Hormonal Induction of Casein Gene Expression Limited to a Small Subpopulation of 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumor Cells

Scott C. Supowit and Jeffrey M. Rosen

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

In the hormonally responsive 7,12-dimethylbenz(a)anthracene (DMBA)- or N-nitrosomethylurea (NMU)-induced mammary carcinomas, regulatory mechanisms have been altered such that these tumors retain their hormonal dependence for growth but possess only a limited ability to synthesize the mammary gland-specific milk proteins. Quantitation of casein mRNA levels revealed that very low levels of casein messenger RNA (mRNA) were expressed in both the DMBA- and NMU-induced tumors growing in virgin animals (0.1 to 0.4% of the maximally induced 8-day-lactating mammary gland). Growth of DMBA-induced tumors in pregnant rats and the treatment of NMU-induced tumor-bearing animals with thioproperazine indicated that the tumor casein mRNA levels were hormone inducible (3.4- and 2.1-fold for the DMBA- and NMU-induced tumors, respectively). However, casein mRNA levels were still only 1 to 2% of those found in the normal mammary gland under the same hormonal environment. Localization of the casein-synthesizing cells in the DMBA-induced tumors by peroxidase-antiperoxidase staining and a specific casein antiserum indicated that the tumor casein mRNA sequences were hormonally inducible (3.4- and 2.1-fold for the DMBA- and NMU-induced tumors, respectively). However, casein mRNA levels were still only 1 to 2% of those found in the normal mammary gland under the same hormonal environment. Localization of the casein-synthesizing cells in the DMBA-induced tumors by peroxidase-antiperoxidase staining and a specific casein antiserum indicated that, in both control and hormone-treated tumors, the vast majority of cells (>95%) were unable to synthesize casein. The hormonal induction of casein mRNA sequences could be correlated with an increase in the number of cells synthesizing casein, which appeared as small clusters of cells throughout the tumors. Therefore, the loss of hormone-regulated differentiated function in these tumors, which maintained hormone-dependent growth, suggests the presence of a defective regulatory mechanism beyond the level of the hormone-receptor complex.

INTRODUCTION

The synthesis of the milk proteins, casein and a-lactalbumin, provides a biochemical marker for studying the mechanisms by which hormones regulate the expression of differentiated function in the normal mammary gland and how these regulatory mechanisms may have deviated in hormone-dependent mammary cancer. Previously, in our laboratory, molecular hybridization and cell-free translation were used to compare total polyadenylic acid RNA populations from hormone-dependent DMBA-induced mammary tumors and normal midpregnant rat mammary tissue (25). Quantitative rather than qualitative differences were observed between these 2 RNA populations with the most striking difference being a 100-fold reduction in the expression of the milk protein mRNA sequences in the tumors. Although these tumors displayed hormone-dependent growth, quantitation of casein mRNA levels in tumors grown in either virgin animals (25) or ovariectomized animals, which had received hormone replacement (19), revealed that usually only 1% of the levels of casein mRNA was present in the tumors as compared to 8-day-lactating mammary tissue. In addition, both DMBA-induced tumors and another hormone-dependent mammary carcinoma induced by the carcinogen NMU (6) have been reported to contain a-lactalbumin at levels which are less than 10% of those found in a 5-day-lactating mammary gland (17). Since these tumors retain hormone-dependent growth, the defect in hormonally regulated milk protein gene expression is apparently not due to a "receptor minus" phenotype observed in most hormone-resistant cell lines and tumors (21).

In order to study this problem in greater detail, we have asked these questions. (a) Can casein mRNA be induced in these tumors by hormonal manipulation of the tumor-bearing animals? (b) Is the attenuated ability of these tumors to synthesize casein due to a very low level of synthesis in the entire tumor cell population, or is there a limited subpopulation of cells that synthesize casein?

In order to determine whether milk protein gene expression can be hormonally induced in both types of tumors, we have measured casein mRNA levels in DMBA-induced tumors growing in 14-day-pregnant animals and in NMU-induced tumors from animals treated with thioproperazine, a drug which increases serum prolactin levels. Localization of the tumor epithelial cells, which were actively synthesizing casein, was accomplished by use of an anti-rat casein antiseraum in conjunction with a PAP staining technique. We report that there is a limited hormonal induction of casein mRNA sequences in both DMBA- and NMU-induced rat mammary tumors and that, in the case of the DMBA tumor, this induction is the result of an increase in a very limited subpopulation of tumor cells.

MATERIALS AND METHODS

Tumor Induction and Treatment. Primary mammary carcinomas were induced in 50-day-old female Sprague-Dawley rats by DMBA (9, 25) or NMU (6). Ten DMBA-treated animals bearing detectable tumors were mated with male Sprague-Dawley rats. Seven females became...
pregnant within 5 days, and of these 7, 5 of the carcinogen-treated animals maintained pregnancy through Day 14. Each tumor was measured twice weekly with calipers so that growth rates could be compared to tumors growing in virgin animals. At this time, the animals were sacrificed, and the tumors were removed. Portions of each tumor to be used for RNA isolation were quick frozen in liquid N₂. Five to seven sections (1 to 2 cm) from different parts of each tumor were fixed in Telley's solution for use in histological and immunocytochemical studies. Five-μm-thick serial sections were made from each dissected tumor section. The normal mammary glands from these pregnant, tumor-bearing animals and tumors growing in virgin animals were removed and treated as described above.

Animals bearing NMU-induced mammary tumors, which were kindly provided by Dr. Paul Kelly, were either ovariectomized 36 hr before sacrifice or treated with i.v. injections of thioproperazine (10 mg/kg twice daily for 3 days before sacrifice). Tumors from these 2 groups and a control group of untreated animals were removed and quick frozen in liquid N₂. Since these tumors were frozen, they were not used for immunocytochemical studies.

**Nucleic Acid Isolation.** Total nucleic acids were extracted from quick-frozen DMBA- and NMU-induced mammary tumors and 14-day-midpregnant mammary glands by a phenol-chloroform-sodium dodecyl sulfate extraction procedure at pH 8.0 (25). DNA and small RNA molecules were removed by 3 extractions with 3 M sodium acetate, pH 6.0. The total RNA samples were precipitated in ethanol, and the resulting pellet was resuspended in water and stored at -70°.

**Quantitation of Casein mRNA Levels.** A specific, full-length 15S casein probe (specific activity, 5 X 10⁶ cpm/μg) was synthesized from a purified 15S casein mRNA fraction as described by Rosen and Barker (18). cDNA titration hybridizations were performed by a modification of the procedures of Young et al. (29) and Pauley et al. (16). Each 25-μl reaction contained 0.73 ng of cDNA, and incubations were carried out for 48 hr to an equivalent CDT of 0.09. The concentration of casein mRNA in each sample was determined by comparing the slope of each hybridization curve with the slope obtained when purified casein mRNA was hybridized to the casein cDNA probe. Slopes were determined by subjecting the linear portion of each hybridization curve to least-squares analysis.

**Casein Detection by PAP Staining.** Samples of DMBA tumors (midpregnant and virgin) and 14-day-midpregnant mammary glands were embedded with Tissue Prep (Fisher) in a Lipshaw Tramitac Tissue Processor following fixation in Telley's solution. Five-μm-thick serial sections of each tissue sample were placed onto microscope slides. For histological evaluation, samples were stained with either trichrome or hematoxylin and eosin. PAP staining was performed according to the technique of Sternberger (22) as modified by Medina et al. (13).

A specific anti-casein rabbit antisera was prepared against an equal mixture (by weight) of each of the major rat casein proteins I, II, and III as described in detail elsewhere (20). Normal rabbit serum, goat anti-rabbit IgG, and the PAP complex were supplied by Cappel Laboratories. Controls for the PAP procedure include use of normal rabbit serum in place of the anti-casein antisera and adsorption of the casein antibodies by incubation with a 100-fold excess of purified, unlabeled casein. Following PAP staining for the detection of casein, the treated samples were photographed using a Zeiss photomicroscope.

**RESULTS**

**Analysis of Tumor Casein mRNA Levels.** In order to study the relationship between hormonally regulated growth and the induction of differentiated function, we measured both the basal and induced casein mRNA levels in 2 different carcino-gen-induced mammary carcinomas. Casein mRNA levels were compared in DMBA-induced mammary tumors growing in either 14-day-pregnant or virgin animals, in NMU-induced tumors from untreated or ovariectomized rats, or in animals treated with thioproperazine, a drug which results in elevated serum prolactin levels. To insure that the DMBA-induced tumors grown in the pregnant animals were hormone responsive, each tumor was measured with calipers starting at Day 1 of pregnancy (data not shown). Each tumor grew rapidly and displayed an enhanced growth rate when compared to tumors grown in virgin animals (19). The growth rates for the tumors in the pregnant animals were comparable to those in virgin rats treated with estrogen and/or prolactin (19). NMU-induced tumors from thioproperazine-treated animals were also hormone responsive in that they continued to grow during the 3-day period of drug treatment.³ Titration hybridizations were performed using a 15S casein cDNA probe and total tumor RNA. The validity of this assay in quantitating levels of specific mRNAs has been documented in detail in previous studies (16, 29). A 3.4-fold increase in casein mRNA levels was observed in DMBA-induced tumors grown in pregnant animals when compared to those from virgin animals (Table 1). The levels of casein mRNA were 1.2 ± 0.4% and 0.35 ± 0.05% of those found in RNA isolated from 8-day-lactating mammary tissue for the tumors grown in the pregnant and virgin animals, respectively. The 8-day-lactating RNA represents a maximally induced state of casein gene expression (18). Thus, even in an identical hormonal environment, the casein mRNA sequences are expressed at 40-fold-lower levels in the tumors as compared to the normal 14-day-pregnant mammary gland which was taken from the tumor-bearing animal (Table 1, 40 to 50% of 8-day-lactating RNA). Tumors grown in pregnant animals displayed enhanced growth rates compared to those grown in virgin animals, probably in response to the elevated serum placental lactogen, prolactin, and steroid hormone levels during pregnancy. However, only a 3.4-fold induction of casein mRNA sequences was observed in these tumors. Thus, they have

* P. Kelly, personal communication.
URf retained an attenuated ability to express these mammary gland-specific gene products.

Casein mRNA levels were also measured by cDNA titration hybridization in primary, NMU-induced mammary tumors, another type of hormonally dependent mammary adenocarcinoma. As shown in Table 1, the casein mRNA levels from control tumors were $0.135 \pm 0.01\%$ of the 8-day-lactating level. This low level of expression is comparable to that found in the DMBA-induced tumors grown in virgin animals. Tumors from ovariectomized animals contained a slightly decreased level of casein mRNA as compared to the control tumors, while a 2.1-fold induction of casein mRNA was observed in the tumors from the thioproperazine-treated animals. Thus, for both types of hormonally dependent mammary tumors, a limited induction of casein mRNA was observed, but maximally induced levels were usually only 0.2 to 2% of the levels found in the 8-day-lactating rat mammary gland.

**Casein Localization in Mammary Cell Populations by PAP Staining.** Titration hybridization using a specific casein cDNA probe revealed that both the DMBA- and NMU-induced mammary tumors synthesize very low levels of casein mRNA which can be increased by hormonal manipulation of the tumor-bearing animal (Table 1). Since casein inducibility appears to be a marker for hormone responsiveness in these tumors, we wanted to determine if the induction of casein synthesis was due to a low level of increased synthesis throughout the entire tumor cell population or the response of only a few maximally induced cells. Immunocytochemical studies were performed using a specific anti-casein antiserum and PAP staining to localize casein in fixed sections of DMBA-induced mammary tumors grown in either pregnant or virgin animals.

A trichrome-stained section of hormone-stimulated midpregnant gland is shown in Fig. 1A. Secretory alveolar and ductal cells as well as fat cells and connective tissue can be seen in the figure. PAP staining with the anti-rat casein antiserum revealed intense staining of the secretory epithelial cells in the midpregnant gland (Fig. 1C). Negatively reacting controls stained with normal rabbit serum are shown in Fig. 1B. The vast majority of the secretory epithelial cells displayed intense staining, and a uniform response was evident (Fig. 1C).

Fig. 2A shows a hematoxylin and eosin-stained section of a DMBA-induced mammary tumor grown in a virgin animal. The tumor epithelial cells are not arranged into secretory units as found in the normal midpregnant gland but rather are found in a random arrangement which is more typical of a mammary adenocarcinoma. In addition, very few fat cells and an increase in connective tissue are observed in these tumors. When casein localization was analyzed in these tumors, only a few cells were positively stained (Fig. 2C, arrow), while the great majority of the epithelial cells contained no detectable casein. The negatively stained control is shown in Fig. 2B.

When comparing casein localization patterns between the normal mammary gland and the tumors, it is necessary to keep in mind the morphological differences that exist between the 2 tissues. Approximately 35% of the cells from a normal mammary gland from a 14-day-pregnant rat are epithelial cells, compared to 80 to 90% of the cells in a lactating animal and only 5% in a virgin animal (18). The majority of the cells from the midpregnant gland are fat cells which do not synthesize milk. In the case of the DMBA-induced mammary adenocarcinomas, the vast majority of cells are epithelial in nature. Thus, the few cells that stain positively for casein synthesis represent only a very small fraction of the tumor epithelial cell population, while in the midpregnant gland, virtually all of the epithelial cells were positive for casein synthesis.

In the mammary tumors taken from 14-day-pregnant animals, a different pattern of casein localization was seen. Instead of a small number of single cells containing casein, as in the tumors grown in virgin animals, there were clusters of positively stained cells containing large quantities of casein randomly located throughout the tumor (Fig. 3C). In examining serial...
that >95% of the cells from tumors grown in pregnant animals were negative for casein synthesis was obtained by a more qualitative approach. At least 5 PAP-stained serial sections from different parts of each tumor were observed under the microscope. The entire tissue section on each slide was examined. The small areas containing clusters of casein-synthesizing cells were scored and compared with the large area of the tissue slice which contained no loci of casein-containing cells. The figure of a representative cluster of casein-positive cells found in the tumors growing in pregnant animals (Fig. 3C) represents an enlargement of a very small section of a large

sections of these tumors where most of the cells are epithelial in nature, there were very few clusters of positively staining cells. The vast majority of the tumor epithelial cells do not appear to synthesize casein, while in the midpregnant gland, the vast majority of the epithelial cells were synthesizing milk proteins. The negatively stained control is shown in Fig. 3B. In tumors grown in pregnant animals, >95% of the cells appear to have lost their ability to synthesize casein, even though the hormonal milieu of pregnancy resulted in an enhanced growth response of the tumors. Since one cannot count cells accurately in 5-μm-thick tissue slices used in this study, our estimate

Fig. 2. Casein localization in DMBA-induced mammary tumors from virgin animals by PAP staining. A, a 5-μm-thick section of fixed DMBA-induced mammary tumor from virgin animals which was stained with H & E; B, negative control of a DMBA-induced tumor section utilizing the anti-rat casein antibody; C, a tissue section stained by the PAP procedure in conjunction with anti-rat casein antibody. Arrow, representative single cell which is synthesizing casein. All other experimental details are described in Fig. 1. s, secretory cells. x 250.

Fig. 3. Casein localization in DMBA-induced mammary tumors from 14-day-midpregnant animals by PAP staining. A, an H & E-stained section of hormonally stimulated DMBA-induced tumor; B, the negative control as described previously; C, positively PAP-stained tumor section. The dark-staining material identifies the cluster of casein-synthesizing cells. Other experimental details are described in Fig. 1. x 250.
tissue slice as do all the other figures. Figures with lower magnification present a larger area of the tissue slice and reveal that there are very few of the casein-positive cell clusters in these tumors (data not shown). It is interesting to note that, in the hematoxylin and eosin-stained sections of these tumors (Fig. 3A), the epithelial cells appear to have more of a tubular or secretory arrangement than is found in tumors growing in virgin animals. Thus, the attenuated ability of the DMBA-induced mammary tumors to synthesize casein is due to a very small percentage of the transformed epithelial cells which retain their ability to synthesize casein and not to a very low level of synthesis by the entire tumor cell population. The hormonal induction of casein mRNA sequences is presumably a result of both an increase in the number of cells which are actively synthesizing casein mRNA as well as an increase in casein mRNA levels in these cells.

DISCUSSION

These studies demonstrate that milk protein gene expression, as measured by both molecular hybridization and PAP staining, is a useful biochemical marker for examining hormone responsiveness and the expression of differentiated function in some types of experimental mammary cancer. Both prolactin and estrogen play important roles in the induction and development of DMBA- and NMU-induced tumors and milk protein gene expression (26, 28). In the normal rat mammary gland, the primary lactogenic hormones are prolactin and placental lactogen. The importance of prolactin in the growth of these mammary tumors has been inferred by in vivo studies in which tumor growth was inhibited by either an anti-prolactin antisem or administration of ergot alkaloids, which have been shown to reduce the levels of serum prolactin (2, 3, 11). The induction of casein mRNA levels in the DMBA-induced tumors grown in pregnant animals may result from the increases in serum prolactin and placental lactogen which occur during pregnancy (14). A similar increase in casein mRNA sequences in the NMU-induced tumors was due to elevated serum prolactin levels, which resulted from thioproperazine treatment. The slight decrease in casein mRNAs in these tumors, observed in ovariecómized animals, was probably the result of both decreased serum estrogen levels and the resulting decrease in prolactin release from the pituitary.

These data are consistent with previous findings in our laboratory, which demonstrated low levels of casein mRNA in DMBA-induced tumors from ovariecómized animals receiving hormone replacement (19). Casein mRNA levels were only 2% or less of those observed in 9-day-lactating mammary gland. The highest levels of casein mRNA were found in prolactin- and estradiol-treated animals as compared to animals given estradiol alone or in the absence of exogenous hormone. Similarly, prolactin-inducible casein mRNA activity has been demonstrated in the transplantable R3230AC mammary carcinoma (15). The maximal levels observed, however, were only 1% of the total mRNA activity. Both DMBA- and NMU-induced mammary tumors have been reported to contain the milk protein, α-lactalbumin, at levels equal to or less than 10% of the levels observed in the 5-day-lactating rat mammary gland. Elevation of serum prolactin by transplantation of a pituitary gland into the kidney capsule of the host increased the α-lactalbumin content of the DMBA-induced tumors but reduced the α-lactalbumin content in the NMU-induced tumors (17). This suggests that differential regulation of the milk protein genes may occur in these tumors, since an increase in casein mRNA in NMU-induced tumors was observed in our studies in thioproperazine-treated rats. This phenomenon has also been observed in the R3230AC mammary tumor line, which showed a prolactin-mediated increase in casein mRNA activity with no effect on α-lactalbumin mRNA activity (17).

The low levels of milk protein synthesis in DMBA-induced mammary tumors can be attributed to the inability of the vast majority of the tumor epithelial cell population to synthesize casein. Casein localization studies clearly indicate that, in tumors from virgin animals, only single cells scattered randomly throughout the tumor are synthesizing casein. Hormonal stimulation of these tumors results in an increase in casein mRNA levels, which can be correlated with an increase in the number of cells which are synthesizing large quantities of casein. These can be seen as clusters of cells which were again located randomly throughout the tumor sections. No such clusters were found in the tissue sections from tumors grown in virgin animals. However, the vast majority of the cells (>95%) from the hormone-stimulated tumors were not induced to synthesize casein. A similar result has been observed in the hormone-responsive R3230AC rat mammary carcinoma, using the same approach and antisera against both casein and α-lactalbumin (7) and using primary cultures of hormone-responsive mouse mammary HAN (24). The hormonal stimulation of HAN by transplantation into pregnant mice induces casein synthesis in only a subpopulation of HAN cells. These cells have been identified in primary cell culture using an indirect immunofluorescence assay (24). The weak induction of casein mRNA levels observed in both preneoplastic and neoplastic tissues is therefore probably a reflection of both the proliferation of cells with the capability of synthesizing casein as well as the hormonal induction of casein gene expression within this minor responsive cell population.

The loss or attenuation of mammary gland-differentiated function in the vast majority of DMBA tumor cells does not appear to be the result of a receptor-minus phenotype, which is observed in most hormone-resistant cell lines (1, 21), since both types of carcinogen-induced mammary carcinomas are hormonally responsive, requiring estrogen and prolactin for their induction and maintenance of growth (10, 28). Several investigators have examined the relationship between hormone dependence and the levels of steroid (8, 12) and peptide hormone receptors (5, 27). Even though these studies are complicated by the observation that a heterogeneity of cell types exists with these tumors (4), it appears that, in the case of DMBA- and NMU-induced mammary tumors, the low level of milk protein synthesis is not due to a defect at the level of the receptor.

Currently, studies are being performed in this laboratory to determine if the attenuated ability of DMBA- and NMU-induced mammary tumors to synthesize casein is due to changes in gene structure (e.g., methylation, rearrangement) in these tumors. We are also utilizing the techniques developed by Suard and Kraehenbuhl (23) to separate and identify cell types within the DMBA-induced tumors in order to better understand the origin and hormonal responsiveness of the subpopulation of casein-synthesizing cells as well as the majority of the cells which have lost their ability to regulate casein gene expression.
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