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Soluble Fragment of P-Cadherin Adhesion Protein Found in Human Milk

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Abstract Classical cadherins such as E- and P-cadherin are transmembrane proteins that mediate specific cell-to-cell adhesion and are important to tissue development and function. Cadherin function can be modulated by various means, including proteolytic cleavage of the extracellular adhesion domain from the cells' surface, yielding large soluble fragments termed (soluble) sE- or sP-cadherin. In people with certain carcinomas, sE-cadherin can be detected at elevated levels in the serum and sometimes can serve as a prognostic marker. Soluble E-cadherin also is found in urine of patients with bladder cancer. In addition to being present in bodily fluids of cancer patients, sE- and sP-cadherin are present in the serum of healthy people, suggesting that shedding of cadherins is a normal event. Here, we report high levels of 80 kDa sP-cadherin in human milk. In the lactating mammary gland tissue, P-cadherin appears to be a protein secreted by epithelial cells, rather than an adhesion protein. This is in contrast to the non-lactating mammary gland where P-cadherin is restricted to myoepithelial cells, and is present at sites of cell–cell contact. J. Cell. Biochem. 85: 180–184, 2002.

Key words: metalloproteinase; Listeria; epithelial cells; mammary gland; myoepithelial cells; protein in serum and urine

Cadherins constitute a family of adhesion proteins [see Takeichi, 1991]. E-cadherin (120 kDa) is expressed by most epithelial cells, whereas P-cadherin (118 kDa) is restricted to basal cell layers, including basal cells of skin and prostate, and myoepithelial cells of the mammary gland [Shimoyama et al., 1989]. Cadherins are transmembrane proteins that interact intracellularly with catenins and the actin cytoskeleton to promote strong cell–cell adhesion [see Wheelock et al., 1996]. They appear to serve as signaling molecules to regulate cell behavior [see Knudsen et al., 1998]. Cadherin activity is regulated by multiple mechanisms, including interaction with catenins, phosphorylation events, and shedding of the extracellular domain.

A soluble 80-kDa fragment representing most of the E-cadherin extracellular domain (sE-cadherin) was originally found in conditioned medium from cultured epithelial cells [Wheelock et al., 1987]. E-cadherin shedding also occurs in vivo, and soluble E-cadherin (sE-cadherin) is present in both serum [Katayama et al., 1994, Shirahama et al., 1996] and urine [Banks et al., 1995]. An elevated level of sE-cadherin correlates with the presence of certain cancers, and has been shown to be a prognostic marker for some cancers [Katayama et al., 1994; Griffiths et al., 1996; Gofuku et al., 1998; Velikova et al., 1998; Chan et al., 2001]. In addition to sE-cadherin, an 80-kDa fragment of P-cadherin (sP-cadherin) is present in serum of individuals with or without breast cancer, although its concentration is 20-fold lower than that of E-cadherin [Knudsen et al., 2000].

Here we report that high concentrations of sP-cadherin are present in human milk. In examining the expression pattern of P-cadherin in lactating mammary tissue, we observed that P-cadherin appears as protein secreted by luminal epithelial cells, rather than a cell–cell adhesion protein. This is in stark contrast to the

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non-lactating gland where P-cadherin is found only at sites of cell–cell contract between myoepithelial cells and is not present in epithelial cells.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies, recognizing the extracellular domain of P-cadherin or E-cadherin (HECD-1), were from BD Transduction Laboratories (San Diego, CA) and Zymed (South San Francisco, CA), respectively.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections of normal human mammary tissue using an avidin-biotin system following the manufacturer's instructions (Vector Laboratories, Burlingame, CA). A heat-induced antigen retrieval method was applied as previously described [Peralta Soler et al., 1999]. Primary antibodies were applied overnight at 4°C in a humidified chamber. Sections were counter-stained with hematoxylin. Tissue sections of human mammary tissue were obtained from archival material of normal breast containing lobular structures at different stages of differentiation [Russo and Russo, 1987]. They represent normal lobular structures of excisional biopsies obtained from women that underwent surgery for a suspicious mass, or from reduction mammoplasties from women that underwent surgical procedure for cosmetic reasons. All the collected samples were approved by the IRB of each hospital from which the specimens were obtained.

Western Immunoblot Analysis

Western immunoblot analysis was performed as described [Knudsen et al., 1995]. Human milk was donated by two lactating female employees of the Lankenau Institute for Medical Research. One woman was nursing a newborn, whereas the other was nursing a child older than 1 year. The milk was collected by the donor, aliquoted, frozen immediately upon collection, and stored at −80°C until it was analyzed. Equivalent volumes of the two milk samples were made 1× in Laemmli sample buffer, heated, resolved by SDS–PAGE, transblotted to nitrocellulose, and probed with cadherin antibodies as described [Knudsen et al., 1995]. Recombinant P-cadherin, combined with densitometry, was used to quantify sP-cadherin in milk, as described for sP-cadherin in serum [Knudsen et al., 2000].

RESULTS

In normal non-lactating breast tissue, P-cadherin expression is restricted to myoepithelial cells that underlie the E-cadherin-expressing luminal epithelial cells [Daniel et al., 1995; Peralta Soler et al., 1999]. This was confirmed by immunohistochemical analysis of normal non-lactating mammary gland tissue [Knudsen et al., 2000; these studies]. In human breast carcinoma, which are thought to arise from luminal epithelial cells, P-cadherin frequently is expressed, which likely represents misexpression. We and others have found P-cadherin expression to be a marker of poor prognosis for breast cancer [Peralta Soler et al., 1999; Gamallo et al., 2001]. As part of our studies on P-cadherin in normal and diseased human mammary gland, we examined P-cadherin expression in normal lactating breast tissue, using a monoclonal antibody to the extracellular domain of P-cadherin, and immunohistochemical methods. The results were unexpected.

First, there appeared to be a general increase in P-cadherin expression in lactating versus non-lactating mammary tissue (not shown). However, more surprising, in lactating tissue the P-cadherin was present in epithelial cells, and its staining pattern was similar to that of a secreted protein (Fig. 1), rather than an adhesion protein. The P-cadherin staining was present in epithelial cells and at their apical face, and did not appear at cell–cell borders, as expected for a cadherin. This observation suggests that P-cadherin is not functioning as an adhesion molecule in these cells in lactating mammary tissue. We also observed P-cadherin (Fig. 1, panel A), but not E-cadherin (Fig. 1, panel B) staining in material present in the lumen of ducts (Fig. 1). This material, which presumably is milk, did not stain with a pan-cadherin antibody directed to the cadherin intracellular domain (not shown), suggesting that the P-cadherin is not full-length.

The immunohistochemical staining, together with the absence of a cadherin intracellular domain, suggested that the P-cadherin is secreted or shed into milk. To test this possibility we performed Western immunoblot analysis of human milk using the cadherin antibodies. In

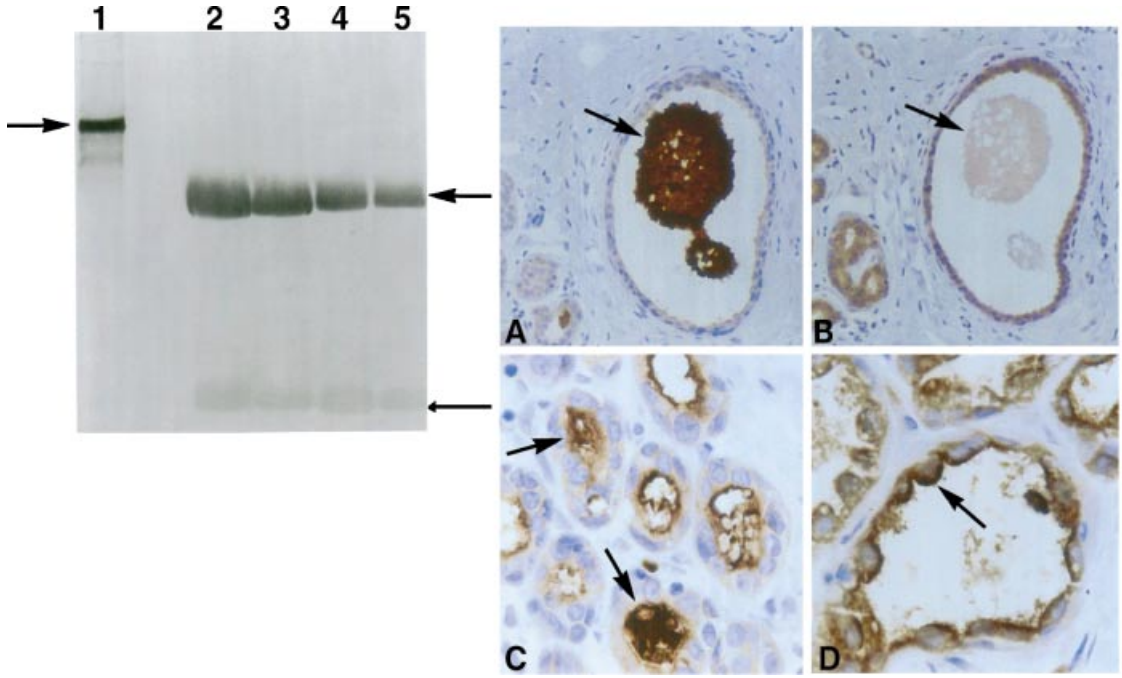

Fig. 1. P-cadherin expression in milk. Western immunoblot analysis (left panel) of human milk and immunohistochemical analysis (right panels) of lactating breast tissue using a monoclonal antibody specific for the extracellular domain of P-cadherin. In the Western immunoblot, recombinant P-cadherin (**lane 1**) is detected as a 120-kDa band (arrow) [Knudsen et al., 2000]. **Lanes 2**, **3**, **4**, and **5** correspond to two different amounts of human milk from two lactating women, with lanes 2 and 4 representing one individual and lanes 3 and 5 the other. Note the presence of a prominent 80-kDa band (arrow) and a minor 35-kDa band (small arrow). Using recombinant P-cadherin as a standard, and densitometry, we estimated the P-cadherin concentration of the 80-kDa fragment in milk to be 100–400 μg/ml, which is at least 100-times higher than in serum [Knudsen et al., 2000]. Immunohistochemistry of P-cadherin in lactating human breast was performed using an avidin-biotin method on archival paraffin-embedded tissue sections. Milk present in the lumen of a duct shows P-cadherin (arrow in **A**) but not E-cadherin staining (arrow in **B**). Type IV lobules (i.e, lactating) show abundant P-cadherin staining in the lumen (arrows in **C**), in addition to cytoplasmic and apical staining in epithelial cells (arrow in **D**).

samples from two different lactating women, we detected an 80 kDa form of P-cadherin, using the extracellular domain antibody (Fig. 1), but no signal with the intracellular domain pan-cadherin antibody (not shown). The molecular weight is identical to that of sP-cadherin previously detected in serum [Knudsen et al., 2000]. No full-length P-cadherin was detected. In contrast to sP-cadherin, sE-cadherin was not detected in milk, nor was its full-length form (not shown). Using recombinant human P-cadherin as a standard, we estimated the P-cadherin concentration in milk to be in the range of 100–400 μg/ml, which is at least 100-times higher than the concentration detected in serum [Knudsen et al., 2000].

DISCUSSION

Large soluble fragments of the extracellular domain of classical cadherins have been reported previously in conditioned medium from cultured cells [Wheelock et al., 1987], in bodily fluids from individuals with and without cancer (see Introduction), and during embryonic chick development [Paradies and Grunwald, 1993]. A soluble 80 kDa form of E-cadherin shed by cultured epithelial cells was described by Wheelock et al. [1987] more than a decade ago. This form of E-cadherin could disrupt cell–cell adhesion. More recent work has shown that sE-cadherin promotes invasive behavior by cultured epithelial cells, perhaps by its ability to disrupt cell–cell adhesion [Noë et al., 2000]. A 90-kDa fragment of N-cadherin (sN-cadherin) is present in developing chick neural retina [Paradies and Grunwald, 1993]. When isolated, sN-cadherin binds cells, and when immobilized it promotes cell attachment, stimulates neurite outgrowth, and initiates intracellular signaling events. An 80-kDa fragment of P-cadherin is present in the serum of women with or without breast cancer [Knudsen et al., 2000]. In addition, a P-cadherin fragment was found in

human sperm lysates [Rufas et al., 2000], and P-cadherin was occasionally detected in a secretory pattern in prostatic tissue [Peralta Soler et al., 1997]. Shedding of VE-cadherin and E-cadherin from endothelial and epithelial cells, respectively, occurs during the induction of apoptosis [Herren et al., 1998; Steinhusen et al., 2001].

Thus, shedding of classical cadherins is a common theme. It occurs under normal and diseased conditions, and accompanies tissue remodeling. The mechanism and regulation of release of the cadherin extracellular domain fragment from the cell surface is not fully understood. However, it is clear that it involves proteases, although whether the enzymes are cadherin specific is not known. There is strong evidence that E-cadherin shedding results from the activity of metalloproteinases, including matrilysin and stromelysin-1, although additional proteases are probably involved as well [Noë et al., 2000].

Although sP-cadherin can be detected in serum, its level is 20-fold below that of sE-cadherin. This is not surprising considering that P-cadherin is expressed by a more restricted cell population than is E-cadherin, which is expressed by most, if not all epithelial cells. However, our observation that sP-cadherin is present at high levels in human milk was unexpected. Our immunohistochemical results suggest that P-cadherin expression is upregulated in the lactating mammary gland, compared to non-lactating tissue. Moreover, it appears to be expressed by luminal epithelial cells in the lactating gland, whereas its expression is restricted to myoepithelial cells in the non-lactating gland. Most surprisingly, the P-cadherin expression pattern in lactating gland is that of a secretory protein, rather than an adhesion protein. This is consistent with the 80 kDa sP-cadherin fragment being present at high levels in milk. Likely, the sP-cadherin in milk is generated by proteolytic cleavage of the extracellular domain of full-length P-cadherin, since no alternatively spliced forms of P-cadherin mRNA have been described [Shimoyama et al., 1989].

The generation of sP-cadherin in lactating mammary gland appears to be specific. That is, E-cadherin appears to be functioning in the expected fashion, as an adhesion protein. E-cadherin was detected by immunohistochemistry at sites of contact between luminal epithelial cells. It was not detected by immunohistochemistry in material present in the ducts of the lactating mammary gland, nor was sE-cadherin detected in milk. Since E-cadherin does not appear to be shed at high levels into milk, and there is no evidence for cadherin-specific metalloproteinases, it is possible that the P-cadherin is proteolytically processed in a vesicle inside the epithelial cells, rather than at the cell surface, and prior to its being secreted into the lumen of the duct. This idea is consistent with the immunohistochemical pattern, which shows P-cadherin staining that appears to be inside and at the apical surface of epithelial cells.

What, if any, function does sP-cadherin perform? If it has a function, it could be in the mammary gland itself. Perhaps the sP-cadherin acts as a signaling protein in luminal epithelial cells, while E-cadherin continues to play its customary role as an adhesion protein. This might play some role in optimizing the secretory function of the epithelium. On the other hand, sP-cadherin in milk might perform some function in the infant. It is unlikely that sP-cadherin in milk would readily encounter P-cadherin-expressing cells in the infant's mouth or gastrointestinal tract, since such cells are presumably basal to the E-cadherin-expressing epithelial cells. Therefore, a signaling role for sP-cadherin in the infant seems unlikely.

We also considered the possibility that sP-cadherin might play a role in preventing bacterial infection in the infant. Human E-cadherin has been shown to be a receptor for the food borne pathogen *Listeria monocytogenes* [Mengaud et al., 1996]. If like E-cadherin, P-cadherin was able to bind the bacteria, sP-cadherin might bind Listeria in the infant's gastrointestinal tract and thereby prevent infection of epithelial cells through E-cadherin. Although this seemed an attractive hypothesis, P-cadherin failed to bind *Listeria monocytogenes* [S. Sousa, M. Lecuit and P. Cossart, Institute Pasteur, personal communication].

In summary, we have observed that P-cadherin expression is upregulated in the normal human lactating mammary gland, compared to the non-lactating gland, and that it behaves like a secretory protein. We also have observed that an 80-kDa fragment of the extracellular domain of P-cadherin is found at a high concentration in milk, where it plays an unknown role.

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