Retinoic Feeding, Hormone Inhibition, and/or Immune Stimulation and the Progression of N-Methyl-N-nitrosourea-induced Rat Mammary Carcinoma: Suppression by Retinoids of Peptide Hormone-induced Tumor Cell Proliferation in Vivo and in Vitro

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ABSTRACT

Female Sprague-Dawley rats were treated at 50 and 57 days of age with 2.5 mg per 100 g body weight of N-methyl-N-nitrosourea (MNU). At 60 days of age, the animals were divided into eight groups (40 rats/group) and treated as follows: (a) controls; (b) immune stimulation (IS); (c) hormone inhibition (HI); (d) HI + IS; (e) retinyl acetate feeding (RA); (f) RA + IS; (g) RA + HI; and (h) RA + HI + IS. IS treatment was accomplished by three i.p. injections (at 1, 3, and 5 weeks after carcinogen treatment) of a mixture of cell particulate preparations from pooled MNU-induced rat mammary carcinomas and Freund’s (complete) adjuvant. HI treatment consisted of daily s.c. injections of tamoxifen (12.5 to 25.0 \( \mu g/100 \) g body weight) and CB-154 (200 to 400 \( \mu g/100 \) g body weight), and RA treatment consisted of daily feeding of retinyl acetate (1.0 mm). Both RA alone and HI alone significantly (\( p < 0.01 \) to 0.001) reduced mammary carcinoma incidence; HI treatment was significantly (\( p < 0.01 \)) more effective than RA treatment. The combination of RA + HI was significantly (\( p < 0.01 \)) superior to either treatment alone; RA + HI treatment virtually completely blocked the development of mammary carcinomas at termination of study (a total of 2 mammary carcinomas was observed in the RA + HI groups of rats at 1 year after carcinogen treatment). IS treatments did not significantly influence mammary carcinoma incidence, either alone or in combination with RA and/or HI.

Female Sprague-Dawley rats given MNU and subsequently treated daily for 4 weeks with the prolactin secretion-stimulating drug haloperidol (0.05 mg/100 g body weight) responded with a significant (\( p < 0.001 \)) increase in mammary carcinoma development when compared with control rats. RA treatment of haloperidol-treated rats significantly (\( p < 0.001 \)) blocked the stimulatory effect of haloperidol on mammary carcinoma development. The addition of insulin (5.0 \( \mu g/ml \)) to the culture media of 2-day organ cultures of MNU-induced rat mammary carcinomas resulted in a significant (\( p < 0.05 \)) stimulation of [\( ^{3}H \)] thymidine incorporation into DNA of the cultured cells. Retinoic acid (1 \( \times 10^{-8} \) M) significantly (\( p < 0.05 \)) blocked the stimulatory effect of insulin on [\( ^{3}H \)] thymidine incorporation into DNA; retinoic acid alone did not significantly affect [\( ^{3}H \)] thymidine incorporation into DNA.

The results of this study demonstrate a striking prevention of the development and progression of MNU-induced rat mammary carcinomas by a combination of two biological response modifiers, i.e., RA and HI. Retinoid-induced suppression of peptide hormone-induced stimulation of mammary carcinoma cells in vivo and in vitro has also been demonstrated, suggesting a mechanism by which retinoids may suppress the progression of rat mammary carcinomas.

INTRODUCTION

In a recent communication (12), we report the effects of the use of 3 biological response modifiers (RA, HI, and IS) singly and in combination, on the chemoprevention of DMBA-induced rat mammary carcinomas. Treatments were initiated beginning 3 days after carcinogen administration. RA alone and HI (tamoxifen + CB-154) alone sharply suppressed the genesis of these tumors, a suppressive effect which was significantly enhanced when the 2 treatments were used simultaneously. IS (cell particulate of DMBA-induced rat mammary carcinomas plus Freund’s complete adjuvant) was only effective in reducing mammary carcinoma incidence when administered to retinoid-fed animals. The combination of RA, HI, and IS was superior to either treatment alone or other doublet combinations of treatment; no mammary carcinomas were observed in the animals treated with all 3 biological response modifiers at the termination of treatment, 20 weeks after DMBA administration.

MNU, when administered to young female rats, like DMBA, is an effective mammary gland carcinogen (2). RA and/or prolactin suppression commencing shortly after MNU treatment sharply reduces the incidence of mammary carcinomas (10). The endocrine responsiveness of the MNU-induced rat mammary carcinoma may be more akin to human breast cancer than the polycyclic aromatic hydrocarbon-induced tumors, i.e., MNU-induced tumors may be more sensitive to estrogens and less responsive to prolactin for growth processes (5, 7). One purpose of the present report is to evaluate the effectiveness of RA, HI, and/or IS in the chemoprevention of MNU-induced rat mammary carcinomas. Are these treatments as impressively effective in reducing mammary carcinoma incidence in MNU-treated animals as they are in animals treated with DMBA? Also, we evaluate a mechanism by which retinoids might inhibit rat mammary gland carcinogenesis, i.e., retinoid-induced suppression of polypeptide hormone-induced mammary tumor cell proliferation. Two peptide hormones, prolactin and insulin, are efficacious stimulators of rat mammary carcinoma cell proliferation in vivo (prolactin) and in vivo.

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vitro (insulin). Therefore, we asked the question, can retinoids block the mitogenic effects of these peptides on these tumors? The impetus for the latter study was derived, at least in part, by the relatively recent report of Todaro et al. (8), who provided evidence that retinoids can suppress the proliferative action of transforming growth factor (a peptide) on mouse fibroblasts in vitro.

MATERIALS AND METHODS

Female Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Mich.) were used in these studies. All animals were housed in a temperature-controlled (24 ± 1°C; S.E.) and light-controlled (14 hr/day) room and were given a diet of Wayne laboratory meal (Allied Mills, Inc., Chicago, Ill.) and water ad libitum. The vitamin A content of the Wayne laboratory meal is 15,000 IU of the vitamin per kg of ration, as specified by the manufacturer.

RA, HI, and/or IS and the Progression of MNU-induced Rat Mammary Carcinoma. At 50 and 57 days of age, 320 rats were treated i.v. with 2.5 mg MNU per 100 g body weight. MNU (Ash Stevens, Inc., Detroit, Mich.) was dissolved in 0.9% NaCl solution (10 mg/ml) acidified (pH 4 to 5) with acetic acid. At 60 days of age, the rats were divided into 8 groups (40 rats/group) and treated as follows: (a) controls; (b) IS; (c) HI; (d) HI + IS; (e) RA; (f) RA + IS; (g) RA + HI; and (h) RA + HI + IS. IS was accomplished by i.p. injections of an equal mixture by weight of cell particulate preparations from 25 pooled MNU-induced rat mammary carcinomas (1.0 mg particulate preparation per injection) and Freund's (complete) adjuvant (Calbiochem-Behring Corp., La Jolla, Calif.) as described previously (12). This mixture (CP of MT + FCA) was administered 3 times at 1, 3, and 5 weeks after the last day of carcinogen injection. Tumor cell particulates were provided by grinding the tumors in sterile, cold 0.9% NaCl solution with a glass homogenizer, centrifuging the suspension for 30 min (10,000 x g), and resuspending the pellet in sterile, cold 0.9% NaCl solution. After several centrifugations and washings, the pellet was resuspended in 0.2% formaldehyde solution, and the suspension was refrigerated (4°C) for 2 days. The suspension was then washed with sterile, cold 0.9% NaCl solution; centrifuged (10,000 x g); and resuspended in sterile, cold 0.9% NaCl solution (1.0 mg particulate preparation per 0.1 ml 0.9% NaCl solution).

HI was accomplished by daily s.c. injections of CB-154, a prolactin secretion-suppressing drug, and an estrogen antagonist, tamoxifen (ICI 46,474). Tamoxifen (tamoxifen citrate) was mixed with gum arabic (1:4 mixture, by weight), and this mixture was added to distilled water containing CB-154. CB-154 solution was prepared by adding the ercp alkaloid to distilled water (final concentration, 2.0 mg CB-154 per ml). The dose of HI for the first 6 months of injections was tamoxifen at 25 μg per 100 g body weight and CB-154 at 400 μg per 100 g body weight; thereafter, and to the end of the study, the dose was halved, i.e., tamoxifen at 12.5 μg per 100 g body weight and CB-154 at 200 μg per 100 g body weight. RA consisted of blending retinyl acetate into the stock diet in the form of stable gelatinized beadlets at a retinyl acetate concentration of 328 mg retinyl acetate per kg of diet (1.0 ml) (328 mg retinyl acetate = 993,939 IU of vitamin A). This dose of retinyl acetate was used for the first 6 months of the study; thereafter, and to the end of the study, the dose was halved, i.e., 164 mg retinyl acetate per kg of diet (0.5 ml). The diet of the rats not given retinyl acetate contained gelatinized beadlets without retinyl acetate. All rats not receiving HI or IS were given injections of the diluent for HI or IS, respectively.

The dose levels of HI and RA for the first 6 months of the study are those which are maximally tolerated by these animals, i.e., these dose levels are slightly below the dose level in which significant reductions in body weight gains begin to be observed. Reducing the HI and RA dose by 50% (second 6 months of the study) was initiated to ensure that significant reductions in body weight gains would not be observed for, as these animals age, less tolerance to RA and HI treatments could conceivably occur.

All rats receiving treatments IS, RA, and RA + IS and control rats were sacrificed 4 months after the last day of carcinogen treatment. HI- and HI + IS-treated rats and RA + HI- and RA + HI + IS-treated rats were sacrificed 9 and 12 months, respectively, after the last day of carcinogen treatment. All rats were weighed and palpated for mammary tumors at biweekly intervals. When mammary tumors reached 2.0 cm in diameter, they were surgically excised, fixed in Bouin's fluid, stained with hematoxylin and eosin, and examined histologically. The excision site was sutured, and the rat was placed back in the experiment. At the termination of the study, the rats were decapitated, and trunk blood samples were collected. Serum prolactin concentrations were determined by radioimmunoassay. All mammary tumors were removed and examined histologically. Mammary carcinoma incidence was analyzed statistically by χ² analysis.

Retinoid Treatment, Prolactin Secretion Stimulation, and Rat Mammary Gland Carcinogenesis in Vivo. Thirty-one female Sprague-Dawley rats were treated with MNU as described above. Eight weeks after the last injection of MNU, the rats were divided into 2 groups, i.e., those to be treated with the prolactin secretion-stimulating drug haloperidol (0.05 mg per 100 g body weight, in a 0.3% tartaric acid solution, injected s.c.; McNeil Laboratories, Inc., Fort Washington, Pa.) and those to be treated with haloperidol and, in addition, RA (328 mg retinyl acetate per kg of diet). The treatments were daily and of 4-week duration. All rats were weighed and palpated for mammary tumors at 0, 2, and 4 weeks after the onset of treatments (8 to 12 weeks after the last carcinogen administration). At the termination of the study (24 hr after the last injection of haloperidol and RA treatment), the rats were decapitated, and the trunk blood samples were collected. Serum prolactin concentrations were determined by radioimmunoassay. All mammary tumors were removed and examined histologically. Mammary carcinoma incidence was analyzed statistically by χ² analysis.

Retinoid and Insulin Treatments and DNA Synthesis of Rat Mammary Carcinomas In Vitro. Seven female Sprague-Dawley rats were treated with MNU as described above. Approximately 16 weeks after carcinogen treatment, when each rat had at least one mammary carcinoma approximately 2 cm in diameter, the tumors were aseptically removed; trimmed of necrotic, hemorrhagic, and connective tissues; and placed in a holding media (Hanks' base Medium 199; containing gentamicin sulfate, 50 mg/liter; Grand Island Biological Co., Grand Island, N.Y.).

Preparation of Tumor Slices for Organ Culture. Slices were prepared with the aid of a Stadie-Riggs tissue slicer and a No. 10 Bard-Parker surgical blade. Each mammary carcinoma provided 5 to 15 large slices ranging from 10 to 15 mm in diameter and 0.1 to 0.3 mm thick. Each slice was processed by simple halving with a surgical blade, each half being subsequently halved until the slices measured approximately 1 x 1 mm. The small slices (1 x 1 mm) were pooled, then placed in 10 x 35-mm Falcon disposable Petri dishes at 10 slices/dish. Each Petri dish contained 2.0 ml of the culture medium.

Each tumor specimen was divided into 4 groups, i.e., a control and 3 experimental groups. Each group (controls and experimental) had 9 small Petri dishes containing a total of 90 small slices. The small Petri dishes were placed in a covered water-saturated larger Falcon disposable Petri dish (15 x 100 mm), 3 small dishes/larger dish. These Petri dishes were then placed in a small gassing chamber and housed in an incubator at 37°C. The chambers were continuously infused with gas (95% O₂:5% CO₂) during the culture period. Each tumor was individually cultured; slices from different tumors were never combined. The large number of randomly selected small slices per group provides reasonable assurance that an equal quantity of epithelium is distributed among the 4 groups at the onset of culture.

The culture medium used in these studies was modified Earle's salts Medium 199 (1250 mg NaHCO₃/liter) obtained from Grand Island Biological Co. Bovine pancreas insulin (22.5 IU/mg; California Biochemical Corp., La Jolla, Calif.) was added to the culture media of Groups 2 and 4 at a concentration of 5.0 μg/ml. All trans retinoic acid (Sigma Chemical Co., St. Louis, Mo.) was added to the culture media of Groups 3 and 4.
at a concentration of $1 \times 10^{-5} \text{M}$. Retinoic acid (in lieu of retinyl acetate) is commonly used in vitro studies, for it is probable that retinoic acid (or a metabolite) is the active form of retinol. Retinol acid was dissolved in 100% ethanol before adding to the culture media, and final media ethanol concentration was <0.01%. The diluent (ethanol) for retinoic acid was added to the culture media of Groups 1 and 2. All media contained gentamicin sulfate at 50 mg/liter. After all additions, the media were passed through a Millipore filter (0.45 μM).

At the end of the second day of culture, 4 hr prior to termination, sterile [methyl-3H]thymidine (40 to 60 Ci/mmol; New England Nuclear, Boston, Mass.) was added to the culture medium at a concentration of 1.125 μCi/ml. Termination of the cultures was designed to facilitate quick removal of the small slices from the media in order to obtain a wet weight for each group, and then storage in 0.9% NaCl solution at $-20^\circ\text{C}$ for DNA extraction and analysis.

DNA Extraction and Analyses of Cultured Slices. For DNA extraction and analysis, the tissues from each group were ground in 0.9% NaCl solution with a Willems Polytron Homogenizer. An equal volume of 20% trichloroacetic acid was added to the homogenate; the resulting precipitate was centrifuged (3000 × g) and washed twice with 10% trichloroacetic acid. The precipitate was then washed twice in sodium acetate in methanol and in chloroform:methanol, once in 100% ethanol, and once in 100% ethyl ether, in that order, to remove lipid and H2O. In all of the foregoing procedures, the preparations were kept constantly cold. The defatted-dehydrated extract was placed in a ventilated fume hood (12 to 18 hr) and then in a vacuum desiccator (24 hr), and was subsequently weighed.

The defatted-dehydrated extract was digested (3 hr at 37°) with repeated stirrings in 0.5 N KOH. The preparation was cooled, precipitated with cold 10% perchloric acid, centrifuged (3000 × g), and washed twice. The precipitate was then incubated for 30 min with constant stirring in hot (70°) 5% perchloric acid in which the DNA was soluble. This preparation was cooled, centrifuged (3000 × g), and washed twice with cold 5% perchloric acid. The supernatant was collected for DNA and [3H]-thymidine analysis. DNA content was quantitatively determined (in duplicate) by the diphenylamine-colorimetric method of Burton (1). Calf thymus DNA (Sigma) was used as a standard. The [3H]-thymidine content was determined by pipetting neutralized aliquots (in triplicate) of the supernatant onto 2.3-cm Whatman No. 3 filter papers. The filter paper was air-dried and placed in a liquid scintillation vial containing toluene PPO-PPOP fluor. The samples were counted in a Beckman LS-100C liquid scintillation counter with a counting efficiency of 56%. Counting efficiency was determined by the percentage counts of a precisely calibrated (dpm), sealed tritium standard. The results were expressed as cpm [3H]-thymidine per μg DNA values of each group was analyzed by the t test for paired observations.

RESULTS

Effect of RA, HI, and/or IS on the Progression of MNU-Induced Rat Mammary Carcinomas. The control rats were sacrificed at 4 months after the last MNU injection because they were inundated with mammary carcinomas (Chart 1): IS, RA, and RA + IS groups were also sacrificed at this time. Total numbers of palpable mammary carcinomas for each group of rats (40 rats/group) at 4 months after the last MNU injection were: control, 70; IS, 76; HI, 2; HI + IS, 4; RA, 41; RA + IS, 91; RA + HI, 0; and RA + HI + IS, 0 (Table 1). No significant difference in mammary carcinoma incidence was observed in controls and IS-treated rats. RA alone significantly reduced mammary carcinoma incidence compared with controls ($p < 0.01$). A further reduction in mammary carcinoma incidence was observed in HI- and HI + RA-treated rats when compared with controls ($p < 0.001$). Mammary carcinoma incidence in RA + IS-treated rats was reduced by 23%, when compared to rats treated with RA alone. However, this reduction did not quite reach the 5% level of statistical significance.

At 9 months after carcinogen treatment, the HI and HI + IS groups of rats were sacrificed. Total numbers of palpable mammary carcinomas in the groups of rats at this time were: HI, 13; HI + IS, 21; RA + HI, 2; and RA + HI + IS, 0 (Table 1). Mammary carcinoma incidence was significantly reduced ($p < 0.01$) in HI + RA-treated rats when compared with HI-treated rats. IS treatment did not significantly affect tumor incidence. At 12 months after carcinogen treatment, the remaining 2 groups of rats were sacrificed. Total numbers of mammary carcinomas observed in these rats at this time were: RA + HI, 2; and RA + HI + IS, 2 (Table 1).

By 10 months postcarcinogen treatment, benign mammary tumors (fibroadenomas) were frequently observed. Prior to this period, the incidence of these tumors among the groups of animals was negligible. Total number of benign mammary tumors.
in the RA + HI group and the RA + HI + IS group was 27 and 43, respectively, at 1 year after carcinogen treatment. Rate of body weight gain among the 8 groups of rats was comparable, and no significant differences were noted. Non-tumor-related mortality was negligible among the 8 groups of animals. Ear duct carcinomas began to appear in the animals of Groups 7 and 8 between 9 and 12 months after carcinogen treatment. When these tumors became quite large, the animal was sacrificed and removed. At this time, all of these animals were free of mammary carcinomas. Mean serum prolactin values (ng/ml) were 2.8 ± 0.7 (S.E.) for the HI + RA groups of rats, with serum prolactin values approximately 10% of that observed in control animals (28.8 ± 19.1).

Effect of Retinoid Treatment and Prolactin Secretion Stimulation on Development of MNU-Induced Rat Mammary Carcinomas. Daily treatment of rats for 4 weeks with the prolactin secretion-stimulating drug haloperidol resulted in a striking increase in the incidence of mammary carcinomas (Chart 2). In this relatively short period of time, mammary carcinoma number increased from a total of 2, at Day 0, to 36 (15 rats), a rate of mammary carcinoma development comparable to rats treated with RA alone (Chart 1). In the same period of time, mammary carcinoma number increased from 1 to only 7 in the group of rats treated with haloperidol and RA; a rate of mammary carcinoma development nearly 4 times that observed in control rats (Chart 1). In the same period of time, mammary carcinoma number increased from 1 to 7 in the group of animals (16 rats) treated with haloperidol and RA; a rate of mammary carcinoma development comparable to rats treated with RA alone (Chart 1). The difference between mammary carcinoma incidence in the haloperidol- and haloperidol plus RA-treated rats is highly significant (p < 0.001). All tumors were mammary adenocarcinomas. Body weight gains were not different among the 8 groups of animals. Mean serum prolactin values (ng/ml ± S.E.) were 250.0 ± 18.3 and 244.7 ± 16.1 for the haloperidol- and haloperidol plus RA-fed groups of rats, respectively; serum prolactin values were approximately 10% times greater than that observed in control animals (28.8 ± 19.1).

Effect of Insulin and/or Retinoic Acid Treatments on [3H]-Thymidine Incorporation into DNA of 2-Day Organ Cultures of MNU-Induced Rat Mammary Carcinomas. The addition of insulin to the culture media resulted in a significant (p < 0.05) increase in [3H]thymidine incorporation into DNA (Chart 3). Each of 7 mammary carcinomas responded positively to the stimulatory effects of insulin. The addition of retinoic acid to the culture media did not significantly affect [3H]thymidine incorporation into DNA. The addition of retinoic acid to cultures containing insulin blocked the stimulatory effect of insulin (p < 0.05); no significant differences in [3H]thymidine incorporation into DNA were observed between controls, retinoic acid-, and retinoic acid plus insulin-treated cultures.

DISCUSSION

In this communication, we show that RA and HI alone significantly suppressed the progression of MNU-induced rat mammary carcinomas; HI was significantly more effective than RA. The 2
treatments combined virtually totally blocked the emergence of mammary carcinomas. One year after carcinogen administration, only 2 mammary carcinomas were observed in the groups of animals (40 rats/group) treated with both RA and HI. In a previous report (12), using the DMBA-induced rat mammary carcinoma model, virtually identical results were obtained. Thus, mammary carcinomas induced by MNU and DMBA are strikingly similar, quantitatively and qualitatively, in their response to RA and/or HI. In the present communication, we could not detect a significant effect of IS (CP of MT + FCA) alone or in combination with RA and/or HI on MNU-induced rat mammary gland carcinogenesis. In the previous study (12), using DMBA, IS (CP of MT + FCA) was effective, but only in RA treated rats, i.e., there were >40% less mammary carcinomas in IS + RA treated rats than in rats treated with RA alone. In the present study, we observed a reduction of mammary carcinoma incidence in IS + RA treated rats, but this reduction (=23%) did not reach the 5% level of statistical probability. Perhaps MNU-induced rat mammary carcinomas are less sensitive to this form of IS. Despite this slight difference in IS responsiveness, it is clear that the 2 models (MNU and DMBA) of human breast cancer have nearly identical responsiveness to 2 biological response modifiers, RA and HI. The nearly complete prevention of mammary gland carcinogenesis, at 1 year post-carcinogen treatment, by using a combination of 2 nontoxic biological response modifiers, is an observation of considerable importance.

The mechanism by which retinoids inhibit mammary gland carcinogenesis is unknown. An intriguing hypothesis, proposed by Todaro et al. (8), is that retinoids may modulate the mitogenic effects of peptide hormones on specific epithelial target organ sites. This hypothesis was derived, at least in part, from the observation that retinoids can antagonize the stimulatory effect of prolactin on mammary carcinoma development. Insulin, another polypeptide hormone, is a potent mitogen of rat mammary carcinomas in vitro. We have shown (11), as have many other laboratories (3, 4, 9), that the addition of insulin to cell or organ cultures of rodent mammary gland carcinomas results in a striking increase in [3H]thymidine incorporation into chemically extracted DNA, an increase in [3H]thymidine-labeled cells, and an increase in number of cells with mitotic figures. In this communication, we show that the addition of retinoic acid to media of organ cultures of rat mammary gland carcinomas blocks the stimulatory effect of insulin on [3H]thymidine incorporation into DNA. Retinoic acid added to the culture media alone did not affect [3H]thymidine incorporation into DNA. Thus, we provide both in vivo and in vitro evidence that retinoids can inhibit the proliferative actions of peptide hormones on rat mammary gland carcinomas. Our results and those of Todaro et al. (8) are similar in many respects to a recent report by Mehta et al. (6) showing inhibition by retinoic acid of prolactin-induced DNA synthesis and lobuloalveolar development of organ cultures of normal mouse mammary gland.

In summary, we have shown that a combination of hormone inhibition and retinoid feeding commencing shortly after carcinogen treatment most impressively blocks the emergence of rat mammary gland carcinomas during a 1-year post-carcinogen treatment period. In addition, we show that retinoids can block the stimulatory effect of polypeptide hormones on in vitro [3H]thymidine incorporation into rat mammary carcinoma DNA (insulin) and on in vivo rat mammary carcinoma development and growth (prolactin), thus providing a mechanism by which retinoids may suppress the progression of carcinogen-induced rat mammary gland carcinomas.

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\textit{in Vivo} and \textit{in Vitro}

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