Production of Fibronectin by Normal and Malignant Human Mammary Epithelial Cells

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ABSTRACT

The production and retention of fibronectin by primary cultures of cells derived from the human breast has been analyzed. Two examples of each of the following cell types were examined: (a) normal epithelium from milk; (b) metastatic breast cancer cells in pleural effusions; (c) fibroblasts; (d) tissue macrophages of milk. Cell-associated fibronectin could be detected by indirect immunofluorescent staining on normal and malignant mammary epithelium and on mammary fibroblasts, but not on milk macrophages. Immune precipitation followed by gel electrophoresis of 35S-labeled cell lysates and conditioned medium confirmed that fibronectin was indeed synthesized by both types of epithelial cells and by fibroblasts, but not by macrophages, and that much of the protein was released into the medium. Quantitative analysis with radioimmune assay of the fibronectin on cells and in media showed that both normal and malignant epithelial cells synthesized levels of protein comparable to that produced by fibroblasts, but only a small fraction (<10%) of the material synthesized was retained by the cells. Growth on collagen-coated plastic increased the percentage of fibronectin retained by normal and malignant epithelium but did not affect retention by fibroblasts.

INTRODUCTION

Many behavioral properties of mammalian cells are related to the structure and function of their membrane. In particular, the change to malignancy (i.e., metastatic growth) is likely to be related to alterations in the cell membrane, which may affect the interaction of the cell with hormones and growth factors and with heterologous and homologous cells (14, 23). Fibronectin, a large glycoprotein found associated with the extracellular matrix and surface membrane of cultured fibroblasts and in basement membranes in vivo (40), is implicated in the adhesion of cells to each other and to a substrate (8, 18, 24). Since it was observed that many transformed fibroblasts show a reduced level of cell-associated fibronectin (11, 13, 15, 27, 36), considerable interest has been focused on this protein, its possible role in normal cell adhesion (35, 41), and its use as a marker for transformation (4, 22, 36).

Much of the work related to fibronectin has been done in fibroblast model systems, although other cell types have been shown to produce this protein in culture (10, 12, 21, 26, 30, 38, 39). The major solid malignant tumors in humans are carcinomas which originate from epithelial cells, and it would be important to determine whether fibronectin is produced by human epithelial cells and whether cell-associated fibronectin is reduced in the corresponding cancer cells, particularly in those showing metastatic growth. Studies following the presence of fibronectin on malignant human epithelial cells, as revealed by indirect immunofluorescent staining, have been restricted to cell lines (22, 30). Such studies have suggested that cells derived from metastatic tumors have less fibronectin associated with their surfaces than do cells derived from normal tissues or primary tumors (30). However, cells which have been in culture for several years may have changed considerably by adaptation or selection and cannot be taken as representative of the original cell type. Moreover, estimates of the level of fibronectin produced and the amount retained by the cells have not been made. A quantitative analysis of the production and retention of fibronectin by primary cultures of human epithelial cells and their malignant counterparts would perhaps provide important information relevant to the role of this protein in epithelial cell function and in the development of carcinomas.

We have made such an analysis for human mammary epithelium, using cells cultured from milk as representative of normal epithelium and cells cultured from pleural effusions taken from breast cancer patients as representative of malignant metastatic cancer cells. For comparative purposes, fibronectin production and release by fibroblasts from the human mammary gland have also been examined.

MATERIALS AND METHODS

Cells

Normal Human Mammary Epithelial Cells. These were cultured from early-lactation milks (2) together with the macrophages found in milk (33, 34). Cells from roughly 20 ml of milk were cultured in a 5-cm dish. After 2 weeks in culture, the epithelial cells form an almost confluent layer, and most of the macrophages have detached.

Milk Macrophages. To obtain pure cultures of the tissue macrophages found in milk (1, 3, 33), the cells obtained from approximately 20 ml of milk were allowed to adsorb to 5-cm plastic dishes for 10 to 15 min. The supernatant was then removed, and the adherent cells, which are the macrophages, were retained.

Malignant Human Mammary Epithelial Cells. The 2 samples of breast cancer cells cultured here came from pleural effusions taken from 2 patients at the ICRF Breast Cancer Unit at Guy’s Hospital. The cells in the effusion were collected by centrifugation, resuspended in medium containing 20% serum and 10% dimethyl sulfoxide, and frozen in liquid N2. For culture, one vial containing 10⁶ clumps of cells was split between four 5-cm plates.

Human Mammary Fibroblasts. These were isolated from reduction mammoplasty tissue by enzyme digestion and separated from epithelial cells as described previously (33).
Culture of Cells

Quantitative Determination of Fibronectin Production by RIA. All 4 cell types (normal and malignant mammary epithelium, tissue macrophages of milk, human mammary fibroblasts) were cultured in 5-cm dishes in the same medium, namely Roswell Park Memorial Institute medium containing hydrocortisone (5 μg/ml), 15% FCS, and 10% human serum. Where dishes were coated with collagen, the same technique was used as described previously (33). Both FCS and human serum had been passed twice through a column of gelatin-Sepharose to remove fibronectin (8). RIA showed the FCS to contain no detectable fibronectin, and the human serum contained less than 0.3 μg/ml. Medium was changed every 4 days, and when the dishes were approximately three-fourths confluent, i.e., approximately 2 weeks after seeding, the medium was again changed, and cells and supernatant were harvested 3 days later for RIA. Cells were collected by scraping into 1 ml of buffer containing 8 M urea, 1% Triton X-100, 0.2% sodium azide, and 1 mM phenylmethylsulfonyl fluoride (26).

Indirect Immunofluorescent Staining with Anti-Fibronectin Serum. Cells were grown as described for use in the RIA assay, except that coverslips were placed on the bottom of the 5-cm dishes; these were removed after 2 weeks when the cultures were three-fourths confluent and stained as described below.

Labeling of Fibronectin with [35S]Methionine. Cells were grown as described above, but using untreated serum (i.e., which had not passed through a column of gelatin-Sepharose to remove fibronectin), and when three-fourths confluent were labeled for 2 days with [35S]methionine (50 μCi/dish) in Roswell Park Memorial Institute medium containing only 0.03 g of methionine per liter and 5% FCS. Medium and cells were harvested as described above.

Purification of Fibronectin and Production of Antiserum

Fibronectin was isolated from human plasma by 2 cycles of affinity chromatography on gelatin-Sepharose as described by Engvall and Ruoslahti (8). The protein was eluted from the column with 8 M urea in 0.05 M Tris buffer, pH 7.2; dialyzed; lyophilized; and redissolved in 10 mM CAPS buffer, pH 11, containing 1 mM CaCl₂ and 0.15 M NaCl for injection (14). Iodination of the purified material followed by gel electrophoresis in 5% urea gel showed it to be a single component with a molecular weight of about 200,000 (see Chart M). A rabbit was given 2 injections (3 weeks apart) of 300 μg of fibronectin in 4 sites s.c. with Freund's adjuvant. The serum from blood taken 3 weeks after the last injection was titrated against iodinated fibronectin, and 1 ml was found to precipitate 0.3 mg of purified protein.

Radioimmune Assay for Fibronectin

Chart 1B shows a standard curve for fibronectin using 0.008 μl of serum to precipitate 5 ng of ¹²⁵I-fibronectin. The range which is detected with accuracy is 2 to 30 ng. Fibronectin does not dissolve readily unless at high pH or in urea. To obtain a solution in PBDSA of known concentration for iodination or for standard curve determinations, fibronectin was sonicated in PBSSA, the solution was centrifuged, and the amount of protein dissolved was estimated by the method of Lowry et al. (20). Antiserum was incubated overnight at 4°C with the various samples of cold fibronectin (final volume, 400 μl in dilution buffer (i.e., PBSA containing 0.13% Nonidet P-40, 0.04% azide, bovine serum albumin (2 mg/ml), and 40 μg of rabbit IgG)). ¹²⁵I-Fibronectin (5 ng, approximately 30,000 cpm) was then added, and the mixture was incubated for a further 24 hr overnight at 4°C. Goat anti-rabbit IgG (40 μg in 20 μl) was added to precipitate the complex. After 24 hr at 4°C, the precipitate was centrifuged and washed with dilution buffer, and radioactivity was estimated. For estimation of fibronectin produced by cells, culture media or diluted cell lysates were added instead of cold fibronectin. The amount of lysate required for estimation of fibronectin was small, and the final concentration of urea was less than 0.1 M. Collagen has been shown not to interfere with the assay (28).

Indirect Immunofluorescent Staining of Cells

Cells or coverslips were fixed in methanol containing 5% acetic acid and then washed and incubated at room tempera-
Fibronectin and Human Mammary Epithelial Cells

RESULTS

Cell Types Cultured from Human Mammary Gland. Normal epithelial cells can be cultured from human milk (2) which contains, in addition, large quantities of tissue macrophages (1, 3, 34). If the epithelial cells are separated from the macrophages, their growth is much improved by cocultivation with killed fibroblast feeders (33). The macrophages themselves, however, can serve as feeders; in the work reported here, unfractionated milk, containing epithelial cells and macrophages, has been cultured to avoid the complication of added fibroblasts which produce large amounts of fibronectin. As will be seen below, the tissue macrophages in milk do not produce fibronectin in culture and therefore can be cocultured with epithelial cells to be assayed for production of this protein. After 2 weeks in culture, more than 90% of the macrophages had detached from the glass and the remaining epithelial cells cover more than three-fourths of the surface of a 5-cm dish under the conditions used here (Fig. 1B). The cells grown from milk have been characterized as epithelial by their ultrastructure (34) and by their positive reaction with a specific antisem (anti-HME) against milk fat globule membrane components (3).

The breast cancer cells studied in this investigation were cultured from pleural effusions taken from breast cancer patients with metastatic disease. In our experience, breast epithelial cells (as characterized by anti-HME serum) can be cultured from 90% of pleural effusions taken from breast cancer patients (31). The 2 samples studied here (PE-7 and PE-25) were selected because the pathologist reported a high percentage of malignant cells, and examination of the cells after 2 weeks in culture showed that more than 80% stained positively with anti-HME serum. PE-7 also produced casein when tested at passage 3. The cells have been used here in primary culture as described in "Materials and Methods," and their appearance under phase is shown in Fig. 1, C and D.

Mammary fibroblasts isolated from reduction mammaplasty tissue were cultured for comparative purposes and are also shown in Fig. 1A.

Indirect Immunofluorescent Staining with Anti-Fibronectin Serum of Cells Cultured from the Human Mammary Gland. In order to determine qualitatively whether fibronectin could be detected on the various cell types derived from the breast, they were stained using indirect immunofluorescence. Normal and malignant human mammary epithelium, mammary fibroblasts, and milk macrophages were cultured in medium containing serum from which fibronectin had been removed by adsorption to Sepharose-gelatin (see "Materials and Methods") and were stained by indirect immunofluorescence with the anti-fibronectin serum. Macrophages, cultured either separately or with epithelial cells, were completely negative (Fig. 2, E and F), and breast fibroblasts showed the typical fibrillar network staining positively with anti-fibronectin serum (Fig. 2, G and H).

Both normal and malignant mammary epithelial cells showed some fluorescence after staining. The milk epithelial cells showed a diffuse staining, which tended to be perinuclear (Fig. 2, A to D). Staining was not more intense at the cell boundaries, and the network similar to that seen in fibroblast cultures was not apparent; although where cell processes could be seen to stretch out from the cells, they showed a stronger fluorescence (Fig. 2, C and D). All of the cells did not stain with equal intensity as can be seen by comparing cells in the field shown in Fig. 2A; the pattern of staining was, however, similar. From Fig. 2, I and J, it may be seen that the malignant mammary epithelial cells grown from PE-7 also show a diffuse pattern of staining but that fluorescence is stronger over some of the nuclei. Control cultures stained with preimmune serum showed no positive fluorescence.

Since the macrophages have no detectable cell-associated fibronectin and the medium in which the cells were grown contained no fibronectin, it seems safe to conclude that the staining on the normal and malignant epithelial cells was due to fibronectin produced and retained by the cell. Whether it is retained in the same way or is functionally different in the normal and malignant epithelial cells we cannot say.

Detection of [35S]-labeled Fibronectin. The synthesis of fibronectin by normal and malignant human mammary epithelial cells was confirmed by labeling cells with [35S]methionine and demonstrating the production of 35S-labeled protein. Radioactively labeled lysate and medium were precipitated with anti-fibronectin serum, and the precipitates were dissociated and run on urea gels. Autoradiographs of the gels showed that, although labeled fibronectin was associated with cell lysates, a considerable amount was found in the conditioned medium from fibroblasts and normal and malignant epithelium (Fig. 3). Macrophages did not produce detectable fibronectin even after 2 weeks in culture (Fig. 3) or when cultured on collagen-coated dishes (see below). To make a quantitative comparison between the amounts of fibronectin retained and produced by the different cell types, cell-associated fibronectin and fibronectin...
released into the medium have been estimated using a RIA.

**Quantitative Determination of Fibronectin Produced and Retained by Cells Cultured from the Human Mammary Gland.**

Chart 1A illustrates the purity of the fibronectin used in the radioimmune assay which, as can be seen from the standard curve shown in Chart 1B, is useful for estimations over a range of 2 to 30 ng. For determination of cell-associated and released fibronectin, cells were collected from cultures which were approximately three-fourths confluent, and the medium, which had been in contact with the cells for 3 days, was harvested. As for the indirect immunofluorescence assay, the cells were approximately three-fourths confluent, and the medium, which of 2 to 30 ng. For determination of cell-associated and released fibronectin, cells were collected from cultures which were approximately three-fourths confluent, and the medium, which had been in contact with the cells for 3 days, was harvested. As for the indirect immunofluorescence assay, the cells were cultured in medium containing serum from which bovine and human fibronectin had been removed (see "Materials and Methods"), and 2 examples of each cell type (milk epithelium, malignant breast epithelium, and breast fibroblasts) were examined. Table 1 shows the values obtained. The first point to note is that both the epithelial cells and the fibroblasts retain only a small fraction of the fibronectin that they produce, most of it being released into the medium. The values for cell-associated material were around 10% for fibroblasts and 2 to 10% for the epithelial cells; there is no significant difference between the normal and malignant epithelium. The second point is that the total amount of fibronectin produced by normal and malignant epithelial cells and by fibroblasts is of the same order and within the range of 20 to 60 /ig/mg cell protein. Both samples of normal mammary epithelium produced protein levels in the lower range, and the levels produced by both normal fibroblasts were in the higher range. The 2 samples of metastatic malignant cells showed more variation. It would be necessary to examine tumor cells from a larger number of breast cancer patients to determine how representative of metastatic breast cancer cells are the 2 examples that we have studied here. However, our results show that breast epithelial cells produce quantities of fibronectin comparable to the levels produced by fibroblasts and that a reduction in the level of the protein synthesized or retained (in vitro) is not invariably associated with the change to malignancy.

**Effect of Culturing on Collagen-coated Dishes on Retention of Fibronectin by Cells.** The data shown in Table 1 were obtained using cells grown on untreated plastic dishes. We have observed better growth of mammary epithelium on collagen-coated dishes and routinely grow milk epithelial cells in this way (32). Because of the known affinity of fibronectin for collagen (8, 18, 24, 40), we examined how growth on collagen-coated dishes affected the retention of fibronectin by epithelial cells. The data shown in Chart 2 suggest that human mammary epithelial cells retain a greater proportion of the fibronectin that they produce when grown on collagen-coated dishes; this applies to cells grown from milk and to the 2 samples of metastatic breast cancer cells examined here. Fibronectin retention by mammary fibroblasts is not significantly affected by growth on collagen-coated dishes.

**DISCUSSION**

Studies on fibronectin production by epithelial cells have been largely limited to an examination of cell-associated fibronectin, using indirect immunofluorescence, and have variously reported production (6, 29, 38) and lack of production (43). There is one report, however, by Quarconi et al. (26) of a quantitative study of fibronectin production and retention by rat intestinal epithelial crypt cells, in long-term culture, which shows a high production of protein but little retention by the cell. Our results with primary cultures of human mammary epithelial cells from breast milk are very similar to those reported by Quarconi et al. in that they show a substantial production of fibronectin but a poor retention of the protein by the cell. Yang et al. (43) have recently reported that human mammary epithelial cells cultured from milk do not produce fibronectin. They base their conclusions, however, on indirect immunofluorescent staining of cells in sparse culture with anti-fibronectin serum. The production of 35S-labeled fibronectin by normal and malignant cells and the data from the RIA presented here make it quite clear that these cells do produce fibronectin. The positive staining with indirect immunofluorescence was increased on cells grown on collagen-coated dishes. Perhaps
the negative results of Yang et al. are due to the use of sparse cultures grown on plastic. Our results are in agreement with those of Smith et al. (30) who, using indirect immunofluorescence, found fibronectin to be present on normal human mammary epithelium cultured from reduction mammoplasty tissue.

A comparison of the pattern of fibronectin production and retention by epithelium and fibroblasts from normal breast shows some similarities and some differences. The 2 cell types behave similarly in that (a) they retain only a small fraction of the fibronectin they produce and (b) they produce similar amounts of fibronectin (19 to 27 μg/mg protein for epithelial cells compared to 49 to 59 μg/mg protein for fibroblasts). Differences are observed, however, in the pattern of distribution of retained fibronectin, as shown by indirect immunofluorescence, and in the effect of growth on collagen on the fraction of fibronectin retained. Such differences, if confirmed over a large number of fibroblast and epithelial cell strains, could perhaps serve to distinguish the 2 cell types in culture.

The increased retention of fibronectin by both normal and malignant cells when grown on collagen-coated plates is a consistent observation. This could mean that the collagen-fibronectin interactions are directly responsible for the improved attachment of fibronectin to the cell. Collagen could also decrease the degradation of surface proteoglycans by the epithelial cells (7), and cross-linking of fibronectin to these molecules could affect its retention by the cell (25). The observation serves as a reminder that epithelial cells grown on plastic exhibit a topography radically different than that of the cells as they are found in vivo, attached to a basement membrane and separated by it from the stroma and blood supply. Basement membranes are known to contain collagen, fibronectin, and sulfated proteoglycans. It is easy to see how, in vivo, because of the tight junctions sealing the surface of the epithelium, fibronectin secreted from the basal part of the cell could be immediately incorporated into the basement membrane or form an attachment with the components thereof. By supplying the cells with collagen in vitro, we may be moving closer to the in vivo situation, which is perhaps even better represented by growth in collagen gels, where there is evidence of basement lamina formation (7).

A comparison of normal and malignant mammary epithelium in primary culture has shown no consistent quantitative difference in the level of fibronectin production and release. In fact, PE-25 showed a higher level of total fibronectin production than did the normal epithelial cells. The tumor cells examined were metastatic, and if a clear reduction in fibronectin production or retention in vitro were invariable markers for metastatic potential we would have detected it.

It should be emphasized that our data relate to the level of fibronectin (detected in RIA by reaction with a specific antiserum) produced and retained by cells. Differences in the way fibronectin is held by the normal and malignant cells or functional differences in the fibronectin produced would not have been detected in our experiments. Such differences could exist and may be relevant to malignant change. In interpreting our data, consideration should also be given to the fact that breast cancer cells from pleural effusions can be cultured more readily than are cells from either primary carcinomas or other accessible metastatic lesions such as skin or lymph nodes (31), and most breast cancer cell lines have been derived from cultures of pleural effusions. Indeed, it is conceivable that the ability of this tumor cell type to attach and proliferate in culture is related to a capacity for the production of fibronectin. Thus, even if the cells from PE-7 and PE-25 are representative of breast tumor cells metastasizing in the pleural cavity, they may not be representative of the original invasive cell or of tumor cells metastasizing to other sites.

The above considerations do not alter the basic conclusion which can be drawn from our results, namely, that the level of production of fibronectin or its retention by the cell in vitro cannot be used as an absolute marker to distinguish cultured breast cancer cells from their normal counterparts. They do suggest, however, that the pattern of fibronectin production and retention should be examined in a range of primary and metastatic breast carcinomas. Since such tumors are difficult to culture, it will probably be necessary to use tissue sections, which might show up any differences occurring in vivo, and would avoid artifacts resulting from cell culture. As discussed above, epithelial cells grown on solid supports in vitro may exhibit behavioral characteristics atypical of the same cells in vivo where interactions with homologous and heterologous cells and with the basement membrane are crucial to normal function. The retention of fibronectin can also be affected by growth factors (5), hormones (9), and tumor promoters (16) and by disruption of the cytoskeleton (19). Presumably, a high proportion of the fibronectin produced by normal mammary epithelial cells in vivo is incorporated into the basement membrane or retained by the cells. Unless culture conditions can be found for normal mammary epithelial cells to mimic this behavior in vitro, it may be difficult to use the in vitro culture system to determine whether changes in fibronectin metabolism are associated with the in vivo change to malignancy in breast cancer.

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Fig. 1. Appearance of cells cultured from human breast. A, fibroblasts; B, milk epithelial cells; C, D, pleural effusions PE-7 and PE-25, respectively. × 100.
Fig. 2. Indirect immunofluorescent staining with anti-fibronectin antiserum. A, C, E, G, I, fluorescence; B, D, F, H, J, phase; A to D, milk epithelial cells; E, F, macrophages; G, H, fibroblasts; I, J, PE-7. $\times$ 400.
Fig. 3. Precipitation of 35S-labeled protein by anti-fibronectin serum from conditioned medium (A) and cell lysates (B). A. Tracks 1, 3, 5, 7, and 9, precipitated with immune serum. Tracks 2, 4, 6, 8, and 10, precipitated with preimmune serum; Tracks 1 and 2, PE-7 (14 days in culture) Tracks 3 and 4, milk macrophages (14 days in culture); Tracks 5 and 6, milk macrophages (4 days in culture); Tracks 7 and 8, milk epithelial cells (14 days in culture); Tracks 9 and 10, human fibroblasts (14 days in culture). B. Lysates were precipitated first with preimmune serum as described in "Materials and Methods" and then with immune serum. Track 1, human fibroblasts (14 days in culture); Track 2, human mammary epithelium (14 days in culture); Track 3, milk macrophages (4 days); Track 4, milk macrophages (14 days); Track 5, milk macrophages (14 days on collagen-coated dishes); Track 6, PE-7 (14 days).
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