ABSTRACT

The protein composition of breast secretions from 99 premenopausal women with benign or malignant breast diseases and from 70 control women without breast pathologies has been studied by using polyacrylamide gel electrophoresis. These fluids have been classified into two types according to their major polypeptide components. Type I fluids are defined by three major distinctive bands at Mr 44,000, 24,000, and 17,000, while those designated Type II present distinctive bands at Mr 80,000, 15,000, and 14,000. Amino acid sequencing and immunoblotting analysis demonstrated that proteins in Type I secretions correspond to Zn-α2-glycoprotein, apolipoprotein D, and gross cystic disease fluid protein-15, while those from Type II fluids have been identified as lactoferrin, lysozyme, and α-lactalbumin. Most women (93%) without breast pathology and most patients (88%) with benign diseases had secretions with a Type I polypeptide pattern. By contrast, a large percentage (57%) of secretions from women with breast carcinoma presented a Type II protein pattern. Further studies with a large number of women will be useful for corroborating the potential clinical interest of breast fluid protein analysis.

MATERIALS AND METHODS

INTRODUCTION

The prevention of breast cancer might be improved by the availability of biochemical markers for detecting the early steps of the disease as well as for discriminating high-risk mastopathies from benign lesions. One approach to identifying these markers is to study the composition of the breast secretions of nonlactating women. In recent years, based on the hypothesis that breast fluid should be indicative of the environment and metabolic activity within the mammary tissue, several groups have investigated the cytological and biochemical constituents of fluid aspirated from the nipple of nonpregnant women (1–19). The cytological studies of these secretions have revealed the presence of alterations in epithelial cells from women with benign and malignant breast diseases (1, 7). In addition, it has been found that women whose breast fluid cytology was reported as atypical hyperplasia are at a 3-fold greater risk of developing breast cancer than the women who did not show atypia (14).

In relation to the biochemical composition of the breast fluid, data are available for the levels of a wide variety of steroid hormones (2, 5, 8–12), lipids (10), cholesterol and cholesterol epoxides (13, 16), lactose (17), and proteins (10, 15). The latter analyses have revealed the presence of all normal classes of serum proteins. However, in spite of the suggestion that some of these proteins could be involved in the development of breast diseases, very little is known about the polypeptide composition of the fluid secretions of nonlactating women.

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2 To whom requests for reprints should be addressed.

In the course of studies directed toward the biochemical characterization of cyst fluid from women with gross cystic disease of the breast, we have found that apoD2 is the major protein component present in the fluid (20). This observation as well as data indicating that this protein (previously designated GCDFP-24) could be a marker of breast tumors in an early stage of development and/or with a low metastatic potential (21) prompted us to search for the presence of apoD in the breast fluid obtained from women with diverse breast diseases.

In this work, we report that breast fluids can be classified into two types according to their polypeptide composition, apoD being one of the distinctive proteins. In addition, we have identified all the major protein components of both types of secretions obtained from women with benign and malignant breast diseases. The possible implications of these findings for differential diagnosis of benign and malignant breast diseases are also discussed.

1To whom requests for reprints should be addressed.
Protein Purification. ApoD, Zn-α2-gp, and GCDFP-15 were purified from the cyst fluid of women with gross cystic breast disease, as a preliminary step for subsequent antisera production. We used the same high-performance liquid chromatography procedure previously described for the purification of apoD (20), with minor modifications directed at improving the chromatographic resolution of the system. Thus, cyst fluid containing about 2 mg of protein was applied to a TSK-3000 SWG column (2.15 x 30 cm) equilibrated and eluted at a constant flow rate of 0.1 ml/min with 0.1 M ammonium acetate, pH 5.0, containing 0.1% SDS. The samples were run at room temperature with a high-performance liquid chromatograph (Waters, Milford, MA) equipped with a model 481 UV detector and two model 510 solvent delivery systems. Measurements were performed at 280 nm in the sensitivity range of 2.0, and 1.0-ml fractions were collected for further analysis by SDS-PAGE.

Antisera Production. Antisera against the proteins purified as described above were produced from New Zealand White rabbits according to the method described by Vaitukaitis (23). The animals received intradermic injections at multiple sites with 50 µg of purified proteins dissolved in phosphate-buffered saline and emulsified with an equal volume of Freund’s complete adjuvant. The rabbits were bled 6 weeks after the injection. After removal of the cells, antisera were divided in aliquots (1–2 µl) of fluids expressed from the nipple of these patients were analyzed by SDS-PAGE. Two different patterns of protein bands were recognized (Fig. 1). The first group, arbitrarily designated Type I, was characterized by the presence of bands whose mobilities (Mr, 67,000, 44,000, 24,000, and 17,000) were identical to those of the major protein components present in cyst fluid from women with gross cystic breast disease. The second group of breast secretions, Type II, contained major bands at Mr, 80,000, 67,000, 15,000, and 14,000. This pattern was closely related to the one obtained by SDS-PAGE of samples of human milk. Moreover, if samples were treated with reducing agents, two additional bands at Mr, 56,000 and 23,000 were detected in all cases. The intensity of some bands was variable in different samples (Fig. 1); however, all three bands characteristic of each of the two patterns were always detected as major protein components in each sample.

Immunological Characterization of Proteins from Breast Secretions. According to SDS-PAGE, the distinctive proteins in Type I breast fluids are closely related to those found in cyst fluid from women with gross cystic breast disease, which have been identified as Zn-α2-gp (26), apoD (20), and GCDFP-15 (26). To investigate the possible identity of proteins from both sources, we raised antisera against purified cyst fluid proteins, which could be further used for Western blot analysis. Thus, proteins in cyst fluid with a low albumin content (27) were separated by size-exclusion high-performance liquid chromatography in the presence of SDS, and the result obtained is shown in Fig. 2a. Analysis by SDS-PAGE of the fraction corresponding to an elution volume of 45 ml revealed that this material represents molecular aggregates of the major protein...
components present in the fluid. These aggregates could be dissociated by extensive heating of the samples with a buffer containing 2-mercaptoethanol and SDS. On the other hand, the SDS-PAGE analysis showed that fractions corresponding to elution volumes of 50–51, 54–55, and 60–61 ml were highly enriched in Zn-α2-gp, apoD, and GCDFP-15, respectively. Each of these pairs of fractions was rechromatographed under the same conditions that resulted in single peaks in all three cases (Fig. 2, b-d). The subsequent SDS-PAGE analysis showed that these peaks contained the above-mentioned proteins in a pure enough form for the generation of specific antibodies against each of them. About 50 μg of purified Zn-α2-gp, apoD, and GCDFP-15 were used to immunize rabbits, and the resulting antisera were used for Western blot analysis. The results obtained in representative experiments are shown in Fig. 3. Antisera against Zn-α2-gp, apoD, and GCDFP-15 reacted with the major bands at Mr 44,000, 24,000, and 17,000 present in Type I breast fluids. Moreover, antisera against these three proteins did not recognize any band in Type II breast fluids (data not shown). A similar strategy was used to demonstrate that major bands in Type II breast secretions correspond to proteins present in human milk. Thus, antiserum against whole milk proteins reacted with the bands present in breast secretions of this type. Moreover, specific antisera against lactoferrin and α-lactalbumin strongly reacted with bands at Mr 80,000 and 14,000, respectively (Fig. 3).

Amino Acid Sequencing of Proteins from Breast Secretions. In order to provide supporting data for the above immunological results, we tried to obtain information regarding the NH2-terminal sequence of proteins from breast secretions. The limited amount of protein available from these breast fluids hampered the gathering of this structural information. This problem was finally overcome by using a microsequencing procedure which involves the blotting of electrophoretically separated proteins onto Immobilon membranes and direct protein sequencing from the relevant bands. The results obtained in the different sequential degradations of proteins present in the two types of breast secretions are shown in Table 1. Interestingly, no amino acids could be identified in at least two separate runs of the major proteins (Mr 44,000, 24,000, and 17,000) in Type I fluids, suggesting that these proteins were blocked at their NH2-terminal end. This result would be in accordance with previous data indicating that Zn-α2-gp (28) and apoD (29) purified from plasma show cyclized glutamine as a NH2-terminal residue. Therefore, in order to provide further confirmation of the identity of the major breast fluid proteins (Mr 44,000 and 24,000), aliquots of breast fluid from patients with breast carcinoma or fibrous mastopathy were treated with pyroglutamate aminopeptidase and then subjected to SDS-PAGE, blotting, and direct sequencing. The resulting sequences in both cases (Table 1) were identical with those reported for Zn-α2-gp (28) and apoD (29), respectively. In addition, a clear amino acid sequence was detected in the band at Mr 17,000 (Table 1). This sequence was identical to the one reported after a glutamine residue located at position 29 in GCDFP-15 (26), indicating that this protein has also cyclized glutamine as a NH2-terminal residue, which is released by treatment with pyroglutamate aminopeptidase. The sequencing analysis of proteins present in Type II breast fluids allowed the identification of at least nine amino acid residues in each of them. In all cases, the NH2-terminal sequences were identical to those corresponding to human milk proteins with the same molecular masses (Table 1). In addition to these proteins characterized as distinctive in each type of secretion, the Mr 67,000 protein present in both types of fluids was identified as albumin by amino acid sequencing. Moreover, if samples were treated with reducing agents, the two additional bands at Mr 56,000 and 23,000 present in all cases were also identified by amino acid sequencing as heavy and light chains of immunoglobulins, respectively (data not shown).

Analysis of Protein Patterns in Breast Fluids from Women with Different Breast Pathologies. A systematic analysis of samples obtained from control women and from patients with different benign and malignant mastopathies revealed that the Type I pattern was found in all breast fluids obtained from patients diagnosed with duct ectasia, fibroadenoma, and gross cystic breast disease. The same pattern was also present in 49 of 58 fluids obtained from patients with fibrous mastopathy. The other samples from this latter group showed Type II pattern (three cases) or a mixed pattern (six cases) characterized by the presence of proteins from both types (Table 2). In addition, most breast secretions (65 of 70) from control women without breast diseases showed the Type I pattern. By contrast, only 9 of 21 fluids from women diagnosed with breast carcinoma presented with the Type I pattern, while the remaining 12 showed Type II. These results indicate that Type II secretions are significantly associated with breast cancer (P < 0.001; χ² test with Yates’ correction).

DISCUSSION

In recent years a number of cytological and biochemical studies of breast fluid aspirated from nonlactating women have been used to better understand the natural history of benign
Table 1 Amino-terminal sequences of distinctive proteins from Type I and Type II breast secretions

<table>
<thead>
<tr>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (kDa)</td>
<td>Sequence</td>
</tr>
<tr>
<td>44</td>
<td>ENQDGRYSLTY</td>
</tr>
<tr>
<td>24</td>
<td>AFHLGKXPNP</td>
</tr>
<tr>
<td>17</td>
<td>DNTRKHHKNN</td>
</tr>
</tbody>
</table>

*a* Proteins were identified by a search of the National Biomedical Research Foundation Data Bank.

Table 2 Distribution of breast secretions on the basis of their protein pattern

<table>
<thead>
<tr>
<th>Breast pathology</th>
<th>Type I</th>
<th>Type II</th>
<th>Mixed type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>65</td>
<td>5</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Duct ectasia</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Gross cystic disease</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Fibrous mastopathy</td>
<td>49</td>
<td>3</td>
<td>6</td>
<td>58</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>9</td>
<td>12</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

and malignant breast diseases. However, the collection of data on the polypeptide composition of these breast secretions has been hampered by the small amounts of fluid which can be obtained from most women. In this work, and on the basis of polyacrylamide gel electrophoresis, we present evidence that breast secretions can be subdivided into two types according to their polypeptide composition. In addition, the use of microsequencing techniques has allowed us to identify the major protein components of both categories of breast fluids obtained from women with benign and malignant breast diseases.

After the finding that apolipoprotein D is the major protein component in the cyst fluid of women with gross cystic breast disease (20), the present work was initially aimed at evaluating the possibility that this protein could also be present in breast secretions from women with different breast diseases. The analysis by SDS-PAGE of a significant number of fluids showed clear differences in the concentration of a protein with electrophoretical mobility indistinguishable from that corresponding to apoD. The finding that these variations could also be extended to the concentration of other proteins present in the breast fluid prompted us to consider the existence of distinctive patterns in the polypeptide composition of breast secretions. We first established two separate categories of fluids. Those designated Type I can be characterized by the presence of distinctive proteins with molecular masses of 44,000, 24,000, and 17,000 kDa, which have been identified as Zn-α2-gp, apoD, and GCDFP-15. These proteins are also the major components in cyst fluid from women with gross cystic breast disease. On the other hand, Type II secretions presented as distinctive proteins those with molecular masses of 80,000, 15,000, and 14,000 kDa, identified as lactoferrin, lysozyme, and α-lactalbumin. Thus, these secretions are closely related to human milk, although they lack significant amounts of β-casein.

The existence of these two different types of secretions could be the consequence of differentiated hormonal stimulation of the mammary epithelium. In relation to this, it is worthwhile mentioning that apoD, Zn-α2-gp, and GCDFP-15 are among the very few proteins which are induced by androgens in human breast cells (30—32). Therefore, it is tempting to speculate that androgens could be the physiological steroids directly involved in the production of Type I breast fluid secretion. On the other hand, the presence of Type II fluids whose composition resembles human milk could be associated with an increased stimulation of the breast epithelium by prolactin. However, since it is well known that the activity of the mammary gland involves complex interactions between many different hormones, other possibilities like inhibitory effects of estrogens on apoD and GCDFP-15 expression (33, 34) should not be excluded.

After proving that breast secretions can be subdivided into different types on the basis of their polypeptide composition,
we tried to establish a correlation between fluid types and the different breast pathologies. Since a very little amount of fluid (less than 1 μl) was sufficient for SDS-PAGE analysis, we were able to type breast secretions from a total of 169 women of 240 in which attempts were made to collect fluids. Interestingly, most non-secretor women (58 of 71) were found among those without breast pathologies. The results obtained show that the Type I breast secretion is present in most control women (93%) as well as in most patients (88%) with benign diseases like fibroadenoma, gross cystic disease, duct ectasia, and fibrous mastopathy. On the other hand, Type II secretion was found in a high percentage (57%) of women with breast carcinoma. It is also remarkable that the three patients with benign diseases and the Type II pattern have presented a histological diagnosis of atypical hyperplasia or sclerosing adenosis, which is indicative of an increased risk of breast cancer (35). Taken together, these results suggest that the Type II pattern is indicative of premalignant or malignant diseases.

The occurrence of Type I breast fluids in some breast carcinomas could be due to the existence of a subset of tumors which retain the ability to synthesize and secrete those proteins characteristic of this pattern (Zn-α2-gp, apoD, and GCDFP-15). In relation to this, it is remarkable that four independent groups have demonstrated the presence of these proteins in about 40–60% of breast carcinomas (36–39), which is in the same range as reported here for breast cancer secretions. The possibility of apocrine differentiation in tumors with Type I secretion was carefully considered, since previous reports indicate that proteins present in these fluids are potential markers of apocrine activity (40, 41). However, we could not confirm such an association, which agrees with recent data from Hurlimann and van Melle (39) for breast tumors expressing Zn-α2-gp. Interestingly, these authors have found that tumors expressing Zn-α2-gp are associated with a favorable evolution. According to these data, it is tempting to speculate that Type I breast fluids could be indicative of this specific subset of carcinomas with good prognosis.

Finally, these results on the association of breast secretion patterns with breast pathologies, although stimulating, should be considered preliminary, since it seems clear to us that studies with a large number of patients should be required to corroborate their possible clinical significance. Similarly, it seems reasonable to conclude that clinical follow-up of women without breast cancer and with Type II fluid could confirm an increased risk for malignant transformation in this subgroup of women. Further studies with a large number of women are in progress to progress the usefulness of breast fluid protein analysis as a rapid and noninvasive method for the screening and prevention of malignant breast diseases.

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PROTEINS IN BREAST SECRETIONS


Identification of the Major Protein Components in Breast Secretions from Women with Benign and Malignant Breast Diseases

Luis M. Sánchez, Francisco Vizoso, Irene Díez-Itza, et al.


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