Immunocytochemical Demonstration of Hormonally Regulable Casein in Tumors Produced by a Rat Mammary Stem Cell Line

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ABSTRACT

The rat mammary epithelial stem cell line, Rat Mammary 25, and the dimethyl sulfoxide-resistant variant derived from it, Rama 259, when injected separately into female, virgin, athymic nude mice formed tumors which grew at approximately the same rate. When these mice were mated, the growth rate of the Rama 259 tumors increased 2.5-fold during late pregnancy and lactation, while that of the Rama 25 tumors was unaffected. The tumors in all cases consisted of glandular-like structures and blocks of cuboidal and elongated cells. Antibodies were raised against the three major caseins in rat milk. Examination of histological sections showed that antibodies immunocytochemically stained some of the cells (5 to 20%) in the glandular regions, a few (1 to 5%) of the cuboidal cells, and none of the elongated cells in Rama 25 tumors from lactating mice. Only a relative few (1 to 5%) of the glandular cells stained in Rama 25 tumors from perphenazine-treated mice, while no cells (<1%) stained in tumors from virgin mice. The casein antibodies failed to stain any cells (<1%) in the Rama 259 tumors even when they stained luminal epithelial cells in the mammary glands taken from the same lactating or perphenazine-treated mice. These results demonstrated that a minor population of cells within the Rama 25 tumors can be hormonally regulated to produce casein in a way similar to that for the majority of the cells in normal mammary glands.

INTRODUCTION

Development of normal mammary glands in rats occurs by processes of both cell multiplication and cell differentiation under the controlling action of mammmotropic hormones which circulate during pregnancy and lactation. These hormones include prolactin, estrogen, glucocorticoids, insulin, and progesterone (1). The same hormones also control the growth of DMBA2-induced tumors of the rat mammary gland (2). The major differentiation-specific protein which is secreted by the rat mammary glands is casein (3), and this can be detected in the differentiated sections of the gland, the alveoli, by immunofluorescent (6, 19) or immunocytochemical (7, 15) techniques using casein antibodies on histological sections of pregnant and lactating animals. In DMBA-induced mammary tumors in pregnant but not in virgin rats, a small proportion of cells also contain immunoreactive casein (15). This suggests that the production of casein is controlled in a similar manner in both normal and tumor cells.

Recent studies in vitro have raised the possibility that mammary epithelial development can take place by a process of cellular interconversions (4, 9, 10). In particular, a tumorigenic cuboidal epithelial stem cell line, Rama 25 (in which 25 is the single-cell clone number), has been isolated from a DMBA-induced rat mammary tumor (4). This cell line can differentiate in culture, forming either droplet and doming (alveolar-like) or elongated (myoepithelial-like) cells (4, 9, 14) which are the main differentiated epithelial cells seen in normal glands and in the early carcinogen-induced tumors of the rat after the animals were mated (18). Differentiation to alveolar-like cells, which was followed by measuring the secretion of small quantities of immunoreactive casein in the tissue culture medium, could be enhanced by dimethyl sulfoxide in combination with the hormones prolactin, hydrocortisone, insulin, and estrogen, while no enhancement was seen with a variant cell line Rama 259 (13). We have now investigated whether the tumors formed by Rama 25 and Rama 259 in nude mice can also make casein in vivo and under what conditions.

MATERIALS AND METHODS

Tumor Induction. The isolations of the single-cell-cloned cell line, Rama 25, and the DMSO-resistant variant cell line, Rama 259, have been described previously (4, 13, 14). Both cell lines were plated at a density of 105 in 50 ml of Dulbecco's modified Eagle's medium, 10% fetal calf serum, insulin (50 ng/ml), hydrocortisone (50 ng/ml) in 600-ml Falcon flasks and grown until just confluent in an atmosphere containing 10% CO2 at 37° for 5 days. The medium was removed, cell monolayers were washed twice with PBS, and the cells were removed by digestion with trypsin-EDTA solutions and collected in ice-cold PBS after 4 centrifugations (13). To induce tumors, 2 × 106 cells in 0.2 ml PBS were injected s.c. into the right inguinal regions of 6-week-old female Institute of Cancer Research C57BL athymic nude (nu/nu) mice (5). Tumor size was recorded as the average of 2 diameters at right angles (13). When the tumors were just under 1 cm in diameter (55 to 56 days postinoculation), groups of the animals were either mated with male BALB/c mice or given daily s.c. injections of 0.2 ml of perphenazine (0.5 mg/ml) in 25% ethanol-75% PBS (3). Mice bearing tumors were sacrificed 1 to 3 days postpartum, after 5 days of injections of perphenazine or after no treatment. Both the tumors and the left inguinal mammary glands were removed and processed for histology. The injection site was directed away from any mammary glands, and the resultant tumors contained no incorporated mammary glands.

Serology. Antisera was raised in rabbits against a mixture of the 3 separately purified caseins (molecular weights, 42,000, 29,000, and 24,000) from rats' milk as described previously (4, 16). Purified IgG fractions were obtained by precipitation with 25% ammonium sulfate followed by chromatography on DEAE-cellulose and were used diluted 1:1000 with 0.5% bovine serum albumin in PBS. The antisera was characterized by the following method. Explants from lactating rat mammary glands were radioactively labeled with [3S]methionine, the extracted material was incubated with antisem serum, immune complexes were isolated with Protein A-Sepharose, and the precipitate was analyzed by polyacrylamide gel electrophoresis. This yielded the M, 42,000, 29,000, and 24,000 proteins corresponding to the 3 rat caseins described by Rosen et al. (8). Prior absorption of the antisera with the pure caseins, but not actin, prevented immune precipitation of the
radioactive caseins. The antitissuease serum also failed to react with proteins in the acidic whey fraction of rat milk or rat α-lactalbumin. In culture, Rama 25 cells made only the M, 42,000 casein component (9, 10), and this component had the same major tryptic peptides as the M, 42,000 component from lactating glands. Sheep anti-rabbit IgG was purified by affinity chromatography and conjugated to alkaline phosphatase (Sigma, type VII) with glutaraldehyde (1). Absorbed antiserum was prepared by incubating the antitissuease serum with purified caseins (1 mg/ml) at 37°C for 3 hr.

**Results**

**Physiological State and Growth Rate of Tumors.** Injection of $2 \times 10^6$ Rama 25 or Rama 259 cells into female nude mice produced tumors in 90 to 95% of the animals within 6 weeks, and the tumors grew at approximately the same rate (Chart 1). Prior to injection, no cells in the monolayer cultures stained with anticasein serum. Two methods were used to stimulate mammary differentiation and alveolar development in those mice bearing tumors: (a) treatment of the mice with perphenazine which stimulates hypersecretion of prolactin (3); and (b) mating. Mammary glands from the tumor-bearing mice which were treated with perphenazine showed only a very moderate ductal growth and a little lobule formation, but this was by no means as extensive as in those glands from pregnant and lactating animals (4). This staining was abolished by absorption of the antiserum with purified caseins (5). Absorbed antiserum stained the luminal regions of many ducts and most alveoli and the intraluminal debris in glands from midpregnant and lactating animals (Fig. 1A). This staining was greatly enhanced (Chart 1). Thus, the ratios of increase in diameter of the tumors between 65 and 85 days and that between 30 and 50 days after injecting the cells (i.e., during pregnancy-lactation and the virgin reproductive states) were 0.95 that between 30 and 50 days after injecting the cells (i.e., during pregnancy-lactation and the virgin reproductive states) were 0.95 for the Rama 25 tumors and 2.6 for the Rama 259 tumors. This increase in rate of growth of the Rama 25 tumors, as measured by the increase in their average diameters, was relatively unaffected after the animals were mated, while that of the Rama 259 tumors was greatly enhanced (Chart 1). Thus, the ratios of increase in diameter of the tumors between 65 and 85 days and that between 30 and 50 days after injecting the cells (i.e., during pregnancy-lactation and the virgin reproductive states) were 0.95 for the Rama 25 tumors and 2.6 for the Rama 259 tumors. This increase in rate of growth of the Rama 25 tumors became significantly different only 9 to 18 days after the animals were mated (Chart 1).

**Physiological State and Casein-producing Tumor Cells.** Immunocytochemical staining of the inguinal mammary glands of female Wistar-Furth rats and nude mice (Table 1) with anticasein serum yielded only a little staining (1 to 5% epithelial cells) for mammary glands from mature, virgin animals (60 to 65 days). However, the antiserum stained the luminal regions of many ducts and most alveoli and the intraluminal debris in glands from midpregnant and lactating animals (Fig. 1A). This staining was abolished by absorption of the antiserum with purified caseins (8).

The Rama 25 and Rama 259 tumors in nude mice consisted of circular, duct-like structures, blocks of cuboidal cells, and elongated cells (Fig. 1, C, F, and G) (11, 13), all derived from the injected cell lines. Antibodies to casein failed to stain any cells in

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4 C. M. Hughes and P. S. Rudland, unpublished results.
amount of casein synthesized by Rama 25 cultures is only about agreement with our previous results in vitro where the maximum amount of casein mRNA is only 1 to 2% of that found in the lactating rats (15) and is also consistent with the finding that the 5% or less reported for these cells in DMBA-induced tumors in occur in blocks of cuboidal cells (11). This proportion of casein-glandular structures and elongated cell regions contain only a cells in the Rama 25-induced tumors stain with the anticasein producing cells in the Rama 25 tumors is similar to the value of serum. This proportion is probably about 1 to 5%, since the mammary gland. However, only a small proportion of the casein, one of the main polypeptide products of differentiation of tumors in the virgin mice (Table 1; Fig. 1E), even though a few luminal epithelial cells stained in mammary glands from the same mice (Table 1). In the mammary glands from the perphenazine-treated mice, many of the alveolar cells and a few of the ductal cells specifically stained with antibodies to caseins; the other cells failed to stain (Table 1). A few (1 to 5%) of the tumor cells in duct-like structures from Rama 25 tumors stained specifically with anticasein serum, while no staining was detected in the Rama 259 tumors. In mammary glands from the tumor-bearing lactating mice, most of the epithelial cells in the normal alveolar structures specifically stained with antibodies to casein (Fig. 1, A and B). In the Rama 25 tumors, many of the cells in duct-like structures also stained with antibodies to casein (Fig. 1C; Table 1), and this staining could be abolished by prior absorption of the antiserum with the caseins (Fig. 1D). A small fraction (1 to 5%) of the blocks of cuboidal cells also stained in the Rama 25 tumors (Fig. 1F), although the vast majority and all the elongated cells (Fig. 1G) did not (Table 1). Duct-like structures (Fig. 1H) and cuboidal and elongated cells from Rama 259 tumors growing in lactating mice completely failed to stain with anticasein serum (Table 1) even though mammary glands in the same mice were extensively stained (Fig. 1A; Table 1).

**DISCUSSION**

These studies demonstrate that the cell line Rama 25 can induce tumors which, in the lactating nude mouse, produce casein, one of the main polypeptide products of differentiation of the mammary gland. However, only a small proportion of the cells in the Rama 25-induced tumors stain with the anticasein serum. This proportion is probably about 1 to 5%, since the glandular structures and elongated cell regions contain only a minority of the tumor cells; most of the cells in these tumors occur in blocks of cuboidal cells (11). This proportion of casein-producing cells in the Rama 25 tumors is similar to the value of 5% or less reported for these cells in DMBA-induced tumors in lactating rats (15) and is also consistent with the finding that the amount of casein mRNA is only 1 to 2% of that found in the mammary glands of lactating rats (15). These results are also in agreement with our previous results in vitro where the maximum amount of casein synthesized by Rama 25 cultures is only about 1% of that found in organ cultures of midpregnant glands grown in a similar hormonal environment (13). Since no cells in the monolayer cultures of Rama 25 stained with antibodies to casein prior to injection and the percentage of cells staining in tumors in the lactating mice and in the monolayer cultures after suitable hormonal treatments (4) were similar in both cases at 1 to 5%, then it is unlikely that any selection for specific cell types had occurred in the nude mouse tumors. However, since Rama 25 was originally obtained from a single-cell clone (4), it is also unlikely that clonal variation in the extent of casein gene expression could have arisen in the epithelial cells of the tumor. It is more likely that much of the observed heterogeneity of casein production among the tumor epithelial cells results from alterations in their local environment. In support of the latter explanation, most of the cells producing casein occur in organized glandular-like structures, and only a very few epithelial cells outside these structures produce casein. Similarly, when Rama 25 cells are grown on plastic dishes in vitro, most of the immunoreactive casein is detected in epithelial cells in organized structures called domes, rather than in the surrounding epithelial cells which form a flat monolayer (4). However, since Rama 25 cuboidal epithelial cells also irreversibly generate elongated cells with some myoepithelial characteristics both in vitro and in the tumors as determined by biochemical, immunocytochemical, and ultrastructural criteria, then differentiation along this pathway yields cells which are incapable of producing casein (4, 9). Therefore, these elongated cells could be considered to have lost the ability to express the casein gene.

Our results also show that the number of Rama 25 tumor cells producing casein is increased both in perphenazine-treated mice and, more especially, in lactating mice. The increase in the number of cells producing casein is difficult to estimate since there are so few (<1%) in Rama 25 tumors from virgin animals. However, there would appear to be at least an order of magnitude increase in the number of cells producing casein in Rama 25 tumors in lactating mice. This is in approximate agreement with the 3.5-fold increase in the amount of casein mRNA in DMBA-induced tumors in lactating compared with those in virgin rats (15) and the 10- to 20-fold increase in the amount of casein secreted into the medium of the Rama 25 cultures after they were treated with mammotrophic agents (13). In tissue culture,
a combination of DMSO and the hormones prolactin, hydrocortisone, insulin, and estradiol induces the maximum rate of differentiation of Rama 25 cells into alveolar-like cells, and the combination prolactin, estradiol, and DMSO is the most important for the production of casein (13). This is consistent with our results in vivo where perphenazine, which is thought mainly to stimulate the circulating levels of prolactin (3), causes a small number of cells to produce casein in the tumors. This increase is amplified when the tumor cells are exposed to the additional hormonal changes, including the increase in estrogens, that occur in the lactating animal. Recently, we have been able to replace the synthetic inducer, DMSO, with the naturally occurring hormonal agents prostaglandin E, (13) or vitamin A (12) in culture. Whether these agents play a similar role in inducing differentiation of Rama 25 cells in vivo is unknown.

In tissue culture, Rama 259, a subclone of Rama 25, which was selected for its ability to survive and grow in media containing 1.8% DMSO, fails to produce casein with any of the inducers and the mamatomorphogenic hormones. Likewise, epithelial cells in the tumors produced by Rama 259 fail to produce casein under the same conditions in which some cells do in Rama 25 tumors. However, although the growth rate, as measured by an increase in the diameter of the tumors, is relatively unchanged for the Rama 25 tumors after mating of the mice, that of the Rama 259 tumors is dramatically increased during late pregnancy and lactation. Histological examination of the tumors shows that the increase in the average diameter of the Rama 259 tumors reflects a real increase in the number of tumor cells, and the relative proportions of cells in glandular-like areas, in blocks of cuboidal or elongated cells are relatively unchanged. The reason for this increase in cell number is unknown, but it is possibly unrelated to the effects of prolactin or estrogens and may be dependent upon other hormones and factors (10) that change during pregnancy and lactation. In conclusion, we have shown that a subpopulation of casein-producing cells can be detected in tumors produced by Rama 25 cells in nude mice, and that their appearance coincides with the physiological state of the mouse when casein-producing cells are seen in mammary glands. The fact that virtually no such cells are seen in tumors produced by a variant subline of Rama 25 which is resistant to casein induction by prolactin and estradiol in vivo suggests that these hormones are responsible, in part, for the production of casein-secreting cells in the Rama 25 tumors in vivo.

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