Casein Production by Human Breast Cancer

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SUMMARY

Casein was measured in the sera of breast cancer patients, in breast cancer tumors, and in breast cancer cells in long-term tissue culture using a sensitive and specific radioimmunoassay. Levels present in breast cancer sera were not elevated above control values. Eight of forty-seven (17%) of the tumor samples tested were positive for casein, the highest level representing 0.003% of the soluble protein. When seven human breast cancer cell lines were assayed for casein, the results were uniformly negative even under conditions of stimulation by lactogenic hormones. In addition, direct immunoprecipitation of labeled cellular protein supported the negative result of the radioimmunoassay. Thus it appears that casein production is not a common characteristic of most human breast cancers.

INTRODUCTION

It has been previously reported that the milk protein, casein, is present in large amounts in the sera of patients with carcinoma of the breast (5). These data imply that neoplastic mammary epithelial cells have the capacity to synthesize a product normally secreted by the mammary gland in the presence of lactogenic hormones. On the other hand, the milk protein, a-lactalbumin, found in rodent systems to be coordinately regulated with casein (14), was not significantly elevated in the sera of breast cancer patients, although it was present in some mammary tumor samples (8). Furthermore, MCF-7, a human breast cancer cell line in continuous tissue culture, has been reported to accumulate a-lactalbumin (15). Determination of the capacity of human breast cancer to synthesize discrete milk proteins in vivo and in vitro would be desirable for a number of reasons: (a) these proteins might be valuable as serum markers in the detection and monitoring of carcinoma of the breast; (b) the relative ability of individual tumors to synthesize these proteins might provide clinical data relevant to therapy; for example, the presence or absence of casein might be correlated with the endocrine responsiveness of the tumor; and (c) the existence of breast cancer cells in culture which were capable of synthesizing milk proteins could provide an excellent model for the study of the regulation of lactogenic functions.

We have developed a sensitive and specific radioimmunoassay for human casein and examined a variety of serum samples, tumor cytosols, and breast cancer cell tissue culture lines for the presence of this milk protein. Furthermore, we have additionally examined the capacity of these cell lines to synthesize casein by measuring incorporation of [3H]proline into material that was precipitable by antibody using a direct precipitation method. Our results indicate that serum samples from breast cancer patients and breast cancer cells in culture seldom contain significant casein levels, while 17% of the breast tumor cytosols tested are positive for casein. Breast cancer cells in culture do not appear to synthesize a protein that is specifically precipitable by anticasein antibody.

MATERIALS AND METHODS

Patient Samples. Blood was collected from patients and normals, and the serum was separated and stored at −70°.

Preparation of Tumor Cytosols. Tumor samples were obtained from surgical specimens or biopsy material and immediately frozen in liquid nitrogen and stored at −70° until used. The frozen tissue was weighed, pulverized and homogenized using a Brinkmann Polytron PT 10 ST (Brinkmann Instruments, Inc., Westbury, N.Y.) in a buffer containing 0.01 M Tris-HCl, 0.0015 M EDTA, and 0.005 M dithiothreitol, pH 7.4. One ml of buffer was added per 100 mg of tissue. The resulting homogenate was centrifuged at 105,000 × g for 1 hr at 4°. The pellet and fat layer were discarded, and the cytosol was used for assay. Many assays were performed on cytosols which had been stored frozen for up to 6 months. Protein concentrations were determined by the method of Lowry et al. (11).

Techniques of Cell Culture. The MCF-7 cell line was established from the pleural effusion of a postmenopausal female with metastatic breast cancer (16). Starter cultures were provided by Marvin Rich of the Michigan Cancer Foundation. The Eves-T cell line was established in this laboratory as previously described (9). The ZR-75-1 cells were provided by Nathaniel Young and Linda Engel of the National Cancer Institute. All other breast cell lines (MDA-231, G-11, HT-39, and BT-20) were provided by Ronald Herberman of the National Cancer Institute. Cells were grown in monolayer culture in MEM1 supplemented with 10% fetal calf serum (North American Biological Co., Miami, Fla.). For hormonal stimulation experiments, the medium was changed to MEM without serum before hormone addition. Cells were harvested in Dulbecco's phosphate-buffered saline, pH 7.4, without calcium or magnesium, by scraping with a rubber policeman and collected by centrifugation at high speed in a Sero-fuge (Clay-Adams, Parsippany, N. J.). Samples of the media were saved for casein determination.

1 The abbreviations used are: MEM, Eagle's minimal essential media plus 2 × glutamine, penicillin, and streptomycin; SDS, sodium dodecyl sulfate.
The cell pellets were resuspended in 0.05 M sodium phosphate buffer, pH 7.5. Cells were sonically disrupted for 5 sec at the lowest setting (Branson Sonicator; Heat Systems-Ultrasonics, Plainview, Long Island, N. Y.), and the resulting sonicate was centrifuged for 30 min at 105,000 x g in a Beckman L5-65 ultracentrifuge. Both the cytosol and pellet were used for casein determinations.

Preparation of the Antibody. Casein used for immunization was prepared from human milk by a combination of acid precipitation and polyacrylamide disc gel electrophoresis. The techniques for acid precipitation have been described elsewhere (13). Approximately 50 µg of acid-precipitated material were applied to a 10% polyacrylamide gel containing 0.1% SDS and subjected to electrophoresis using a Tris-glycine-SDS buffer, as described elsewhere (12). Fig. 1a illustrates the electrophoretic pattern of the acid-precipitated material. Fig. 1b shows the migration of human casein after purification by diethylaminoethyl chromatography, according to the method described by Groves and Gordon (4). This 25,000-molecular weight species, corresponding to caseins I through VI of Groves and Gordon, was used for immunization (approximately 150 µg/animal). Unstained gels were scanned at 280 nm using a Beckman gel scanner, and the protein band migrating in the position of the 25,000-molecular weight species was excised and homogenized in complete Freund's adjuvant using a Brinkmann Polytron. The resulting emulsion was injected intradermally into the back of a castrated male sheep in 20 µl.

Preparation of Radiolabeled Casein. Acid-precipitated casein was chromatographed on diethylaminoethyl, and casein Fractions I through VI and Kappa casein were isolated. The identity of Fractions I through VI was verified by electrophoresis on 10% polyacrylamide gels containing 6 M urea. Fig. 2 illustrates the casein fraction used for iodination corresponds to casein IV described by Groves and Gordon (4). Labeling was effected by a modification of the procedure of Hunter and Greenwood (6). One mCi of Na125I (Amersham/Searle, Arlington Heights, Ill.) was reacted with 5 µg of casein IV in 0.05 M sodium phosphate buffer, pH 7.5. Radioactive casein was purified by chromatography on Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 0.05 M phosphate buffer containing 0.5% bovine serum albumin, pH 7.5. The specific activity of the preparation was generally 150 µCi/µg. The same casein (Fraction IV) was used as the competing antigen in determination of the standard curve.

Radioimmunoassay Conditions. A double antibody equilibrium system was used in the radioimmunoassay. The incubation mixture consisted of 0.05 ng of labeled casein (approximately 10,000 cpm), 0.1 ml of sample, and a 1:50,000 dilution of antiserum, in a final volume of 0.4 ml of 0.05 M phosphate buffer with 0.5% bovine serum albumin, pH 7.5. When serum samples were assayed, the antiserum was first absorbed against a 10-fold excess of normal male serum (1 ml antiserum plus 9 ml normal male serum) before final dilution in the assay mixture; an additional 0.1 ml of the male serum was added to each tube of the standard curve. The mixture was allowed to stand overnight at 4°C. Then 0.1 ml of a 1:100 dilution of normal sheep serum was added to each tube. After vortexing, 0.1 ml (0.1 mg) of goat antihuman gamma globulin (Miles Yeda, Ltd., Rehovot, Israel) was added, and the mixture was allowed to stand for 18 hr at 4°C. One ml of cold phosphate buffer was then added to each tube, and the precipitate was collected by centrifugation at 1000 x g for 30 min. The supernatant was decanted, and the pellet was counted directly in a Micromedics Model MS588 gamma counter.

Sensitivity and Specificity of Radioimmunoassay. The sensitivity and specificity of the radioimmunoassay are illustrated in Chart 1. The assay as described is capable of measuring human casein at 1 ng/ml and does not appear to cross-react significantly with other milk proteins, bovine casein, or human placental lactogen.

Radioimmunoassay of Serum Samples. The values obtained with normal and patient sera are shown in Chart 2. Since 107 of 113 (95%) normal samples had values ≤ 1.7 ng/ml, this value was chosen as the upper limit of normal. The highest value obtained with normal serum was 2.6 ng/ml. In samples obtained from lactating women, the lowest value was 2.5 ng/ml with a range extending to 76 ng/ml and a mean of 15.4 ng/ml.

Among patients with cancer, there were elevations in 3 of 31 (9.7%) with metastatic breast carcinoma, 2 of 20 (10%) with pancreatic carcinoma, 10 of 19 (52.6%) with colon cancer.
labeled cell cytosol proteins were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, several peaks of radioactivity were apparent. However, after immunoprecipitation with casein antibody, approximately 5% of the radioactivity remained in the precipitate, and there were no discrete peaks of labeled protein present. However, such a technique would be sensitive only if the labeled casein accounted for at least 0.1% of the cytosol protein.

**Radioimmunoassay of Tissue Culture Cell Cytosols, Pellets, and Media.** Table 1 shows the results of assays for human casein in breast cancer cell line cytosols. None of the cytosols were able to displace the radiolabeled casein. Since protein concentrations in each case were 0.5 mg/ml and the assay is sensitive to 1 ng/ml, any casein present would account for less than 0.0002% of the cytosol protein. When cytosol protein concentrations were increased by 10-fold to 5 mg/ml, we observed significant displacement of the labeled casein. However, the final calculated value for soluble protein then fell to less than 1 ng/ml or less than 0.0001%. Furthermore, such displacement was also seen when the cytosols were prepared from normal rat kidney (NRK), Buffalo rat liver (BRL-62), or HTC cells, suggesting that the displacement observed represented a nonspecific

**Chart 1.** Standard curve and specificity data for casein radioimmunoassay. $B/B_0$, ratio of radioactivity precipitated in the presence of added unlabeled antigen ($B$) to that precipitated in the absence of unlabeled antigen ($B_0$). h, human.

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**Chart 2.** Casein levels in the sera of normals and patients with various malignant and nonmalignant diseases.

carcinoma, 2 of 12 (16.6%) with gastric carcinoma, 1 of 8 (12.5%) with renal carcinoma, 3 of 19 (15.8%) with melanoma, 2 of 12 (16.7%) with sarcoma, and 0 of 6 with astrocytoma. The highest elevation (6.1 ng/ml) was 3.6 times the upper limit of normal in a patient with gastric carcinoma.

The highest elevation (11.3 ng/ml) in the nonmalignant disease reference group was in a patient with renal failure. In general, there was a high proportion of elevation in patients without evident cancer. Most of these patients had inflammatory conditions.

**Radioimmunoassay of Tumor Cytosols.** Cytosol preparations derived from primary or metastatic breast tumors were assayed for the presence of casein. Of 47 samples tested, 5 (10.6%) were positive and 3 (6.3%) were borderline when calculated per mg of soluble protein (Chart 3). The highest level of soluble protein observed was 30 ng/mg or 0.003% of the total soluble protein.

**Immunoprecipitation of Labeled MCF-7 Cytosols.** Chart 4 illustrates the results of the immunoprecipitation of labeled MCF-7 cytosol proteins with anticasein serum. When...
protein effect or cross-reactivity with some component common to these cells. The 105,000 × g pellets and the medium in which the cells were grown were also assayed for casein. Pellets were first redissolved in the assay buffer with the aid of sonic disruption. Both were negative within the limits of the assay. To rule out the possibility that the homogenization procedure was destroying any casein present, carrier casein was added to a cell preparation before homogenization. The casein was recovered in the cytosol. Lastly, an attempt was made to stimulate casein synthesis in the MCF-7 cell line with lactogenic hormones. Table 2 shows that none of the hormones or hormone combinations tested had any observable effect on the levels of casein in the cells. Estradiol, 5α-dihydrotestosterone, insulin, and dexamethasone have previously been shown to elicit a variety of responses in these cells at the concentrations used (9, 10). Furthermore, the triple hormone combination of placental lactogen, insulin, and dexamethasone has been determined to be maximally effective in stimulating casein synthesis in rodent mammary glands in organ culture (14).

**DISCUSSION**

Casein biosynthesis is normally activated only in the fully differentiated breast cancer tumors. Whether or not this group with tumors positive for casein represents a clinically or pathologically significant subpopulation of breast cancer patients remains to be determined.

Lastly, we examined breast cancer cell lines in tissue culture. We were unable to demonstrate the presence of casein either by direct immunoprecipitation or by a competitive protein-binding assay with this antibody in 7 human breast cancer lines in continuous culture. In addition, no combination of lactogenic hormones used in this study resulted in detectable casein production.

Our data are consistent with the idea that neoplastic mammary cells in culture no longer retain the hormonal responsiveness necessary for initiation of casein synthesis. The fact that both breast cancer cell lines (10) as well as many mammary tumors (8) fail to respond to prolactin may explain the failure of the cell lines to accumulate casein. However, we cannot rule out the possibility that the conditions we used were not optimal for induction of casein synthesis. Perhaps hormones found to be sufficient for lactation in rodents are inadequate in humans.

In summary, we have developed a sensitive and specific antibody to human casein and used it to measure casein levels in the sera of patients with breast cancer, in mammary tumors, and in breast cancer cells in tissue culture. None of the serum samples for individuals with breast cancer had clinically significant casein levels; 17% of the breast tumors assayed were positive. No human breast cancer cell line tested appeared to accumulate casein. From these data we conclude that significant casein synthesis is not a general characteristic of human breast cancer, when measured with our antibody. The absence of detailed specificity data in the report of Hendrick and Franchimont (5) prevents us from commenting on the validity of their assay.
REFERENCES

Fig. 1. Polyacrylamide-SDS disc gel electrophoresis of acid precipitated casein before (a) and after (b) diethylaminoethyl chromatography.

Fig. 2. Polyacrylamide-urea gel electrophoresis of acid-precipitated casein before (a) and after (b) diethylaminoethyl chromatography.
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