Expression of Differentiation-specific Proteins in Preneoplastic Mammary Tissues in BALB/c Mice


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ABSTRACT

The levels of milk-specific mRNAs (α-casein, whey acidic protein) and proteins (casein, α-lactalbumin) were examined in the DIM series of mammary preneoplastic outgrowth lines and tumors. The constitutive production of casein protein was variable among the preneoplastic DIM outgrowth lines maintained in virgin mice. Outgrowth line DIM-2 consistently expressed a very low level of casein mRNA and protein but no detectable α-lactalbumin protein. Outgrowth line DIM-4 expressed a high level of casein protein and no α-lactalbumin; however, by transplant generation 9, the levels of casein mRNA and protein were significantly decreased but remained greater than those found in DIM-2. Outgrowth line DIM-3 expressed high levels of casein protein during all transplant generations examined and sporadically exhibited detectable amounts of α-lactalbumin. The level of β-casein mRNA in DIM-3 was 7 times greater than seen in DIM-4 outgrowths, but still only 2% of that measured in the normal mammary gland from lactating mice. The majority of tumors derived from the DIM outgrowth lines expressed very low levels of β-casein mRNA and total casein protein, although occasionally tumors were observed with very high levels of casein expression. Immunoblot analysis of cellular extracts indicated that α, β, and γ-caseins were expressed in the three outgrowth lines to varying degrees. Whey acidic protein mRNA attained barely detectable levels in the best of cases. Only outgrowth line DIM-3 responded to a normal lactogenic stimulus (lactation) with significantly increased levels of milk-specific products. As determined by avidin-biotin peroxidase staining, the percentage of alveoli expressing β-casein and α-lactalbumin proteins in lactating mice increased from 20 and 0%, respectively, in virgin mice to 85 and 40%, respectively, in lactating mice. Similarly, the levels of mRNA for β-casein and whey acidic protein increased 8- and 5.5-fold, respectively, in lactating mice. These results indicate that the cell populations selected by serial transplantation of preneoplastic mammary tissues fall into at least three categories with respect to expression of mammary-specific differentiation products: uninducible, inducible, constitutive. The DIM-3 outgrowth line appears to represent a highly inducible cell population. As concluded in earlier investigations, there was no correlation between secretory activity, morphology of the outgrowths, and tumor-producing capabilities in virgin mice. The effect of a normal lactogenic stimulus on the tumor potential of the DIM-3 line is currently under investigation.

INTRODUCTION

Mammary preneoplastic outgrowth lines represent transplantable populations of cells derived from HAN or ductal hyperplasias which are at greater risk for neoplastic transformation than normal mammary gland epithelial cells (1–3). One class of preneoplasias, the HAN, generates transplantable lines which are morphologically similar to the mammary glands of early to midpregnant mice. However, the differentiated alveolar cell phenotype of the HAN outgrowths is maintained in the hormonal milieu of virgin mice, a milieu which supports only the ductal cell phenotype in normal mammary gland of BALB/c mice (1). Thus, it is apparent that the altered regulation of hormone-mediated differentiation, like the acquisition of immortality, is a cardinal feature of mammary preneoplasias (1, 2, 4, 5). The two cardinal features of mammary preneoplasias are independent of the etiological agents involved in the induction of the primary HAN, since HAN outgrowth lines have been established from murine mammary tumor virus-infected (6, 7), hormonally stimulated (8, 9), and chemical carcinogen-treated BALB/c mice (10, 11) as well as from BALB/c mammary epithelial cell lines established in vitro monolayer cell culture (12).

Although the mammary outgrowth lines are morphologically differentiated, studies on the extent of functional differentiation of these lines have yielded ambiguous results. Whereas BALB/c primary HAN in situ exhibit apparent milk protein synthesis in response to the appropriate hormonal stimulation (2, 9, 13), BALB/c preneoplastic outgrowth lines exhibited very low or nonexistent levels of casein mRNA and protein and were variably responsive to hormonal modulation of these products, depending on the outgrowth line (11, 14–16). The early experiments were performed on outgrowth lines that had been maintained for many transplant generations covering a span of 3–10 years; thus it was unclear how biological progression with transplant passage affected the properties of the outgrowth lines. A recent study by Smith et al. (16) suggested that early transplant generations of C3H/Sm preneoplastic outgrowth lines maintained a constitutive level of casein synthesis and were hormonally inducible for both casein and α-lactalbumin synthesis. It was with these results in mind that we examined the extent of functional differentiation in a series of newly established and characterized BALB/c preneoplastic outgrowth lines (12).

One of the ultimate aims of this research is to determine if a normal physiological stimulus for mammary differentiation can inhibit the neoplastic transformation in preneoplastic outgrowth lines. Experiments in the rat mammary system have demonstrated that a single pregnancy and lactation can inhibit the expression of chemical carcinogen-induced transformed cells in the mammary gland (17–20). The only comparable work in the mouse mammary system is a study by Zelilmaker (13) which demonstrated that prolonged lactation can inhibit nodulogenesis and tumorogenesis in C3H × O20 F1 and BALB/cF3H mice. Prior experiments had demonstrated that primary HAN in situ will exhibit apparent milk protein synthesis constitutively in virgin mice as well as in mice under a lactogenic stimulus (13, 16, 21). Additionally, mammary glands of parous rats and mice are less sensitive to the tumorigenic effects of 7,12-dimethylbenz(a)anthracene than are mammary glands in virgin rodents (22). One hypothesis presented to explain these results states that hormones cause differentiation of mammary cells (normal, preneoplasias or carcinogen-altered cells) into a terminal stage (nonproliferative) cell (18, 19). This hypothesis has not been fully tested yet. The experiments described herein...
provide a first characterization of the extent and degree of functional differentiation in a series of mammary nodule outgrowth lines. The subsequent aim will be to examine the effect of a lactogenic stimulus on the inhibition of tumor-producing capabilities of the preneoplastic cell populations.

MATERIALS AND METHODS

Mice and Tissues. Female BALB/cMed mice were bred and maintained in a closed conventional mouse colony at Baylor College of Medicine. The mice were housed 4–6 to a cage in temperature- and light cycle-controlled rooms and fed Wayne Lab-Blox and water ad libitum.

The origin and characteristics of the DIM series outgrowths have been described (12). Briefly, the outgrowth lines were derived from cells of the COMMA-D cell line which were injected into mammary gland-free fat pads of 3-week-old syngeneic mice. The DIM outgrowth lines have different morphological and tumorigenic characteristics (12). DIM lines 1, 2, and 3 exhibit an alveolar morphology and have high tumor-producing capabilities (>70% tumor incidence at 5–7 months) from transplant generations 2–10. In contrast, line DIM 4 had a ductule morphology and a lower tumor-producing capability (~40% tumor incidence at 10–13 months) between transplant generations 2 and 6 before progressing to an outgrowth line with characteristics similar to those of the other DIM lines. The outgrowth lines were maintained in virgin female BALB/c mice by serial transplantation into mammary gland-free fat pads every 12 weeks as described in Ref. 2. For the experiments described herein, samples of the outgrowths were taken 12–16 weeks after transplantation. Samples of mammary tumors which arose from the outgrowth lines were taken 4–12 months after transplantation.

Antiserum. The antiserum used in these experiments were rabbit polyclonal antisera against mouse casein, α-lactalbumin, and MFGP. The specificities of the two different antisera prepared against mouse caseins have been described previously (6, 23). The majority of the experiments were performed with antiserum described in Ref. 23. This antiserum recognizes the major mouse caseins (α1, α2, β, γ) with no discernible reactivity against transferrin or α-lactalbumin. The second anti-casein antiserum recognizes the major mouse caseins (16) and was used on 20% of the outgrowths to confirm the reactivity of the first anti-casein antiserum. The α-lactalbumin antiserum was a gift from Dr. Barbara Vonderhaar, National Cancer Institute, NIH. It recognizes mouse α-lactalbumin and has been described in Ref. 24. These three antisera were used at dilutions 1:500–1:1000. The anti-MFGP antiserum was a gift from Dr. Frank Stockdale, Stanford University, California. The specificity of this antibody is described in Ref. 26.

Immunohistochemistry. The avidin-biotin peroxidase method as described by Hogbin and Smith (27) was used in the majority of these experiments. Briefly, the samples were reacted sequentially with primary antisera (1:500–1:2000), goat anti-rabbit biotinylated IgG (1:500), and avidin-biotin complex (1:80). The biotinylated IgG and avidin-biotin complex were purchased for Vector Laboratories. Each incubation occurred for 30 min in a humidified chamber maintained at room temperature and was followed by a wash in Tris-saline buffer, pH 7.6, for 20 min. The reaction product was visualized by incubation with diaminobenzidine (Sigma Chemical Co., St. Louis, MO) for 6 min at room temperature. The slides were counterstained in hematoxylin, covered with coverslips using Permount and analyzed under a light microscope at ×16–×40.

The immunoperoxidase method as described in Ref. 26 was used with the rat monoclonal antibody. The monoclonal antibody was used at a dilution of 1:50 and the goat anti-rat peroxidase-conjugated antibody was used at a dilution of 1:200.

Each sample was analyzed for the percentage of alveoli which exhibited positive staining (i.e., brown precipitate). Since the reaction product was diffuse and covered both the plasma membrane and alveolar lumina, it was impractical to determine the exact number of positive cells in each positive alveolus; thus the data were expressed as percentage of positive alveoli. In tumors, individual cells could be discerned as positive or negative. A semiquantitative scale was devised based on percentage of positive alveoli as follows: (+), 0–5% alveoli positive; 1+, 6–25%; 2+, 26–50%; 3+, 51–75%; 4+, >75%. The scale was based on 100–200 alveoli/section counted randomly. Each histological section represented a separate sample.

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The controls for specific staining included the use of virgin mammary gland (casein negative, α-lactalbumin negative), pregnant mammary gland (casein positive, α-lactalbumin negative), lactating mammary gland (casein positive, α-lactalbumin positive), omission of the primary antibody or second antibody, and the simultaneous staining of positive and negative samples. Blocking experiments with the antisera to casein and α-lactalbumin have been described previously (16, 23, 24). A blocking experiment with purified mouse MFGP as antigen eliminated positive staining.

Protein Immunoblot. This procedure was carried out as described previously (16, 23). Proteins were placed in pH 6.8 solutions containing 4% SDS, 20 mM Tris-HCl, 3.5% β-mercaptoethanol, 1% Trasylol, 8 mM urea, and DNase/RNase; incubated at 80°C for 3 min; placed in sample buffer; and then subjected to electrophoresis in one-dimensional gels of 15% acrylamide in the presence of 0.1% SDS. Forty µg of protein were loaded in each lane except for the lactating controls where only 20 µg of protein were loaded. The gel was then soaked for 15 min in 25 mM Tris-192 mM glycine-20% methanol, pH 8.3. The proteins in the gel were transferred electrophoretically to nitrocellulose as described in Ref. 28. Electrophoresis was carried out at 0.5 V for 20 min followed by maximal voltage for 45 min in buffer (25 mM Tris-192 mM glycine-20% methanol, pH 8.3).

The electrophoretic blots (unstained) were soaked overnight in 0.25% gelatin in 100 mM NaCl-10 mM Tris-HCl-0.1% Nonidet P-40, pH 7.4 (blocking buffer). The protein blots were stored at −25°C after being wrapped while still moist in clear plastic film (Saran Wrap). The sheets were immersed in blocking buffer for 30 min at room temperature, rinsed in blocking buffer, and then exposed to primary antiserum diluted (1:500) in blocking buffer overnight at 4°C with constant agitation. The blots were subsequently washed in 100 ml of blocking buffer, pH 7.2, for five changes and then exposed to secondary antibody, 125I-protein A at 0.5 µCi/lane diluted in blocking buffer overnight at 1 h at 37°C with shaking. The protein blots were then washed in buffer, dried, and placed on Kodak XAR-5 film for 24–48 h.

Nucleic Acid Probes. β-Casein and WAP plasmids were generous gifts from Dr. Jeffery Rosen and have been characterized previously (29). The identity of the probes was verified by Northern blot analysis.

RNA Extraction. The tissue RNAs were extracted by a modification of the method of Chirgwin et al. (30). Briefly, the tissues were homogenized in 10 volumes of 4.5 mM guanidinium thiocyanate, 28 mM sodium citrate, 0.6% Sarkosyl, 0.1 M β-mercaptoethanol, and 0.1% Sigma antifoam A (pH 7.0). For normal tissues and tumors, nucleic acids were precipitated at −20°C by the addition of 0.0286 volume 1 M acetic acid, 0.125 volume water, and 0.8438 volume ethanol. The resulting pellet was dissolved in 7.5 mM guanidine hydrochloride-25 mM sodium citrate-5 mM dithiothreitol (pH 7.0). RNA was selectively precipitated by the addition of 0.025 volume 1 M acetic acid and 0.5 volume ethanol. The precipitation step was repeated twice and then the RNA pellets were washed in absolute ethanol to remove all traces of guanidine hydrochloride. The RNA was dissolved in 50% dimethyl sulfoxide-10 mM Tris (pH 7.5)-1 mM EDTA (pH 7.5)-0.1% SDS, incubated for 5 min at 45°C, and finally precipitated with 0.1 volume 2.75 M sodium acetate (pH 5.5) and 2 volumes ethanol.

For hyperplastic outgrowths, the guanidine thiocyanate homogenate was layered over a cushion of 5.7 M cesium chloride and centrifuged for 20 h at 36,000 rpm in an SW40 rotor using a Beckman L8-70 preparative ultracentrifuge. The resulting pellet was rinsed well in cold absolute ethanol and then dissolved in water.

RNA concentration was determined by absorbance at 260 nm and protein contamination was monitored by the ratio of absorbance at 260 nm to the absorbance at 280 nm.
Blot Hybridizations. For slot blots, RNAs were denatured with formaldehyde and spotted onto nitrocellulose paper using a Minifold II apparatus according to manufacturer's directions (Schleicher and Schuell, Keene, NH). For Northern blots, RNAs were denatured with glyoxal (31), separated by electrophoresis through a 1.6% agarose gel, and transferred to nitrocellulose paper (Schleicher and Schuell) according to the method of Thomas (32). Before prehybridization, the blots were rinsed in boiling 10 mM Tris (pH 8.0) to uncouple the glyoxal from the RNA.

Hybridizations were performed according to the method of Thomas (32) with some modifications. Blots were baked at 80°C in a vacuum for 2 h and then placed in individual sealed bags and prehybridized with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 250 μg/ml sonicated denatured salmon sperm DNA, and 0.5% SDS at 42°C overnight. Hybridizations were carried out in the same buffer with 10⁶ cpm/ml of an α-32P-labeled (3000 Ci/mmol; ICN Radiochemicals, Irvine, CA), nick-translated probe (Bethesda Research Laboratories, Gaithersburg, MD) for 20 h. The blots were first washed in four changes of 2x SSC-0.1% sodium pyrophosphate at room temperature for 15 min and then in two 15-min changes of 0.1x SSC-0.1% SDS-0.1% sodium pyrophosphate at 50°C. Finally, the blots were rinsed in 0.1x SSC, air dried, and exposed to either Kodak XAR or XRP film at −80°C with DuPont Cronex lightening-plus intensifying screens.

Analysis of RNA-DNA Hybridizations. The following measures were taken for accurate quantification of data: (a) hybridization of RNAs from DIM tissues and controls was carried out concurrently so that hybridization parameters (e.g., specific activity of the probe, duration of hybridization, volume of hybridization solution) would not introduce error into the calculations; (b) both the DIM and control tissues should produce autoradiographic signal within the linear response range of the film in the same autoradiographic exposure of a blot. Thus the same exposure could be used for the measurement of signal generated by both tissues. Because the level of expression of milk protein genes was so much greater in the control than in the DIM tissues the only way to meet this criterion was to blot different amounts of RNA for the controls and the DIM tissues. Thus 10 times more RNA was blotted from DIM tissues than from lactating mammary glands; (c) samples were applied in 2-fold dilutions; (d) autoradiograms which contained hybridization signal within the linear response range of the film were scanned with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX). Multiple exposures were examined to ensure data collection within the linear response range of the film, which was defined by those slots which demonstrated the best linearity with dilution; (e) areas under curves were quantified using an electronic digitizer (model 1224) from Nuponics Corp. (Landsdale, PA), which generated a numerical value to represent the signal intensity. This value was adjusted to account for the differences in amounts of RNA dotted and then expressed as a percentage of the appropriate control value.

RESULTS

Immunohistochemical Analysis of Milk Protein Expression in Mammary Preneoplasias. The expression of the milk proteins casein and α-lactalbumin in DIM outgrowths was detected using the avidin-biotin peroxidase assay. The frequency and degree of positive immunostaining for casein are illustrated in Fig. 1 and Table 1. The three outgrowth lines maintained in virgin mice expressed casein to different degrees. The line DIM-2 exhibited only a low level of casein expression with 12 of 13 samples exhibiting a RSI of less than or equal to 1+. Only 1 sample, a TG-3 sample, exhibited a high level of expression. The mean RSI for all DIM-2 samples was 0.65 or equivalent to only 1–5% of positive alveoli. The outgrowth line DIM-3 exhibited a fairly high level of casein expression with the majority of the samples (14 of 17) greater than or equal to 1+ response. The mean relative staining index of 2.3 indicates that over one-third of all alveoli exhibited casein production. The outgrowth line DIM-4 possessed a very high level of casein expression with 8 of 13 samples showing a maximum response (4+).

The staining pattern for casein within a positive alveolus was the same in all 3 outgrowth lines. Casein protein appeared in the apical portion of the cytoplasm of the cell, along the luminal border of the plasma membrane and within the lumina (Fig. 2, A and B). We did not quantitate the amount of casein per tissue sample; however, it was evident that the amount of casein in the outgrowths was approximately equivalent to that seen in the 15-day control pregnant tissues but much less than the amount seen in the 5-day lactating controls since the immunostaining midpoint titration was 1:1000 for the preneoplastic and pregnant samples and 1:8000 for the lactating samples.

The frequency of positive staining did not vary over the transplant generations examined for lines DIM-2 (TG 3–10) and DIM-3 (TG 2–10). However, although all of the samples examined for line DIM-4 (TG 3–8) were positive for casein, the strong immunostaining seen in line DIM-4 (Fig. 2B) over transplant generations 3–6 (a 3.9+ response) was markedly diminished in samples taken from TG-8; (a 1.4+ response). The four samples with the lowest degree of staining were all from TG-8. The drop in immunostaining was coincident with alterations in other biological properties of line DIM-4 (12).

In order to demonstrate the species of individual caseins expressed by the preneoplastic tissues, 5 samples each of the 3 outgrowth lines were examined by immunoblot analysis (Fig. 3). DIM-2 samples (TG-8, 10, 13) expressed relatively high levels of α-, β-, and γ-caseins (Fig. 3, Lanes e and f). DIM-2 (Fig. 3, Lane d) and DIM-4 (Fig. 3, Lanes g and h) expressed detectable levels of β-casein; however, α- and γ-caseins were difficult to visualize. The relative expression of the caseins was consistent in each of the 5 samples of DIM-3 and DIM-4 outgrowths and in 4 of the 5 DIM-2 outgrowths (TG-8, 10, 13). The same amount of protein (40 μg) was loaded in each lane; thus it was evident that DIM-3 was expressing a consid-
Fig. 2. Immunohistochemical (avidin-biotin peroxidase) staining of DIM outgrowths and tumors. The slides were originally photographed at x40, except B which was photographed at x16 and magnified a further x6 in the final print. A, B, E, samples reacted with anti-casein antiserum at 1:1000 dilution. A, alveoli in DIM-3 preneoplastic outgrowth exhibiting a strong reactivity to antiserum. The staining is localized in the lumina and apical portion of the cytoplasm (arrow). There is no staining observed in adipose, fibroblast, or vascular tissues. B, longitudinal section of a duct in DIM-4 outgrowth exhibiting extensive staining in lumina and cytoplasm. This was a TG-3 sample. C, DIM-3 preneoplastic outgrowth stained with antiserum to α-lactalbumin (1:500). A single alveolus was positive in the entire section (arrow). The staining for α-lactalbumin was confined to the cytoplasm which appears dark in the photograph. The cells that did not express α-lactalbumin appear as normal contrast in this print. D, DIM-2 preneoplastic outgrowth stained with antiserum to milk fat globule proteins (1:2000). The staining is seen as a sharp line along the plasma membrane (arrow). The vast majority of cells organized in well-differentiated alveoli or ducts in all outgrowth lines and normal mammary gland samples exhibited this staining pattern. E, A DIM-4 tumor exhibiting relatively weak reactivity. A few cells in the solid grouping of cells in the lower right show distinct cytoplasmic staining (arrow) whereas the majority of cells in this tumor were negative for casein (arrowhead).
The expression of β-lactalbumin and murine MFGP in the 3 outgrowth lines was tested by transplanting the lines into mice which were subsequently mated (Table 2). Of the 10 paired DIM-2 and DIM-4 samples examined, none of the outgrowths, which were taken from mice in their 3rd day of lactation, exhibited a detectable increase in immunocytochemical staining for β-casein. The inability of DIM-2 and DIM-4 outgrowths to respond to a lactogenic stimulus was also manifested by the absence of lipid droplets in the cytoplasm of the outgrowths maintained in lactating mice. In contrast, the three samples of DIM-3 outgrowths responded to the lactogenic stimulus with an increased expression of β-casein protein.

The effect of a sustained lactogenic stimulus on the expression of casein in the DIM outgrowth lines was tested by transplanting the lines into mice which were subsequently mated (Table 2). Of the 10 paired DIM-2 and DIM-4 samples examined, none of the outgrowths, which were taken from mice in their 3rd day of lactation, exhibited a detectable increase in immunocytochemical staining for β-casein. The inability of DIM-2 and DIM-4 outgrowths to respond to a lactogenic stimulus was also manifested by the absence of lipid droplets in the cytoplasm of the outgrowths maintained in lactating mice. In contrast, the three samples of DIM-3 outgrowths responded to the lactogenic stimulus with an increased expression of β-casein protein.

The expression of α-lactalbumin and murine MFGP in the 3 outgrowth lines is shown in Table 1. The values for casein illustrated in Fig. 1 are included for comparison. It was evident that the synthesis of α-lactalbumin was rare in the 3 outgrowth lines maintained in virgin mice, although when an occasional alveolus was positive, the staining pattern was easily detectable and localized entirely in the cytoplasm (Fig. 2C), a pattern identical to that seen in lactating control tissues. It should be noted that, in our samples, the 15-day pregnant mammary gland was uniformly negative for α-lactalbumin staining.

A lactogenic stimulus was incapable of inducing a significant staining response for α-lactalbumin in DIM-2 and DIM-4 outgrowths. DIM-3 outgrowths, which responded to a lactogenic stimulus with an increased expression of β-casein protein, also exhibited an increased expression of α-lactalbumin (Table 2).

In contrast to the staining observed with both casein and α-lactalbumin, the expression of MFGP in pregnant, lactating, and preneoplastic samples was uniformly positive (Table 1). The plasma membrane of the luminal epithelial cells was uniformly stained (Fig. 2D); however, myoepithelial cells located by the basement membrane were uniformly negative.

Analysis for Milk-specific mRNAs. Levels of β-casein and WAP transcripts were determined by slot blot analysis of RNAs from pooled DIM preneoplastic outgrowths and individual tumors and used as a quantitative measure of functional differentiation (Fig. 4). The levels of β-casein and WAP mRNAs shown in Fig. 4 were tabulated as a percentage of the levels detected in the normal lactating glands shown in Table 3. The levels of transcripts in normal lactating mammary glands (2 samples) were defined as the average of the levels detected in the axillary glands of lactating mice transplanted with DIM.
FUNCTIONAL DIFFERENTIATION IN MAMMARY PRENEOPLASIAS

<table>
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<tr>
<th>Group</th>
<th>Tissue</th>
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This was an increase similar to that detected for β-casein transcripts. DIM-3 outgrowths taken from lactating hosts expressed 1.1% of the level of WAP transcripts expressed in normal lactating glands.

Mammary-specific Protein and RNA Expression in Adenocarcinomas. The results in Table 1 provide information on the immunohistochemical detection of mammary-specific proteins in mammary adenocarcinomas derived from the 3 outgrowth lines. The expression, as determined immunohistochemically, of casein, α-lactalbumin, and milk fat globule protein was nonexistent or greatly diminished compared to the preneoplasias (Fig. 2E). Positive staining was seen in the cytoplasm of isolated or small clusters of tumor cells. In several cases, mammary adenocarcinomas, type B, were adjacent to preneoplastic outgrowth cells with the latter cell type negative and the former cell type positive. The decrease in immunohistochemically detected protein was not surprising. Other investigators have reported that the expression of mammary specific proteins such as MMTV env proteins and milk fat globule protein was decreased in mammary adenocarcinomas, particularly in cases where the adenocarcinomas lost their alveolar differentiation phenotype and cell polarity (34-36).

Similarly the relatively high level of constitutive β-casein mRNA expression in DIM-3 outgrowths was reduced 3- to 10-fold in DIM-3 tumors. DIM-4 tumors also demonstrated a reduction in β-casein transcript levels compared with DIM-4 outgrowths; thus negligible levels of transcripts were detectable. The exception was a DIM-4 tumor which expressed β-casein mRNA at a level comparable to that in a midpregnant gland (Table 3).

The majority of DIM tumors contained WAP transcripts at a level approximately 0.1% of lactating. The exception was the one DIM-4 tumor which also expressed high levels of β-casein mRNA (see above). Levels of WAP transcripts in this tumor were 0.8% of the level in lactating glands and 3% of the level in midpregnant glands.

DISCUSSION

Neoplastic transformation of the mouse mammary gland is a multistage process which involves intermediate cell populations which are direct precursors of the malignant neoplasms (1-3). The most thoroughly characterized of the preneoplastic cell populations are the HAN and the transplanted alveolar outgrowth cells. The preneoplastic outgrowth cells with the former cell type negative and the latter cell type positive. The decrease in immunohistochemically detected protein was not surprising. Other investigators have reported that the expression of mammary specific proteins such as MMTV env proteins and milk fat globule protein was decreased in mammary adenocarcinomas, particularly in cases where the adenocarcinomas lost their alveolar differentiation phenotype and cell polarity (34-36).

Similarly the relatively high level of constitutive β-casein mRNA expression in DIM-3 outgrowths was reduced 3- to 10-fold in DIM-3 tumors. DIM-4 tumors also demonstrated a reduction in β-casein transcript levels compared with DIM-4 outgrowths; thus negligible levels of transcripts were detectable. The exception was a DIM-4 tumor which expressed β-casein mRNA at a level comparable to that in a midpregnant gland (Table 3).

The majority of DIM tumors contained WAP transcripts at a level approximately 0.1% of lactating. The exception was the one DIM-4 tumor which also expressed high levels of β-casein mRNA (see above). Levels of WAP transcripts in this tumor were 0.8% of the level in lactating glands and 3% of the level in midpregnant glands.

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FUNCTIONAL DIFFERENTIATION IN MAMMARY PRENEOPLASIAS

preneoplastic lines were not inducible for increased β-casein, α-lactalbumin, or WAP synthesis by a normal physiological lactogenic stimulus. The refractoriness to complete functional differentiation was also observed at the morphological level with the absence of cytoplasmic lipid droplets. These results are interpreted to mean that the majority of the cells were arrested at a defined differentiation stage, i.e., the early to midpregnant stage. The arrested cell phenotype may be the consequence of the transformation of this cell type (i.e., blocked ontogeny) or a common arrest point for cells transformed at several stages in their differentiation pathway.

The exception was the DIM-3 outgrowth line which was inducible for transcripts and/or proteins of all 3 milk-specific genes. It was interesting that the increases of β-casein and WAP transcripts were of the same same magnitude (6–7-fold) suggesting a coordinate response in the responsive subpopulation of DIM-3 cells. The hormone-induced WAP expression in DIM-3 outgrowths was significant because it has been very difficult to demonstrate modulation of this transcript in vitro (37). The demonstration that WAP expression can be modulated in vivo in the DIM-3 cell population suggests that optimum in vitro conditions have not been obtained yet.

Morphologically, there was little difference between the alveoli populating DIM-2 and DIM-3 outgrowths, except the latter exhibited a high degree of casein synthesis. DIM-4 represented a population in progression from ductule to alveolar phenotype and the change in casein synthesis was correlated with changes in several other biological parameters (12). Thus, between passages 5 and 8, DIM-4 outgrowths also exhibited an increase in background tumor potential and tumorigenic response to 7,12-dimethylbenz(a)anthracene, a decrease in ovarian dependence for growth, as well as a decrease in casein synthesis and a change in morphology. It was apparent that the expression of casein was independent of these other biological properties. These results are in accord with the earlier results of DeOme et al. (38) who concluded that secretory capability, tumor potential, and glandular morphology are independent assortable properties in mammary preneoplasias. In those experiments, the nature of the secretion was not analyzed. It would appear that one component of the proteinaceous secretion observed in the lumina was casein.

Experiments utilizing other outgrowth lines indicate that the alveolar cells may be arrested at different points. For instance, BALB/c outgrowth lines D2a, C3, C4, C5, CD-8, MH-1, and MH-5 exhibit very low or nonexistent levels of casein in mRNA and protein and are noninducible for these macromolecules under in vitro culture conditions, whereas outgrowth lines D1 and MH-9 are inducible for casein mRNA (11, 14–16). The levels of casein proteins were not clearly established. On the other extreme, outgrowth lines derived from C3H/Sm mice exhibit constitutive expression of casein and α-lactalbumin and are further inducible for these proteins (16). These data support the idea that the functional differentiation capabilities of different preneoplastic outgrowth lines reflect the developmental potential of the cells at transformation. It is interesting that the normal mammary gland of virgin C3H mice exhibits a significant degree of lateral alveolar buds whereas the normal mammary gland of virgin BALB/c mice exhibits only ductal epithelium. The differentiation capabilities of the preneoplastic cells may reflect the differentiation potential of the normal cells at time of transformation. Although the hypothesis presented here resurrects the blocked ontogeny hypothesis (39), it is important to state that definitive resolution requires the ability to characterize cell stages and lineages with a set of defined and stable markers. Antibodies to cell stage-specific proteins are starting to be described; therefore such a study appears feasible (40, 41).

It was of interest that a normal differentiation stimulus was ineffective in stimulating complete functional differentiation in 2 of the 3 preneoplastic outgrowth lines. This would imply that a lactogenic stimulus would be ineffective in altering the tumor potential of the DIM-2 and DIM-4 outgrowth lines but may inhibit the tumor potential of the DIM-3 line. An experiment to test this idea utilizing both BALB/c and C3H/Sm outgrowth lines is currently in progress. Do the data also imply that other modes of stimulating functional differentiation will be incapable of modulating the tumor potential of mammary preneoplastic cell populations? As far as we are aware, this question has not been examined systematically for agents that either enhance or block differentiation. Such experiments would be worth pursuing particularly in view of the known responsiveness of the rat mammary gland to differentiation-mediated inhibition of chemical carcinogen-induced tumorigenesis (17–20).

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