrounding the lumena. In the case of the breast cancer cell lines, four out of seven formed acini in Matrigel, and none of those had lumena. However, two of the four that form acini expressed CEACAM1, demonstrating that expression of CEACAM1 alone is not sufficient to confer lumen formation. We conclude that CEACAM1 expression is necessary but not sufficient for lumen formation. In the breast tumour cell lines that expressed CEACAM1, the sub-cellular distribution was similar to that of the mammary tumours, where the staining was distributed throughout the cytoplasm and not found exclusively at the membrane. Since CEACAM1 is a type 1 membrane glycoprotein, we speculate that it remains trapped in endosomes in these cells, and is prevented from transmitting its cell–cell signals that are crucial for lumen formation (Kirshner et al. 2003).

CEACAM1 expression in other tumour cell lines

We also tested the expression of CEACAM1 in other types of cancer cell lines grown in Matrigel and its potential effect on lumen formation. All cells tested formed colonies in Matrigel, but none formed lumena (Table 2). However, only the colon cancer cell line HT29 expressed CEACAM1, thus making it hard to judge if these other cell lines behave like the breast cancer lines. We were especially interested in the prostate cell line, since down-regulation of CEACAM1 expression has been correlated with the progression from normal to malignant (Kleinerman et al. 1995a), and normal prostate cells can form acini with lumena when grown in a 3D culture (Hudson et al. 2000). Studies are now underway to determine if CEACAM1 transfected LNCap cells form lumena when cultured in Matrigel.

Function of CEACAM1 in cultured mammary epithelial cells

Previously, we showed that transfection of CEACAM1 into MCF7 cells restores lumen formation when the cells are grown in Matrigel and lumen formation requires apoptosis of the central cells in the colonies (Kirshner et al. 2003). In those studies, we hypothesized that expression of CEACAM1 triggers apoptosis. For example, when MCF7 cells transfected with a CEACAM1-GFP fusion protein are grown
Figure 2. Expression of CEACAM1 in DCIS and carcinomas. Immunohistochemistry staining of tissues with CEACAM1 specific antibody 4D1C2. (A) Ductal carcinoma in situ with a cribriform pattern showing strong membrane staining along the lumena as well as staining of the central proteinaceous material (100×). (B) High-grade comedo type ductal carcinoma in situ, shows only faint cytoplasmic staining (100×). (C) Moderately differentiated ductal carcinomas with tubular formation often expressed CEACAM1 on the membrane surface of the abortive lumena (200×). (D) Most of the invasive ductal carcinomas, especially those growing as solid nests and sheets without lumena, showed weak cytoplasmic staining (400×). (E) Though rare, poorly differentiated invasive ductal carcinoma occasionally showed strong cytoplasmic staining throughout the tumor. (F) Invasive lobular carcinomas were generally negative for CEACAM1. Note the membrane staining of the residual normal ducts (200×).

in Matrigel and stained with the mitochondrial dye, JC1, CEACAM1-GFP is seen between the cells at 7 d and in the lumena at 12 d (Figure 4A and B). Since the JC-1 dye stains the mitochondria of healthy cells red and apoptotic cells green, one cannot distinguish whether the central green cells are apoptotic, just express CEACAM1-GFP or both. However, when MCF7 cells are transfected with CEACAM1 (no GFP fusion protein) and stained with JC1, the central cells stain green (Figure 4C). Further evidence that the central cells are apoptic is shown by annexin V staining superimposed on the phase contrast photo of an isolated 12 d colony (Figure 4D). Based on these published results, we ask why the central cells apoptose while the peripheral cells do not? One answer consistent with a large body of data on the function of integrins and ECM is that only the peripheral cells contact ECM and thus are spared. The literature suggests that loss of contact of epithelial cells to ECM is sufficient to cause death or anoikis; however, control cells, that do not express CEACAM1 and are located in the centre of colonies, are perfectly healthy (Figure 3B). Thus, a major function of CEACAM1 appears to be induction of apoptosis in the context of the presence or absence of ECM. The mechanism of interaction between integrin subunits and CEACAM1 is addressed in a later section.

The lumenal epithelium of the mammary gland is polarized with microvilli and budding vesicles facing the apical surface of the gland (Gomm et al. 1997). The polarized pattern with microvilli and budding vesicles is faithfully reproduced when normal mammary epithelial cells are cultured in Matrigel (Huang et al. 1999). Similarly, when MCF7 cells transfected with CEACAM1 are grown in Matrigel and the lumen is examined by EM, microvilli with budding
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Figure 3. Expression of CEACAM1 in mammary cell lines grown in Matrigel. Immunohistochemistry staining with anti-CEACAM1 antibody 4D1C2 of cells grown in Matrigel for 12 d. (A) MCF10F cells (400×). (B) MCF7 cells (400×). (C) BT474 cells (100×). (D) MDA-MB-231 cells (200×).

Figure 4. CEACAM1 transfected MCF7 cells form lumena by apoptosis of the central cells. This figure is reprinted with the authors’ permission from Kirshner et al. (2003). MCF7 cells were transfected with either CEACAM1 or CEACAM1-eGFP fusion protein and grown in Matrigel for 7 or 12 d. Cells were counterstained with either JC1 or FITC-annexin V. (A) MCF7/CEACAM1-eGFP, 7 d, counterstained with JC1. (B) MCF7/CEACAM1-eGFP, 12 d, counterstained with JC1. (C) MCF7/CEACAM1, 12 d, counterstained with JC1. (D) MCF7/CEACAM1, 7 d, counterstained with FITC-annexin V and superimposed on the phase contrast image.

vesicles are observed (Figure 5A). Since Frangsmyr et al. (1995) had previously demonstrated CEACAM1 expression on the tips of microvilli in colonic epithelia, we speculated that CEACAM1 would also be localized to the microvilli of polarized breast epithelial cells. Applying immuno-electron microscopy (immuno-EM) staining to MCF/CEACAM1 cells grown in Matrigel during the course of lumen formation, CEACAM1 was found between the cells of the 5-d acinus (Figure 5B), and redistributed to the luminal surface by 12 d in culture, where it was found exclusively on microvilli (Figure 5C). Furthermore, vesicles staining positive for CEACAM1 were found both in the cytoplasm of the epithelial cells and in the central lumena (Figure 5B–D, and see Figure 6D). No CEACAM1 staining was found in the MCF7/vector cells grown in Matrigel (Figure 5E). Thus, we conclude that CEACAM1 plays a role in cell–cell adhesion in the early stages of mammary morphogenesis, and shifts to a purely apical pattern of expression once mature glands are formed. In mature alveolar cells CEACAM1 expression is limited to the microvilli that appear to produce small, micron sized vesicles that have abundant amounts of CEACAM1 on their surfaces. Based on this evidence, we predicted that CEACAM1 should be found in the lipid fraction of breast milk.

CEACAM1 in breast milk

Breast milk was centrifuged to remove cells and the supernatant separated into the lipid and non-lipid fraction. Breast milk is known to contain abundant alveolar cells that are shed into the milk (Cross & Mercer 1993). Staining of these cells revealed an intact nucleus and one or more membrane enveloped lipid vesicles (Figure 6A–D). The lipid vesicle found in these cells was often as large as the nucleus and was filled with triglycerides. RT-PCR analysis of the
Table 2. Expression of CEACAM1 in various cell lines.

| Cell line      | Type   | Colony formation | Lumen formation | CEACAM1 expression in Matrigel (IH) | CEACAM1 expression on plastic (IB) |
|----------------|--------|------------------|----------------|-------------------------------------|-----------------------------------|
| HMEC           | Breast, n | +    | +              | ++++ m                             | +                                 |
| MCF10F         | Breast, n | +    | +              | +++++ m                            | +                                 |
| MCF7           | Breast, t | +    | −              | −                                  | −                                 |
| BT-474         | Breast, t | +    | −              | ++++ m, c                          | +                                 |
| MDA-MB-231     | Breast, t | −    | −              | −                                  | −                                 |
| MDA-MB-468     | Breast, t | −    | −              | ++++ c                             | +                                 |
| SK-BR3         | Breast, t | +    | +              | + m, c                             | −                                 |
| T-47D          | Breast, t | +    | −              | ++ m                               | +                                 |
| ZR-753         | Breast, t | +    | +              | ++ m, c                            | −                                 |
| HT29           | Colon, t  | +    | −              | ++++ m, c                          | +                                 |
| LNCap          | Prostate, t | +    | −              | −                                  | −                                 |
| A2780          | Ovarian, t | +    | −              | −                                  | −                                 |
| HeLa           | Cervical, t | +    | −              | −                                  | −                                 |
| Panc-1         | Pancreatic, t | +    | −              | −                                  | −                                 |
| MiaPaCa        | Pancreatic, t | +    | +/−            | −                                  | −                                 |

IH – immunohistochemistry, IB – immunoblotting, n – normal, t – tumour; m – membrane, c – cytoplasmic staining pattern.

150% make colonies. 2Myoepithelial phenotype. 3Loose colonies.

mRNA isolated from the alveolar cells revealed that both the CEACAM1-4L and -4S isoforms were present (Figure 7A and B). Western blot analysis of the lipid and non-lipid fractions of milk from a second donor revealed that CEACAM1 was found only in the lipid fraction (Figure 7C), in agreement with the prediction that it is on the surface of lipid vesicles. We concluded that only the CEACAM1-4 isoform was present in this specimen based on a molecular size comparison to CEACAM1 isoforms expressed in Jurkat cells. However, it was also possible that CEACAM1 was cleaved from its...
CEACAM1 expression in mammary gland morphogenesis

Previous studies on the 3D model of mammary morphogenesis have focussed on the role of ECM–integrin interactions (for a review see Bissell et al. 2002). It seems likely that the integration of these signals with the cell–cell adhesion signals conveyed by molecules such as CEACAM1 (or E-cadherin) are essential for establishing the complete morphogenic programme. An obvious possibility is a direct association between integrins and CEACAM1 receptors on the cell surface. Indeed, Brummer et al. (2001) have shown that when the phosphorylated form of the long cytoplasmic domain of CEACAM1 is used as bait for interacting proteins in neutrophil lysates, β3 integrin was isolated. However, extension of this finding to breast epithelial cells, to other integrin subunits, and especially to the short cytoplasmic domain isoform of CEACAM1 is lacking. As a first step in assessing the level of CEACAM1 involvement in integrin–ECM communication, a gene array analysis was performed comparing the expression of cell adhesion and ECM proteins in MCF7/vector and MCF7/CEACAM1-4S cells grown on plastic versus Matrigel (Table 3). After normalization for housekeeping genes, no gene regulation (up or down) was seen in CEACAM1 versus vector transfected cells grown on either plastic or Matrigel. Aside from several housekeeping genes, the gene array showed that several genes like CEA and E-cadherin are highly expressed in MCF7 cells. However, the mRNA levels for these genes were unchanged before and after growth of control and transected cells on Matrigel. Based on the limited selection of genes probed, it appears that CEACAM1 alone is responsible for reverting breast cancer cells to a normal phenotype.

Since the role of integrins in a 3D model of mammary morphogenesis is well established, including changes in the surface levels of the β1 and β4 subunits (Weaver et al. 1997), we speculated that either the protein or its cell surface levels must be regulated in CEACAM1 transfected MCF7 cells. To examine this possibility, cell surface levels of various integrins was measured on control and CEACAM1 transfected cells grown on plastic or in Matrigel (Figure 8A). Quite surprisingly, no or very low cell surface expression of integrins was seen in either control or CEACAM1 transfected cells grown on plastic (Figure 8A, blue and purple traces); however, when the cells were grown in Matrigel, the cell surface levels of all the integrins tested increased dramatically (Figure 8A, yellow and red traces). Since mRNA levels of the integrins are unchanged, we conclude that exposure of the cells to the ECM in Matrigel induces their cell surface expression. Comparison of the control versus transfected cells grown in Matrigel revealed that integrins α1 and β1 were down-regulated in MCF7/CEACAM1-4S compared to the vector controls (yellow vs. red traces in Figure 8A), while

**Figure 7.** CEACAM1 is found in human milk. (A) RNA purified from the alveolar cells isolated from fresh breast milk. (B) RT–PCR using the RNA from (A) for CEACAM1 expression compared to RNA from human colon carcinoma cell line HT-29. The two bands correspond to CEACAM1-4L and CEACAM1-4S. (C) Immunoblot analysis of CEACAM1 in breast milk. Lanes 1 and 10, Jurkat/CEACAM1-4L; lanes 2 and 9, Jurkat/CEACAM1-4S; lane 3, CEACAM1-3sol; lane 4, CEACAM1-4sol; lane 5, aqueous fraction of milk; lane 6, lipid fraction of milk; lanes 7 and 8, whole milk. Proteins in lanes 1–7 were probed with anti-CEACAM1 mAb T84.1, recognizing the N domain of CEACAM1, and proteins in lanes 8–10 were probed with anti-CEACAM1-L pAb 22.9, recognizing the long cytoplasmic domain of CEACAM1.
murine mammary morphogenesis. Klinowska et al. had previously shown that the down-regulation of αV-integrin subunits for paired α/integrin subunits. In this respect, αVβ1 binds laminin-1 and α2β1 binds collagen IV, the major components of basement membrane that surround the alveolar structures.

Confocal microscopy was performed to evaluate the possible direct association of CEACAM1 with the above integrin subunits (Figure 8B). A strong co-localization of CEACAM1 with α2 and β1, and a weaker association with α1, and α3 was observed. The co-localization occurred on the periphery of the colonies where contact with the basement membrane occurs. Even though the predominant location of CEACAM1 is between the cells during the early stages of morphogenesis, clearly it also has a basal–lateral localization that allows direct interaction with the integrin subunits and ECM. This suggests that CEACAM1-4S and β1 integrin participate in a common pathway leading to lumen formation. These findings further support our hypothesis that

Table 3. Matrix adhesion genes expressed by CEACAM1 transfected MCF7 cells.

| Gene                  | Vector/ plastic | Vector/ Matrigel | CEACAM1/ plastic | CEACAM1/ Matrigel |
|-----------------------|-----------------|------------------|------------------|------------------|
| Caveolin-1            | 2+              | 2+               | 2+               | 2+               |
| CD44                  | 2+              | 2+               | 2+               | 2+               |
| E-Cadherin            | 4+              | 4+               | 4+               | 4+               |
| CEA                   | 4+              | 4+               | 4+               | 4+               |
| Collagen IV           | 2+              | 2+               | 2+               | 2+               |
| Cystatin C            | 1+              | 1+               | 1+               | 1+               |
| α1-Catenin            | 2+              | 2+               | 2+               | 2+               |
| β1-Catenin            | 2+              | 2+               | 2+               | 2+               |
| β2-Catenin            | 1+              | 1+               | 1+               | 1+               |
| Cathepsin D           | 4+              | 4+               | 4+               | 4+               |
| Cathepsin-L           | 1+              | 1+               | 1+               | 1+               |
| α1-integrin           | 1+              | 1+               | 1+               | 1+               |
| α2-integrin           | 1+              | 1+               | 1+               | 1+               |
| α3-integrin           | 1+              | 1+               | 1+               | 1+               |
| α5-integrin           | 1+              | 1+               | 1+               | 1+               |
| αv-integrin           | 1+              | 1+               | 1+               | 1+               |
| β1-integrin           | 2+              | 2+               | 2+               | 2+               |
| β2-integrin           | 1+              | 1+               | 1+               | 1+               |
| β3-integrin           | 2+              | 2+               | 2+               | 2+               |
| β3-integrin           | 1+              | 1+               | 1+               | 1+               |
| Laminin-γ             | 1+              | 1+               | 1+               | 1+               |
| Hyaluronidase         | 1+              | 1+               | 1+               | 1+               |
| MMP14                 | 2+              | 2+               | 2+               | 2+               |
| MMP15                 | 1+              | 1+               | 1+               | 1+               |
| MMP26                 | 3+              | 3+               | 3+               | 3+               |
| NRCAM                  | 2+              | 2+               | 2+               | 2+               |
| PECAM                  | 2+              | 2+               | 2+               | 2+               |
| PAI-1                  | 4+              | 4+               | 4+               | 4+               |
| Thrombomodulin-1       | 1+              | 1+               | 1+               | 1+               |
| Thrombomodulin-2       | 1+              | 1+               | 1+               | 1+               |
| TIMP-1                 | 1+              | 1+               | 1+               | 1+               |
| TIMP-2                 | 3+              | 3+               | 3+               | 3+               |
| House Keepers*         | 4+              | 4+               | 4+               | 4+               |

*The following housekeeping genes were included: GAPD, cyclophilin A, ribosomal protein L13a, and β-actin.

all other subunits showed no changes. Weaver et al. (1997) had previously shown that the down-regulation of β1 and up-regulation of β4 integrin correlates with the induction of lumen formation in T4–2 cells arising from spontaneously transformed HMT-3522 cells, and that β1 function blocking antibodies restore lumen formation to the transformed T4–2 cells grown in Matrigel. While the corresponding α subunit was not identified in their studies, it is interesting to speculate that it may be α1, since it is down-regulated together with β1 in our study. Integrin β1 has been shown to be critical for murine mammary morphogenesis (Klinowska et al. 1999) where implanted blocking antibodies prevented mammary gland development. Since down-regulation of β1 correlates with restoration of lumen formation in mammary tumour cells grown in Matrigel, we speculate that the absolute levels of β1 are the critical factor, especially in terms of competition with other β subunits for paired α subunits. In this respect, αVβ1 binds laminin-1 and α2β1 binds collagen IV, the major components of basement membrane that surround the alveolar structures.

Figure 8. Effects of ECM on integrin expression in MCF/CEACAM1-4S cells. (A) MCF7/vector and MCF7/CEACAM1-4S cells were grown on plastic or in Matrigel (2 d) and cell surface expression of integrins was assessed using FACS analysis. On the histograms, black peaks correspond to untransfected MCF7 cells used as a negative control, and blue to MCF7/vector cells grown on plastic and yellow, in Matrigel. The purple peaks correspond to MCF7/CEACAM1-4S cells grown on plastic and red, in Matrigel. The yellow peaks (indicated by arrows) are under the red peaks for α2 and α3. (B) MCF7/CEACAM1-4S-ectoGFP cells were isolated from a 2 day Matrigel culture and stained with anti-integrin antibodies (red channel). Colocalization is indicated by the overlap of the two channels (yellow) on the periphery of the colonies where contact with ECM is established.
CEACAM1-4S communicates with the ECM during the initial stages of mammary gland development.

**Discussion**

CEACAM1 is constitutively expressed in the epithelium of a variety of normal tissues, including colon, bladder, prostate, and breast. The early down-regulation of CEACAM1 in >90% of colon cancers (Neumaier et al. 1993) and its ability to render prostate, bladder, and breast cancer cells less tumourigenic (Kleinerman et al. 1995b, 1996, Luo et al. 1997) have led some investigators to propose that CEACAM1 is a tumour suppressor gene. However, the gene is only down-regulated in about 30% of breast cancers (Riethdorf et al. 1997), casting some doubt on this assignment. This discrepancy prompted us to re-examine the pre-malignant and malignant breast tissues for further clues. Strong CEACAM1 expression on the luminal surfaces of hyperplastic ductal cells in benign proliferative breast disease is similar to that seen in the normal breast. Minimal expression was seen in the limited number of atypical hyperplasia cases examined. Cribriform DCIS, which retains luminal structures stain strongly for CEACAM1, while those types that do not (solid and comedo types of DCIS) showed only cytoplasmic staining. Most invasive ductal carcinomas (80%) were positive and only those that had tubular formation expressed the protein on luminal or membrane surfaces, otherwise cytoplasmic staining predominated. We conclude that, while a drop in CEACAM1 expression and alteration of its staining pattern correlates with the differentiation state of both the pre-malignant and invasive lesions, by classical definition, CEACAM1 is not a tumour suppressor gene. It is especially noteworthy that no genetic changes involving the CEACAM1 gene have been reported.

The finding of CEACAM1 in breast milk was a direct result from our ultrastructure studies on breast epithelial cells grown in Matrigel. These polarized cells produce abundant vesicles that are secreted from the tips of microvilli into alveolar lumena. As predicted by these results, the CEACAM1 positive vesicles are found in the lipid fraction of milk and the molecular size of CEACAM1 is identical to that found in intact cells. Others have speculated that lipid droplets in milk are surrounded by membranes to increase their absorption in the infant intestinal tract (Cross & Mercer 1993). If this is correct, CEACAM1 is an ideal molecule for this purpose, since it is found on intestinal epithelia, is a cell–cell adhesion molecule, and is known to cause internalization upon ligation with bacteria (Chen et al. 2001, Muenzner et al. 2001). Further studies involving uptake of milk lipid vesicles by cultured intestinal epithelial cells are planned.

The finding that transfection of CEACAM1 into CEACAM1 negative breast cancer cells restores lumen formation in Matrigel culture is exciting. It appears that CEACAM1 plays an essential role in the morphogenic process. However, we found several breast cancer cell lines that express CEACAM1, but fail to form a lumen when cultured in Matrigel. This means that CEACAM1 is essential but not sufficient for lumen formation. It is possible that these cells are either lacking another essential component of the programme or express an additional gene that overrides the programme. In the first case, other candidate essential genes may include the integrins which bind ECM and help to establish polarity. While this possibility has not been fully explored, we have shown that CEACAM1 associates with the β1 integrin subunit that previously has been shown to be essential for murine mammary gland development (Klinowska et al. 1999). CEACAM1 also lowers the cell surface expression level of the β1 integrin subunit in transfected MCF7 cells, a finding that correlates well with studies on reversion of malignant breast cancer cells with anti-β1 integrin antibodies (Weaver et al. 1997). A possible explanation of these apparently contradictory results is that the β1 subunit may compete for other essential α subunits. Since α1 was also down-regulated in our study, we believe that there may be a competition among α1β1 and α2β1 which bind laminin 1 and collagen IV, respectively, both components of the basement membrane that surrounds the alveolar cells and establishes polarity. The other possibility to be explored is that additional genes expressed suppress the morphogenic program. A likely candidate gene is Her2 which overrides the growth inhibitory signalling in breast cells. In support of this idea, both CEACAM1 and Her2 are expressed in BT474 cells (Table 2), and studies with Her2 transfected cells demonstrate continued growth disrupting lumen formation (Muthuswamy et al. 2001).

In summary, CEACAM1 expression, found in an apical location in normal breast glands, is altered in both pre-malignant and malignant disease. It is secreted into milk on vesicles, and while its function there is unknown, it may play a role in intestinal absorption of lipid. Using a 3D model of morphogenesis, we have shown that CEACAM1 is required for lumen formation in CEACAM1 positive MCF10F cells, and transfection of CEACAM1 into the MCF7 breast cancer cell line restores lumen formation. The mechanism of lumen formation involves apoptosis of the central cells and requires both ECM–integrin and CEACAM1 interactions.

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