Enhancement of Epidermal Carcinoma Formation by Prolactin in Mice

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ABSTRACT—Long-term administration of prolactin (PRL) markedly enhanced the induction of epidermal squamous cell carcinomas by a chemical carcinogen, 3-methylcholanthrene [(MCA) CAS: 56-49-5], in Swiss male albino mice. DNA radioactivity and quantitative estimation of autoradiographs with the use of [3H]thymidine revealed a significant increase in DNA content (twofold) and in the percentage of labeled neoplastic nuclei in mice treated with PRL plus MCA (48.50%) as compared to that in mice treated with MCA alone (23.50%). Ultrastructural and cytologic studies revealed the predominance of a trabecular-hepatoid type of epidermal carcinoma with advanced nuclear irregularities, glycogen granules, mirror-image cells, and phagolysosomes in PRL-MCA-treated carcinomas as compared to characteristic squamous neoplastic cells with enlarged nuclei, tonofilaments, and keratin formation in epidermal carcinomas following treatment with MCA alone. These findings demonstrate that PRL is an important hormone in epidermal neoplastic cell growth and differentiation.—JNCI 1985; 74:1335-1346.

It has been previously reported that PRL plays an important role in mammary carcinogenesis (1-4). An excess of PRL secretion (or hyperprolactinemia) is of fundamental significance in the induction and development of mammary tumors. Estrogens act mainly by stimulation of PRL secretion and in conjunction with PRL in mammary tumorigenesis (1, 2). Hypothalamic lesions, pituitary grafts, or drugs (biogenic amines) that suppress PRL secretion can either stimulate or inhibit the development of spontaneous or carcinogen-induced mammary tumors in rats or mice (5, 6). Although most data suggest that PRL plays an important role in DMBA-induced mammary tumors in rats (2, 7-9), the role of PRL is less important in N-nitroso-N-methylurea-induced mammary tumors in rats, which are histologically similar to DMBA-induced tumors (10, 11).

Although PRL has been shown to stimulate the growth of different human mammary carcinoma cell lines, the role of PRL in the induction and progression of human breast cancer is still controversial (12, 13). PRL in conjunction with estradiol increased the incidence of uterine squamous cervical carcinomas induced by a local application of MCA in mice, but bromocriptine (a PRL inhibitor) reduced it (14). It has also been found that PRL stimulates DNA synthesis in cell cultures of rat mammary tumors, namely, in conjunction with insulin or cortisol (15). However, little is known regarding the role of PRL in tumorigenesis of nontarget tissues, such as liver or skin, and its mechanism of action on neoplastic cells. The present study reports the long-term effects of PRL on tumor induction, DNA synthesis in neoplastic cells, and ultrastructural and cell surface changes in squamous cell carcinomas induced by a chemical carcinogen, MCA, in mice.

MATERIALS AND METHODS

Experiments were done on cesarean-derived barrier-sustained Swiss male albino mice weighing 30 g each. The mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. The animals were divided into 4 groups of 10 mice each. Group 1 was control mice that received only the diluent (acetone). Group 2 consisted of mice treated topically on their dorsal skin with the chemical carcinogen MCA (CAS: 56-49-5) for tumor induction. MCA was dissolved in acetone solution (0.3%) and applied at a dose of 0.2 ml (or 600 µg) by being pipetted twice a week for 6 months on a marked and shaved area of the dorsal skin of each mouse. Group 3 was made up of mice that were treated simultaneously with MCA as above and given im injections of 50 µg rat PRL diluted in 0.1 ml physiologic saline solution (0.9% NaCl), twice a week for 6 months. The injections were given in the posterior leg muscle and far from the sites of MCA application. Rat PRL was donated in vials of 10 mg by National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases-rat PRL-B-2 at an activity of 20 IU/mg (Crop Sac assay) and was prepared by Dr. A. Parlow (Pituitary Hormones and Antisera Center, Harbor-University of California at Los Angeles Medical Center, Los Angeles, CA). Group 4 mice were treated im with rat PRL only, at 50 µg twice a week for 6 months, as above.

At the end of the experiments (6 mo) and 2 hours prior to sacrifice, all mice received im 5 µCi [3H]dThd (sp act, 28.5 Ci/mmol)/g body weight for the study of DNA synthesis in neoplastic cells. The tumors were counted monthly, and their sizes were also measured. DNA

ABBREVIATIONS USED: ANOVA = analysis of variance(s); DMBA = 7,12-dimethylbenz[a]anthracene; [3H]dThd = tritiated thymidine; H & E = hematoxylin and eosin; MCA = 3-methylcholanthrene; PRL = prolactin.

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radioactivity was measured, and pulse labeling of DNA content in control and neoplastic cells was performed in at least 10 specimens, which were removed from each experimental group. Sections were fixed in Bouin’s fluid, dehydrated in ethanol, and embedded in Paraplast. Measurements were done with the use of a nuclear scintillation system with an efficiency of 40% and [3H]dThd as an internal standard. DNA labeling was expressed as the mean percent of the control’s labeling, plus or minus the standard error.

Autoradiographs in which [3H]dThd was used were made on tumor specimens and control epidermis that were fixed in Bouin’s fluid, dehydrated in ethanol, and embedded in Paraplast. Sections were covered with Kodak NTB2 nuclear emulsion (Eastman Kodak Co., Rochester, NY) by a dipping procedure and exposed for 1 month in a darkroom. The filmed sections were processed in D19 developer and Kodak rapid fixer and stained with H & E.

Quantitative estimation of autoradiographs was performed by counts of the labeled cells with a Zeiss light microscope (objective, × 10; ocular, × 10) from 2,000 consecutive epithelial nucleated cells in the tumor proliferative compartments as well as in the basal layers of control epidermis. The percentage of labeled cells was also recorded. Electron microscopy was performed on small tissue specimens from tumors and from control mouse dorsal skin. The specimens were removed, diced, fixed for 3–4 hours in 3% cacodylate-buffered glutaraldehyde, postfixed for 2 hours in 1% phosphate-buffered osmium tetroxide (OsO4), dehydrated, and embedded in a mixture of Epon-Araldite. Thin sections (≤600 Å) were cut with the use of an LKB ultratome III equipped with a diamond knife, mounted on grids, and stained with uranyl acetate and lead citrate. They were examined under a Hitachi HS-8 electron microscope (Hitachi Ltd., Tokyo, Japan).

Scanning electron microscopy was performed on larger specimens removed from tumors, then fixed in 3% cacodylate-buffered glutaraldehyde for 24 hours, dehydrated, and embedded with the use of the critical point method with Freon 13. Specimens coated with gold (≤200 Å) were viewed in an Autoscan electron microscope (Electron Technology, Inc., Hayward, CA) at 20 kV.

RESULTS

Tumors were first detected at 3 months in both MCA-treated groups, and their incidence was markedly enhanced following a simultaneous treatment with PRL at any interval (text-fig. 1). At the time of sacrifice (6 mo), the number, size, and weight of the tumors were almost double in the MCA-PRL-treated mice as compared to those of tumors in the mice treated with MCA alone. Thus we found a total of 78 carcinomas in the MCA-PRL-treated mice as compared to only 12 large tumors weighing 2,500±300 mg in the mice treated with MCA alone; also, 32 tumors of medium size (8×6×5 mm) with an average weight of 2,165±230 mg were found in the MCA–PRL-treated mice as compared to 18 tumors weighing 1,280±150 mg in the mice treated with MCA alone. Twenty small tumors (4×3×3 mm) with an average weight of 825±75 mg were found in the MCA–PRL-treated mice as compared to only 10 tumors of the same size and weighing 550±69 mg in the mice treated with MCA alone. ANOVA between groups 2 and 3 showed a highly statistically significant difference (P<.001). No tumors were observed in mice treated with PRL alone or in control mice treated with diluent only. However, a mild epidermal hyperplasia was seen in mice treated with PRL alone. No significant changes in the body weights of treated mice as compared to the body weights of control mice were observed.

DNA Synthesis

Measurements of DNA radioactivity also revealed a marked increase (twofold to threefold) in DNA synthesis in the nuclei of neoplastic cells following treatment with PRL plus MCA as compared to the DNA synthesis in tumor cells treated with MCA alone or the DNA synthesis in control epidermal cells (text-fig. 2). ANOVA showed a highly statistical difference (P<.001) between groups 1, 2, and 3 and also a significant statistical difference (P<.02) between groups 1 and 4.

Light microscopic autoradiography revealed interesting findings with regard to the distribution of [3H]dThd in the neoplastic nuclei, which was mainly at the periphery of the tumors; no labeled cells could be seen in the horny or epidermal pearls of the squamous cell
carnicomas following administration of MCA alone (fig. 1). A marked autoradiographic distribution of [3H]dThd in the neoplastic cell nuclei was observed in the carcinomas following PRL and MCA treatment. The autoradiographic reaction was heavier and diffusely distributed in these tumors, exhibiting a more trabecular and hepatoid pattern (fig. 2). Quantitative estimation of autoradiographs revealed significant changes in [3H]dThd incorporation in the neoplastic cell nuclei. Thus a more than twofold increase in DNA synthesis was observed in the tumor cells following a concomitant administration of PRL and MCA (48.50%) as compared to findings for cells treated with MCA alone (23.50%), findings for cells treated with PRL alone (12.50%), and findings for cells of control mice (5.50%). ANOVA between all groups (1-4) showed P < .001 between groups 1, 2, and 3 and P < .02 between groups 1 and 4.

**Tumor Pathology**

Tumor diagnostic examination was made on thick plastic sections (≈0.5 μm) with the use of the light microscope. The examination revealed the predominance of characteristic squamous cell carcinomas with several epithelial horny pearls (fig. 3) in MCA-treated mice. These carcinomas were composed of prickle cells and concentric keratinized layers.

Different structural types of epidermal carcinomas, predominantly trabecular and hepatoid composed of anastomotic cellular cords with intense basophilic cells and hyperchromatic nuclei, could be seen in MCA–PRL-treated mice. Mitoses were occasionally seen (fig. 4). Only a mild, diffuse epidermal cell hyperplasia and parakeratosis occurred in mice treated with PRL alone (fig. 5). No hyperplasia and tumors were observed in control mice treated with diluent (acetone) only.

**Ultrastructural Studies**

Electron microscopic observations revealed typical ultrastructural features of the epidermal carcinomas following treatment with MCA alone. Thus there was a characteristic ultrastructural pattern of squamous cell carcinomas, with polyhedral and large prickle cells, enlarged nuclei containing nucleoli, large populations of tonofilaments and polysomes, mitochondria, and few desmosomes; also, keratinized cells could be seen (fig. 6). Neoplastic cells were separated by narrowed intercellular spaces, giving the appearance of solid tumors.

A different ultrastructural pattern was seen following PRL–MCA treatment, the pattern was predominantly acinar and trabecular with intensely basophilic cells, enlarged and hyperchromatic nuclei, increased numbers of polysomes, mitochondria, nuclear abnormalities, and abundant rough endoplasmic reticulum. Neoplastic cells were separated by widened intercellular spaces (fig. 7).

No tonofilaments or desmosomes could be seen following administration of PRL plus MCA. Sometimes nuclear features such as typical mirror-image cells could be seen in the PRL–MCA-treated carcinomas. Advanced nuclear and nucleolar alterations with bizarre nuclear outlines or polylobulated nuclei occupying almost the entire cytoplasm could also be seen. Occasionally seen were large phagolysosomes, occupying almost one-third of the cytoplasm, increased numbers of swollen mitochondria and concentric myelinated figures similar to nebenkern, and β-glycogen particles.

Scanning electron microscopy better visualized the cytoarchitecture and cell surface pattern of the neoplastic cells. Thus tumor cells of MCA-treated mice were flattened and polyhedral with sparse and tiny microvilli and were separated by narrowed intercellular spaces and desmosomes (fig. 8). By comparison, neoplastic cells of MCA–PRL-treated mice were fully rounded with numerous, thick, and coalescent microvilli and blebs on their surfaces. Tumor cells were separated by widened intercellular spaces and were devoid of desmosomes (fig. 9). Engulfment of neoplastic cells by their neighbor cells or apoptosis could be seen in MCA–PRL-treated specimens. An intense stromal reaction with dispersed rounded tumor cells, covered by thick and coalescent microvilli and large blebs and separated by erythrocytes and an abundant network of connective fibrils, could also be seen following treatment with MCA plus PRL (fig. 10).

**DISCUSSION**

The present findings clearly demonstrate that long-term PRL administration markedly enhanced the epidermal carcinoma formation and DNA synthesis in the squamous neoplastic cells, increased the cellular atypicality, and changed the cytodifferentiation of squamous neoplastic cells. Thus the incidence and development of carcinomas chemically induced by MCA in mice as well as their cellular differentiation toward a more trabecular and hepatoid pattern with mitoses and a large population of lysosomes are markedly accelerated by PRL. No
tumors or epidermal hyperplasias were seen in control mice treated with solvent only.

There have been extensive studies that demonstrate the important role of PRL in mammary tumorigenesis, both in the development of spontaneous mammary tumors in mice and rats as well as in the induction and development of DMBA-induced mammary carcinomas in rats. It was also documented that these are PRL-dependent tumors; thus an increase in PRL secretion frequently promotes tumor growth, whereas a decrease or inhibition of PRL secretion by anti-PRL ergoline derivatives is usually followed by tumor regression (5, 6, 11).

A synergistic effect of estradiol and PRL that increased the incidence of MCA-induced uterine cervical carcinomas was previously reported in mice. Simultaneous administration of bromocriptine reduced the tumor incidence and invasion (14). However, no information is available on whether PRL plays an important role in controlling the incidence and growth of other tumors, namely, tumors occurring in nontarget tissues of the hormone. Although the study regarding the role of hormones in neoplastic cell differentiation is a fascinating field, the mechanism by which PRL influences the growth and differentiation of neoplastic cells is not yet fully understood. Since most investigations were done with the use of PRL in association with other hormones, such as estrogens, insulin, or hydrocortisone, it is difficult to evaluate the action of PRL alone on tumor cell growth, differentiation, or DNA synthesis in neoplastic cells.

It seems likely from the present investigations that PRL exerts its tumor enhancement effects by stimulating DNA synthesis in the epidermal neoplastic cells. Thus a significant increase in DNA synthesis, almost twofold, was revealed by DNA radioactivity and autoradiographic studies in PRL–MCA-treated tumor cells (48.50%) as compared to the level in cells treated with MCA alone (23.50%). The PRL–MCA-treated cells had a ninefold increase in DNA synthesis as compared to the level in epidermal cells of controls (5.50%). These findings agree with those previously reported in cell cultures from rat mammary carcinomas and normal epithelial cells. The addition of ovine and rat PRL at 50 μg/ml increased the DNA synthesis in the epithelial cells about 10%, but when higher concentrations (500 μg/ml) were added, DNA synthesis was increased to a maximum of 30%. PRL with insulin was relatively inactive in promoting DNA synthesis of the nonepithelial cells (15). Thus PRL is a mitogenic hormone and acts on tumor cell growth and differentiation, possibly by stimulating DNA synthesis. PRL, a pituitary hormone, stimulates DNA synthesis, tumor cell growth, and differentiation not only in target cells, such as epithelial mammary cells, but also in other epithelial cells, such as epithelial cells. Thus PRL may facilitate the expression of a “malignant” phenotype and, consequently, may drastically change the cytodifferentiation of epidermal neoplastic cells (16).

Recently, the detection of PRL receptors in human breast cancer was correlated with the histology and presence of other hormones (12, 13). In addition, a correlation between the number of PRL receptors and the responsiveness of DMBA-induced mammary tumors in rats was determined (17). PRL receptors were also found in other experimental and human carcinomas (human prostatic adenocarcinoma, Dunning R3327H rat prostatic carcinoma, hamster renal adenocarcinoma) and in normal breast tissue, prostatic tissue, and liver (7, 18). A good correlation between the concentrations of PRL receptors and estrogen receptors and a favorable response to endocrine ablation was reported in rat mammary tumors (2). No information is available regarding the presence of PRL receptors in normal epidermal cells or epidermal carcinomas. The marked changes in DNA synthesis and in cell surfaces as well as a mild epidermal hyperplasia induced by PRL suggest that PRL acts similarly to other cocarcinogens (4, 16).

Overall, the present findings strongly suggest that PRL regulates the tumor growth, tumor invasiveness, and neoplastic cell differentiation in chemically induced squamous cell carcinomas by acting on DNA synthesis and by inducing surface changes in epidermal neoplastic cells.

REFERENCES

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FIGURE 1.—Light microscopic autoradiograph showing a peripheral distribution of $[^3$H$]d$T$hd$-labeled neoplastic nuclei in a squamous cell carcinoma following MCA treatment. No labeled cells can be seen in the horny pearls. Paraplast section, covered with NTB$_2$ nuclear emulsion and stained with H & E. Bar = 40 $\mu$m. $\times$ 400

FIGURE 2.—Heavy autoradiographic distribution of $[^3$H$]d$T$hd$-labeled neoplastic nuclei in an anaplastic, invasive carcinoma in mouse treated with PRL plus MCA. Paraplast section, covered with NTB$_2$ nuclear emulsion and stained with H & E. Bar = 40 $\mu$m. $\times$ 400
FIGURE 3.—Squamous cell carcinoma with several and characteristic horny pearls. Mouse treated with MCA alone. Plastic thick section (0.5 μm) stained with toluidine blue. Bar = 40 μm. × 400

FIGURE 4.—Anaplastic, invasive carcinoma exhibiting a hepatoid and trabecular pattern with several mitoses (arrows). Mouse treated with PRL plus MCA. Plastic thick section (0.5 μm). Bar = 40 μm. × 400
FIGURE 5.—Mild and diffuse epidermal cell hyperplasia with parakeratosis occurred in mice treated with PRL alone. Plastic thick section (0.5 μm) stained with toluidine blue. Bar = 40 μm. X 400

FIGURE 6.—Electron micrograph showing characteristic squamous neoplastic cells with enlarged nuclei (N) and nucleoli (Nc), several tonofilaments (T), desmosomes (d), and keratin formation (K). Intercellular spaces (Is). Mouse treated with MCA alone. Epon-Araldite, uranyl acetate, and lead citrate stains. Bar = 1 μm. X 8,000
Figure 7.—Electron micrograph showing atypical and small tumor cells with nuclear abnormalities (N), abundant rough endoplasmic reticulum (Rer), mitochondria (M), and dense granules (dg). Cells are separated by widened intercellular spaces (Is) and capillary (C). Mouse treated with PRL and MCA. Epon-Araldite, uranyl acetate, and lead citrate stains. Bar = 1 μm. × 11,000
FIGURE 8.—Scanning electron micrograph showing typical polygonal cells covered by sparse, thin microvilli (Mv) and separated by narrow intercellular spaces (Is) and desmosomes (d). MCA-treated mouse. Critical point, gold coating. Bar = 1 μm. × 8,000
Figure 9.—Scanning electron micrograph showing rounded cells covered by elongated, thick, and numerous microvilli (Mv) and blebs (B). Cells are separated by widened intercellular spaces (Is). PRL-MCA-treated carcinoma. Critical point, gold coating. Bar = 1 μm. × 8,000
FIGURE 10.—Scanning electron micrograph showing intense stromal reaction. Neoplastic cells migrating into stroma; they are covered by several thick microvilli (Mv) and blebs (B). Cells are separated by erythrocytes (E) and connective fibers (Cd). Critical point, gold coating. Bar = 1 μm. × 8,000