Biochemical and Ultrastructural Features of Human Milk and Nipple Aspirate Fluids

Manuela Malatesta,¹ Ferdinando Mannello,^{1*} Giuseppe Bianchi,² Maurizio Sebastiani,³ and Giancarlo Gazzanelli¹

¹*Istituto di Istologia ed Analisi di Laboratorio, Facoltà di Scienze M.F.N., Università Studi, Urbino, Italy*

²*Laboratorio Analisi, ASL 2, Ospedale Civile, Urbino, Italy*

³*Centro di Senologia, AUSL 1, Pesaro, Italy*

Breast duct epithelium produces, secretes, and metabolises several biologically important compounds, which are found in breast secretions obtained in physiologic and pathologic conditions (milk and nipple aspirate fluids, respectively). In order to preliminarily evaluate the ultrastructural morphology of the cells found in Type II nipple aspirate fluids (NAF) and correlate it with the biochemical profile of the extracellular fluid present in these breast secretions and in human milk, we analyzed 72 NAFs from nonlactating premenopausal women affected by various breast diseases and 10 normal milk samples. Although several constitutive proteins were detected in all samples examined, the preliminary biochemical analyses and electrophoretic profiles revealed characteristic

behaviours for several biologic constituents, suggesting a possible basic mechanism of production by breast epithelial cells during both physiologic and pathologic conditions. The ultrastructural analysis of milk cellular components give preliminary evidence of the apocrine secretion mechanism peculiar of breast gland, whereas Type II NAF cells appeared as biosynthetically active cells, showing a possible modified secretion mechanism. Our multidisciplinary approach seems to support the hypothesis that cellular and biochemical behaviour of Type II NAF may be an useful tool to identify aberrated breast epithelial cells in nonlactating women that might be prone to premalignant transformation. *J. Clin. Lab. Anal.* 14:330–335, 2000. © 2000 Wiley-Liss, Inc.

Key words: biochemistry; breast secretion; electron microscopy; milk; nipple

The evaluation of the breast directly through analysis of either tissue, individual cells, or extracellular fluid generally requires a surgical biopsy. The utilisation of breast secretions represents instead a quick, repeatable, non-invasive method of obtaining both breast epithelial cells and extracellular fluid (1). In this respect, breast fluid has attracted considerable interest to assess the metabolic characteristics of the underlying mammary tissue (2,3).

Breast secretions may be obtained both in physiologic (as milk during lactation) and in pathologic conditions (as nipple aspirate fluid from breast diseases) (4). Previously, classification of the nipple aspirate fluids (NAF) has been proposed on the basis of their protein profiles (5): Type I NAF, found in healthy control women and in patients diagnosed with benign breast diseases, was characterized by the presence of proteins whose mobilities are identical to those of the major protein components detected in gross breast cyst fluids; Type II NAF, found in a high percentage of women affected by breast carcinoma, showed a polypeptide pattern in which peculiar proteins found both in human milk and breast tumour cytosol extracts.

The breast is unique among secretory glands of the human

body in which many substances in its secretions are retained, concentrated, and metabolized by breast epithelium (4); this peculiar secretory activity permits toxic, mutagenic, and carcinogenic substances, as well as hormones, proteins, and growth factors, to reach breast epithelium where they may be taken up and metabolically activated to produce direct/indirect intracellular effects on breast epithelium (2,6,7).

Because of the attractiveness of NAF as a non-invasive method for evaluating malignant breast diseases (1,8), we have undertaken this preliminary study in an attempt to correlate the ultrastructural morphology of the cells found in Type II NAFs with the biochemical profile of the extracellular fluid present in these breast secretions, comparing the features with those of milk, the physiologic female breast secretion.

Grant sponsor: Assessorato alla Sanità of the Regione Marche, Italy.

*Correspondence to: Dr. Ferdinando Mannello, Ph.D., D.Sc., Istituto di Istologia ed Analisi di Laboratorio, Facoltà di Scienze MFN, Via Zeppi, snc, 61029 Urbino PU, Italy. E-mail mannello@uniurb.it

Received 17 May 2000; Accepted 18 July 2000

MATERIALS AND METHODS

Samples

After informed consent was obtained, human breast milk samples were collected aseptically from 10 healthy lactating women and stored at -30°C until analysis (within 3 weeks). NAFs were obtained by manual compression of the four periareolar breast quadrants from 72 nonlactating women (21–42 years of age). With regard to breast pathology, there were 49 women with benign breast diseases (BBD), diagnosed on the basis of clinical, mammographic, echographic, and cytological studies as epithelial hyperplasia, sclerosing adenosis, macrocyst, intraductal papilloma, and periductal fibrosis. Women with infiltrating ductal carcinoma ($n = 8$) were diagnosed by surgical biopsy. In the study were also included 15 healthy control women who had no significant clinical findings or complaints referable to the breast. Women reporting pregnancy and lactation at least 2 years prior to the study as well as those who had been medically, hormonally, and/or surgically treated during the previous 6 months were also excluded from this study.

Biochemical Determinations

Milk and NAFs (from 1 to 500 μl) were thawed and centrifuged at 12,000g for 15 min at 4°C , and, after the top lipid layer was removed, the clear supernates were analysed. Electrolyte concentrations were measured by an ion-selective electrode technique (9). All biochemical analyses were performed utilizing commercially available kits with automated multiparametric analyzers (Boehringer Mannheim, Milan, Italy) (10).

Polyacrylamide Gel Electrophoresis

All samples were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels (1 mm thickness) at 50 mA for 45 min, after they had been treated with denaturing sample buffer in both nonreducing and reducing conditions (containing dithiothreitol and β -mercaptoethanol). Our protocols for running and staining minislab gels, followed throughout, have been detailed elsewhere (11).

Electron Microscopy

The extremely reduced sample volume of most NAFs, and consequently the low amount of epithelial cells, often made it impossible or problematic to use them in electron microscope analysis. For this reason, ultrastructural investigations were carried out on four well-preserved and representative samples of Type II NAF previously selected by homogeneous biochemical analyses. Immediately after collection, both milk and NAF samples were processed for electron microscopy as detailed elsewhere (10). Briefly, both milk and NAFs were fixed for 1 hr at 4°C in 0.1 mol of Sorensen phosphate-buff-

ered 5% glutaraldehyde and 4% paraformaldehyde, pH 7.4; the cells were post-fixed with 1% OsO_4 and 1.5% $\text{K}_4\text{Fe}(\text{CN})_6$ for 1 hr, washed again, and finally embedded in 1.5% agar agar. The samples were then dehydrated through graded concentrations of acetone and embedded in Epon 812. Ultrathin sections were observed in a Zeiss EM 902 electron microscope operating at 80 kV.

Statistical Analysis

All data, reported as the mean $\pm$ SEM value of at least three independent experiments, were statistically analysed by the Stat-View package, version 4 (Abacus Concepts, Berkeley, CA) on a Macintosh Power PC (Apple, Cupertino, CA). Significance was established at the $P < 0.05$ level.

RESULTS

According to the previously reported classification (5), through SDS-PAGE we recognized two main groups of NAF samples: Type I NAF (58 out of 72) detected in all healthy control women and in $\sim 90\%$ of women affected by BBD, and Type II NAFs (14 out of 72), found in all women bearing breast carcinoma and in $\sim 10\%$ of women affected by BBD. The polypeptide composition of Type I NAF was similar to that found in breast gross cystic fluid (10), whereas Type II NAFs showed an electrophoretic profile characterized by some constitutive proteins found in human milk as well as by polypeptides with peculiar electrophoretic mobility undetectable in milk (Fig. 1A). In fact, in human milk the main protein is represented by lactoferrin (M_r 80,000), whereas in Type II NAF the major protein band shows an electrophoretic mobility identical to albumin (M_r 67,000); on the other hand, other distinctive proteins were found in milk (with a peculiar molecular mass and electrophoretic mobility) that were undetectable in Type II NAFs.

The difference in protein profile between Type II NAF and milk was particularly evident when the SDS-PAGE was stained with enhanced silver stain (Fig. 1C). This procedure allowed us to detect in milk samples, in addition to the peculiar presence of β -casein, a protein doublet at M_r 15,000 and 14,000 whose electrophoretic mobility and behaviour to reducing agents were identical to lysozyme and α -lactalbumin, respectively. Moreover, the treatment of NAFs with reducing agents yielded more electrophoretic bands with higher molecular mass than those found in the absence of reducing agents, as well as the presence of additional protein bands, with electrophoretic mobility identical to the heavy and light chains of immunoglobulins; by contrast, some peculiar proteins of human milk were unaffected by treatment with reducing agents (Fig. 1B). This protein behaviour is probably due to the cleavage of disulfide-bridge-dependent polypeptide associations by reducing agents, thus resulting in an expanded random-coil structure of lower electrophoretic mobility than that of the structurally more

Fig. 1. Protein profiles of milk and NAF samples. Electrophoresis was carried out on 12% SDS-PAGE minislab gels and stained with Coomassie Brilliant Blue. The relative mobilities and molecular masses of standard proteins were expressed in kilodaltons (myosin, β -galactosidase, phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme). (A) Aliquots of human milk (lane 1) and Type II NAF (lane 2) were treated with denaturing nonreducing sample buffer. (B) Type II NAF and milk proteins were separated before (lanes 1 and 2) and after (lanes 3 and 4) treatment with denaturing sample buffer containing reducing agents. (C) Protein behaviour of Type II NAF and milk polypeptides separated as unreduced (lanes 1 and 2) and reduced (lanes 3 and 4) and then visualized with the enhanced silver stain.

compact, unreduced form of the specific protein. A further biochemical characterization of Type II NAF in comparison to milk revealed a different behaviour for selected biochemical analytes (Table 1).

The electron microscopic analysis revealed that the cellular material contained in milk seems to be strongly different from that found in our representative Type II NAFs. In fact, in milk we observed large amounts of membrane-bound vacuoles of various sizes and contents. These vacuoles always occurred as clusters, constituted by one or more vacuoles of remarkable size containing a weakly electron-dense core and a peripheral electron transparent layer, and by numerous small vacuoles containing either electron-dense or electron-transparent homogeneous material and surrounding the large vacuoles. These vacuoles clusters were found to be occasionally connected to cytoplasmic fragments containing organelles such as endoplasmic reticulum cisternae, ribosomes, and mitochondria (Fig. 2). No cell containing nuclei was found.

On the other hand, in our selected Type II NAFs we found erythrocytes and clusters of morphologically similar cells that were roundish in shape (Fig. 3A) with corrugated surface caused by numerous short cytoplasmic protrusions which could strictly interdigitate with those of the lining cells (Fig. 3D). The nuclei, generally one per cell and in central position, were very irregular in shape because of deep indentations; they appeared quite euchromatic and contained one or two reticulated nucleoli. Moreover, clusters of interchromatin granules and many perichromatin granules were clearly visible in the nucleoplasm. In the cytoplasm, the rough endoplasmic reticulum consisted of flattened cisternae of various lengths, while the smooth endoplasmic reticulum appeared as tubules and vesicles of different sizes. Large amounts of ribosomes appeared scattered throughout the cytoplasmic

TABLE 1. Biochemical profile of NAF and milk samples ($\bar{X} \pm \text{SE}$)

Analyte	Type II NAF (n = 14)	Milk (N = 10)
Na (mmol/l)	119.64 $\pm$ 8.82	83.89 $\pm$ 11.4
K (mmol/l)	9.53 $\pm$ 3.87	37.17 $\pm$ 4.77*
Total proteins (g/l)	37.88 $\pm$ 6.54	12.23 $\pm$ 5.93*
Albumin (g/l)	4.55 $\pm$ 1.33	0.52 $\pm$ 0.11*
Immunoglobulins (μ g/l)	8.11 $\pm$ 2.24	24.95 $\pm$ 6.82*
Lysozyme (g/l)	1.92 $\pm$ 0.36	0.49 $\pm$ 0.05*
Casein (g/l)	0.12 $\pm$ 0.02	3.76 $\pm$ 0.42*
Lactose (g/l)	11.43 $\pm$ 4.28	71.44 $\pm$ 7.27*
α -Lactalbumin (g/l)	1.94 $\pm$ 0.07	5.65 $\pm$ 0.86*
Prolactin (mg/l)	136.24 $\pm$ 9.55	63.94 $\pm$ 7.38*
Total cholesterol (g/l)	8.49 $\pm$ 3.65	0.24 $\pm$ 0.08*
Alk. Ph. (IU/l)	166.37 $\pm$ 31.43	95.45 $\pm$ 6.83*
LDH (IU/l)	972.14 $\pm$ 59.65	155.17 $\pm$ 12.49*
CEA (mg/l)	310.49 $\pm$ 19.45	23.48 $\pm$ 3.97*
PSA (μ g/l)	48.75 $\pm$ 9.23	3.23 $\pm$ 0.68*

**t*-test with $P < 0.05$ showing significant difference.

Fig. 2. Morphological aspect of cells found in human milk. A cellular fragment (C), containing many RER cisternae (arrows) and various membranaceous structures, is connected to a large secreting vacuole (asterisk), surrounded by numerous small vacuoles (bar = 1 μ m).

matrix (Fig. 3B), while the Golgi complexes, not numerous but well developed, were generally located in a perinuclear position. The smooth endoplasmic reticulum was abundant, appearing as tubules and vesicles of different sizes. The mitochondria were numerous, ovoidal and small in size, with transverse or longitudinal cristae (Fig. 3C). Clusters of large electron-transparent vacuoles frequently occurred in the inner cytoplasmic region, often surrounding the nucleus (Fig. 3B). A few lipid droplets and residual bodies were randomly distributed.

DISCUSSION

The human breast duct epithelium is able to produce and/or retain, concentrate, and metabolise several endogenous and exogenous compounds (i.e., hormones, enzymes, proteases, growth factors, pesticides, cholesterol epoxides, etc.) (4,12), which are present in breast fluids obtained both from physiologic (milk) and in pathologic (NAF) conditions (2). It has been suggested that the prolonged exposure of breast epithelial cells to high concentrations of these compounds, due to their relatively slow turnover (6), may lead to metabolic and

morphologic alterations through autocrine and/or paracrine mechanisms (3).

Previous studies on NAFs obtained from women affected by different breast diseases have revealed two peculiar NAF types, characterized by distinct biochemical composition: Type I, containing a protein profile similar to that found in breast gross cystic fluids (10), and Type II, characterized by a peculiar protein behaviour with some polypeptides detected also in human milk (5). Interestingly, Type II NAF has been predominantly found in women who diagnosed with malignant breast diseases (2,8,13).

The biochemical profile of Type II NAF obtained in the present preliminary study confirms the presence of some unidentified constitutive milk proteins (5,6), suggesting a probable analogous basic mechanism for production of these polypeptides by breast epithelial cells, according to a previous hypothesis (14). The preliminary finding of several unidentified peculiar proteins of "high breast cancer risk" Type II NAF (undetectable in milk) is also in agreement with previous observations (5,15). The isolation and characterization of these proteins, as well as the evaluation of their potential clinical utility, will be the scope of our further studies, currently in progress in our laboratories.

However, Type II NAF contains significantly higher concentrations of some enzymes, proteases and tumour-related antigens than those found in human milk (Table 1), suggesting that possible metabolic alterations in ductal breast epithelial cells may occur in women affected by breast diseases (13).

In fact, it has been previously suggested that the prolonged exposition of breast epithelial cells to high concentrations of biologically active compounds present in NAFs, mainly due to their relatively slow turnover (6), may lead to both metabolic and morphologic alterations through autocrine and/or paracrine mechanisms, similarly to those prone to breast cancer (16). Accordingly, the electron microscope analysis of cellular component of representative milk and Type II NAF samples reveals quite different morphological patterns. In fact, in our milk samples only secreting vacuoles and cellular fragments devoid of nuclei have been found, giving evidence of the apocrine secretion mechanism, peculiar of the normal breast gland. On the other hand, in our representative Type II NAF samples we found clustered intact cells showing morphological characteristics typical of highly metabolizing cells: the well-developed rough endoplasmic reticulum and Golgi complexes as well as the large amount of free ribosomes reveal a remarkable protein synthesis and processing rate. Our preliminary observation is in agreement with previous findings of a high protein and protease content in Type II NAF (5,6,9,14). Moreover, according to our preliminary biochemical observations and the previously reported data (2,13), the well-developed smooth endoplasmic reticulum suggests that an active lipidic metabolism may also occur.

Fig. 3. Ultrastructural characteristics of cells found in Type II NAFs. (A) The cells show an irregularly shaped nucleus (N) and a cytoplasm rich in electron-transparent vacuoles (V). The cell surface is characterized by numerous short cytoplasmic protrusions (arrow) (bar = 1 μ m). (B) In the cytoplasm are visible many RER cisternae (arrowheads), a well-developed Golgi complex (arrow) and numerous smooth tubules and vacuoles (thin arrows) (bar = 0.5 μ m). (C) Numerous small mitochondria (arrows) and

In the nucleus, the presence of chromatin, mostly occurring in its dispersed potentially active form, as well as of reticulated nucleoli, typical of metabolically active cells (17), strongly suggests high RNA synthesis and processing rates, according to previous studies on NAF cells (1,8). Accordingly, the irregular nuclear shape, increasing the nucleus–cytoplasmic interface, could facilitate the intense molecular trafficking between the two cellular compartments. Finally, the presence of numerous mitochondria is probably related to the energy needs due to the high rate of metabolic activity.

Taken together, our preliminary biochemical data and ultrastructural feature on selected Type II NAF samples allow us to hypothesize that the normal secretion mechanism of breast epithelial cells could undergo to a substantial modification; in particular, the absence of cellular fragments suggests the lack of an apocrine secretion mechanism, probably substituted by a merocrine mechanism. Moreover, the presence of cells associated in clusters could be related to a high rate of exfoliation of breast ductal epithelial cells, probably due to the intense proliferative activity observed in some pathologic conditions (3,10,16).

Our preliminary study suggests that Type II NAF might originate from hyperproduction followed by abnormal secretion by biosynthetically active cells of the terminal breast ductal–lobular unit, through molecular mechanisms probably absent in physiologic condition (1,3,10). This hypothesis could support the notion that Type II NAFs may represent useful tools to identify the biosynthetically active stage of aberrated breast ductal–lobular units (8). This behaviour could also be a hormone-mediated response, as demonstrated in several pathological mammary conditions (6,13,14).

In conclusion, during the development of the breast diseases, the lack of the physiologic control of secretion/reabsorption mechanism (4) and/or the prolonged exposition to the action of several (exogenous and/or endogenous) biologically active compounds through autocrine/paracrine mechanisms (12,13,16) may lead with age to the aberration of breast epithelial cells (8). In this respect, more detailed biochemical/molecular and ultrastructural analyses of NAFs could be adjunctive promising non-invasive methods to identify women at higher risk of neoplastic transformation (1).

Studies are in progress to expand our preliminary findings, in order to characterize the peculiar proteins found in “high risk” Type II NAF samples and to demonstrate their potential clinical utility.

large amounts of free ribosomes (arrowheads) are present in the cytoplasm (bar = 0.5 μ m). (D) The short cytoplasmic protrusion occurring along the cell surface appears to be strictly interdigitated (arrows) in clustered cells (bar = 0.5 μ m).

REFERENCES

1. Sauter ER, Ross E, Daly M, Klein-Szanto A, Engstrom PF, Sorling A. Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk. *Br J Cancer* 1997;76:494–501.
2. Petrakis NL. Nipple aspirate fluids in studies of breast disease. In: Srivastava S, Lippman SM, Hong WK, and Mulshine JL, editors. *Early detection of cancer: molecular markers*. Armonk, NW: Futura Publishing Co.; 1994, p 217–225.
3. Petrakis NL. Studies on the epidemiology and natural history of benign breast disease and breast cancer using nipple aspirate fluid. *Cancer Epidemiol Biomarkers Prev* 1993;2:3–10.
4. Haagensen CD. *Diseases of the breast*. Philadelphia: W.B. Saunders & Co.; 1990.
5. Sánchez LM, Vizoso F, Díez-Itza I, López-Otín C. Identification of the major protein components in breast secretions from women with benign and malignant breast diseases. *Cancer Res* 1992;52:95–100.
6. Petrakis NL. Physiologic, biochemical and cytologic aspects of nipple aspirate fluid. *Breast Cancer Res Treat* 1986;8:7–19.
7. Petrakis NL, Lim ML, Miike R, Lee RE, Morris M, Lee L, Mason L. Nipple aspirate fluids in adult nonlactating women: lactose content, cationic Na, K, and Na/K ratio, and coloration. *Breast Cancer Res Treat* 1989;13:71–78.
8. Sauter ER, Ehya H, Babb J, Diamandis EP, Daly M, Klein-Szanto A, Sigurdson E, Hoffman J, Malick J, Engstrom PF. Biological markers of risk in nipple aspirate fluid are associated with residual cancer and tumour size. *Br J Cancer* 1999;81:1222–1227.
9. Mannello F, Malatesta M, Sebastiani M, Gazzanelli G. Molecular forms and ultrastructural localization of prostate-specific antigen in nipple aspirate fluids. *Clin Chem* 1999;45:2263–2266.
10. Malatesta M, Mannello F, Sebastiani M, Cardinali A, Marcheggiani F, Gazzanelli G. Ultrastructural characterization and biochemical profile of human gross cystic breast disease. *Breast Cancer Res Treat* 1998;48:211–219.
11. Mannello F, Troccoli R. Improved electrophoretic map of breast cyst fluid proteins. *Cancer Detect Prev* 1992;16:107–112.
12. Rose DP. Hormones and growth factors in nipple aspirates from normal women and benign breast disease patients. *Cancer Detect Prev* 1993;16:45–51.
13. Sartorius OW. The biochemistry of breast cyst fluids and duct secretions. *Breast Cancer Res Treat* 1995;35:255–266.
14. Vizoso F, Sánchez LM, Díez-Itza I, Tuya AF, Ruibal A, López-Otín C. Relationship between serum prolactin levels and protein composition of breast secretions in nonlactating women. *J Clin Endocrinol Metab* 1994;79:525–529.
15. Schultz GS, Ebner KE. α -Lactalbumin levels in human mammary tumors, sera, and mammary cell culture lines. *Cancer Res* 1977;37:4489–4492.
16. Osborne CK, Arteaga CL. Autocrine and paracrine growth regulation of breast cancer: clinical implications. *Breast Cancer Res Treat* 1990;15:3–11.
17. Schwarzscher HG, Wachtler F. The nucleolus. *Anat Embryol* 1993;188:515–536.