Chemopreventive Efficacy of Combined Retinoid and Tamoxifen Treatment following Surgical Excision of a Primary Mammary Cancer in Female Rats

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ABSTRACT

Dietary N-(4-hydroxyphenyl)retinamide (4-HPR; 3 mmol/kg diet) and s.c. injections of the antiestrogen, tamoxifen (Tx; 10 Ìg or 20 Ìg per rat, thrice weekly) were used together as adjunct chemopreventive therapy in groups of ovariectomized female Sprague-Dawley rats that each received an i.v. injection (50 mg/kg b.w.) of the mammary gland carcinogen N-methyl-N-nitrosourea (MNU). Treatment was started immediately following the surgical excision of the first (primary) mammary carcinoma from each MNU-treated rat and was continued for 180 days. When compared to the effect of treatment with 4-HPR or Tx (30 Ìg/wk) alone, the combination treatments significantly enhanced terminal survival and reduced nonrecurrent mammary cancer incidence and multiplicity. Data showing the incidence of rats bearing the first through fifth additional cancers to appear following surgical resection of a primary lesion demonstrate that combined treatment with 4-HPR/Tx was immediately and consistently more efficacious than either agent per se in suppressing subsequent tumor appearance. This effect was apparently related to the dose of Tx. These results suggest that combined treatment with 4-HPR/Tx is superior to that of either agent alone in blocking progression of incipient neoplastic lesions at both early and later stages of the process.

INTRODUCTION

Cancer chemoprevention can be experimentally defined by a reduction in cancer incidence, multiplicity, mortality or, in certain systems, an increase in cancer latency. With regard to carcinogen-induced mammary tumorigenesis in female rats, fulfillment of one or more of the aforementioned criteria in a study can be construed as a positive result. Although the anticanerogenic efficacy of any single agent in experimental models of human mammary cancer may be of clinical significance, the eventual clinical application of promising agents may require a combination of chemopreventive agents which when given together show greater activity against experimental cancer than is usually observed when single agent treatments are used. In our laboratory, we have used combination treatment with agents of proven anticanerogenic efficacy as a primary experimental approach toward the eventual goal of total cancer prevention. This strategy has yielded positive results in vivo using a number of treatment modalities, particularly in combination with retinoids (1, 2).

The MNU-induced rat mammary carcinoma system is a multiple tumor model for human breast cancer. Following the administration of a carcinogenic dose of MNU, a female Sprague-Dawley rat will develop multiple, discrete primary mammary cancers with varying latent periods (3). The different latent periods of the induced tumors suggest that, at the time when a first mammary carcinoma becomes palpable, other nonpalpable lesions are present in the mammary tissue. A high percentage of the latter lesions will eventually develop into frank mammary cancers. Because of its multicentric nature, and the presence of microscopic lesions in the mammary gland at the time of first tumor appearance, the MNU model presents a unique opportunity to study the effectiveness of chemopreventive agents at later stages of the neoplastic process. The efficacy of combined retinoid administration and endocrine ablation in suppressing the appearance of new cancers following surgical resection of a first mammary carcinoma was originally described in 1983 (4). In that study, the oral administration of retinyl acetate to rats that were bilaterally ovariectomized was highly effective in reducing the incidence and cumulative number of new carcinogen-induced mammary cancers when compared to treatment of intact animals with a diet placebo. Bilateral ovariectomy alone, or retinyl acetate alone had intermediate efficacy as inhibitors of mammary carcinogenesis. However, like other natural vitamin A compounds, retinyl acetate is unsuitable for chronic pharmacological administration to humans, due to the accumulation of a hepatotoxic level of retinyl esters in the liver (5).

In contrast, the synthetic retinoid 4-HPR, although slightly less potent than retinyl acetate as an anticanerogenic agent in the rat mammary gland (6), is markedly less toxic than retinyl acetate when chronically fed to rats. Furthermore, metabolites of 4-HPR have been found to accumulate in the rat mammary gland but not the liver (6). Similar to retinyl acetate, the chemopreventive effectiveness of 4-HPR can be enhanced by its use in combination with other agents or treatments. For example, the combined modalities of bilateral ovariectomy and dietary administration of 4-HPR were synergistic in suppressing mammary tumorigenesis when compared to either treatment alone (7). The latter results were subsequently extended by McCormick and Moon, who showed that the concomitant administration of 4-HPR with the synthetic antiestrogen, tamoxifen, provided enhanced protection against MNU-induced mammary tumorigenesis when compared to treatment with either agent alone (8). However, in the latter study, the combined activity of 4-HPR plus tamoxifen was not clearly synergistic.

The coadministration of 4-HPR with tamoxifen comprises an experimental design with potentially valuable clinical application. The chemopreventive activity of 4-HPR is currently under investigation in Stage I breast cancer patients who have a high risk of developing disease in the contralateral breast (9). At present, tamoxifen is the adjunct treatment of choice for postmenopausal women who are afflicted with estrogen receptor positive breast cancer (10). If the chemopreventive efficacy of nontoxic tamoxifen therapy can be augmented in experimental animals by its combined administration with 4-HPR, the implications for possible clinical utilization of such a treatment protocol are obvious. Thus, the present study was designed to assess the anticanerogenic efficacy of combined adjunct treatment with 4-HPR and tamoxifen when their administration to MNU-induced female rats was begun following the surgical
removal of a primary mammary carcinoma, a time when numerous, nonpalpable lesions are present in the mammary glands.

MATERIALS AND METHODS

Experimental Animals. Virgin, female Sprague-Dawley [Hsd:(SD)BR] rats were received from Harlan/Sprague-Dawley (Indianapolis, IN) at 31 days of age and maintained in isolation. A total of 320 rats was used in the study. Animals were housed in groups of two to three in polycarbonate cages containing hardwood bedding. The animal room was illuminated for 12 h each day, and maintained at a temperature of 22 ± 1°C and 50% relative humidity. Except as indicated below, animals were allowed free access to food and water throughout the study.

Diet and Chemopreventive Agents. The basal diet for the study was Wayne Lab Meal (Allied Mills, Chicago, IL), which contains 8.25 mg retinyl palmitate per kg. Retinoid-supplemented diets contained 4-HPR (Cilag AG, Schaffhouse, Switzerland) at 1173 mg (3 mmol) per kg diet. Prior to mixing into the basal diet, 4-HPR was dissolved in absolute ethanol:triiodotrianoin (1:3, 50 g/kg diet), with 0.5 ml DL-α-tocopherol and 0.5 ml Tenox 20 (Eastman Chemicals, Kingsport, TN) added per kg diet as antioxidants. Placebo diet contained the 4-HPR vehicle only. Fresh batches of diet were prepared weekly and stored at ~20°C prior to use. As analyzed by high-performance liquid chromatography (11), the retinoid was completely stable under the storage conditions used, and when 4-HPR-supplemented diet was left at room temperature for 4 days. All diet materials and animal cages were changed twice weekly.

Tx (Stuart Pharmaceuticals, Wilmington, DE) was dissolved in ethanol:sesame oil (1:9). The material was administered by s.c. injection (0.1 ml) thrice weekly, at doses of 10 or 20 ng per rat per injection. As prepared for use. As analyzed by high-performance liquid chromatography (11), the retinoid was completely stable under the storage conditions used, and when 4-HPR-supplemented diet was left at room temperature for 4 days. All diet materials and animal cages were changed twice weekly.

Control animals received an i.v. injection of the NaCl solution only. The material was administered by s.c. injection (0.1 ml) thrice weekly, at doses of 10 or 20 µg per rat per injection. Crystalline MNU (Ash-Stevens, Detroit, MI) was freshly prepared by dissolution to a concentration of 12.5 mg/ml in 0.85% NaCl solution acidified to pH ~5.0 with glacial acetic acid. Control animals received an i.v. injection of the NaCl solution only. Procedures for carcinogen preparation and injection have been previously described in detail (3).

Commencing 4 weeks after MNU administration, animals were palpated three times per week to monitor mammary tumor appearance. The data of appearance and location of every palpable tumor were recorded. When the first (primary) mammary tumor in each rat was established (0.3-0.5 cm in diameter, confirmed by two observers), the tumor was surgically excised under light ether anesthesia, and a representative section was fixed in 10% buffered formalin. In the few cases where more than one palpable mammary tumor appeared at the same time, all tumors were removed prior to assignment of each rat to a treatment group. After surgery, animals were immediately placed via the jugular vein. Crystalline MNU (Ash-Stevens, Detroit, MI) was freshly prepared by dissolution to a concentration of 12.5 mg/ml in 0.85% NaCl solution acidified to pH ~5.0 with glacial acetic acid. Control animals received an i.v. injection of the NaCl solution only. Procedures for carcinogen preparation and injection have been previously described in detail (3).

Results of the study were tested for statistical significance by ANOVA, using the % method and compared by log rank analysis (13). The statistical significance of differences between mean tumor multiplicities was assessed using analysis of variance (ANOVA). Differences in mean body weight at termination of the study were tested for statistical significance by ANOVA, using untransformed individual weights. An appropriate discrimination method for making unplanned comparisons (GT2-method) was used to define statistically significant intergroup differences in mean tumor multiplicities and body weights, as suggested by Sokal and Rohlf (14). Differences in percentage survival and cancer incidences were tested for significance by χ² analysis. In all cases, statistical significance was ascribed to a comparison only when a p < 0.05 was attained.

RESULTS

Initial group sizes for MNU-treated rats were as follows: Group 1, 40; Group 2, 40; Group 3, 40; Group 4, 39; Group 5, 40; Group 6, 39; Group 7, 39. Rats which received NaCl solution were placed into two groups of 19 and 20 each. It is important to note that all seven MNU-treated experimental groups were filled within 154 days after carcinogen treatment: Group 1, 140 days; Group 2, 147 days; Group 3, 147 days; Group 4, 133 days; Group 5, 154 days; Group 6, 133 days; Group 7, 133 days. Both control groups (8 and 9) were filled within 133 days after the rats received NaCl solution. The effective number of rats shown for each carcinogen-treated group in Table 1 was derived by striking from the study those animals which produced a benign first tumor. The overall incidence of rats bearing benign tumors at some point in the study ranged from 8% (Group 6) to 23% (Group 2), which does not represent a statistically significant difference in this parameter. Further, the mean cancer multiplicities given in Table 1 do not include cancers which were considered to be recurrent at the primary site. In this experiment, 73 of 914 (~8% overall) cancers were determined to be recurrent and were thus struck from the final data analysis.
The data presented in Table 1 show that the terminal survival of MNU-treated rats that received 4-HPR and tamoxifen (30 μg/wk) was significantly greater than that of placebo-treated controls or those groups which received either 4-HPR or tamoxifen alone. Rats which received 4-HPR plus tamoxifen at 60 μg/wk or restricted rations of placebo diet also survived to a significantly greater extent than did the full-fed controls. It should be noted that only one rat (Group 5) died in a tumor-free state during the entire study. Postmortem examination of rats which received NaCl solution and diet containing 4-HPR plus injections of tamoxifen at the high dose revealed no external signs of agent-induced toxicity. In particular, liver morphology and coloration appeared no different than that observed in the group which received NaCl solution and placebo diet.

In parallel to their effect on survival, in groups of rats which received 4-HPR with either dose of tamoxifen, significant reductions in overall mammary cancer incidence were evident when compared to the placebo-fed controls or the group which received only 4-HPR (Table 1). In contrast, combined treatment with 4-HPR/Tx was insufficient to significantly reduce cancer incidence relative to the food-restricted controls. However, the combination of 4-HPR plus 60 μg/wk Tx significantly reduced cancer incidence compared to Tx alone when given at 60 μg/wk.

When compared to either the placebo-fed controls or those rats which received 4-HPR alone, mean cancer multiplicity was significantly lower in the groups which received 4-HPR plus either dose of tamoxifen (Table 1). The enhanced effectiveness of the combination of 4-HPR plus tamoxifen on tumor multiplicity appeared to be directly related to the dose of tamoxifen (Fig. 1). Thus, relative to the full-fed controls, tumor multiplicity was reduced 25% by single-agent treatment with 4-HPR or the low dose of tamoxifen, while treatment with the high dose of antiestrogen reduced tumor multiplicity by approximately 50%. The administration of 4-HPR with tamoxifen at 30 μg/wk or 60 μg/wk reduced tumor multiplicity by 67% and 75%, respectively, representing an apparently additive effect of the two agents. A comparison of the data for the group which received the high dose of tamoxifen plus 4-HPR with those of the food restricted, placebo-fed group, shows that tumor multiplicity in the former was reduced by 62% relative to the latter group.

Log rank analysis of the incidence curves generated for the first cancer to appear following excision of the primary carcinoma demonstrated that treatment with 4-HPR plus tamoxifen at either dose significantly lengthened the latency period, compared to that observed in rats treated only with 4-HPR (Group 1) or the controls (Group 7). However, the use of ANOVA to make a statistical comparison of mean tumor latencies showed no significant intergroup differences.

The data in Table 2 show the effect of combined treatment with 4-HPR and tamoxifen on the incidence of rats bearing multiple cancers following the excision of the first carcinoma. When the first through fifth subsequent cancers are considered, it is apparent that 4-HPR and the lower dose of tamoxifen were equally effective in inhibiting the appearance of those lesions. In the placebo-fed food restricted group, the incidence of rats bearing subsequent lesions was virtually identical at all points.
The present results show that, when compared to treatment with either 4-HPR or tamoxifen as a single agent, combined treatment with the two compounds following removal of a primary cancer was significantly more effective in suppressing the postsurgical appearance of additional new mammary tumors. This was especially evident with regard to the incidence of cancers which appeared relatively later after chemopreventive treatment. These observations parallel those of Jordan and Allen (17), who showed that the latency of 7,12-dimethylbenz(a)anthracene-induced mammary tumors was directly related to the dosage of tamoxifen (1-4000 µg/wk, s.c.) when it was administered to rats for the period encompassing 30 to 60 days after carcinogen treatment.

The validity and interpretation of the data gleaned from any cancer chemoprevention study are predicated upon observations made in the absence of any significant negative responses (e.g., reduced weight gain) to the treatment regimen. Several lines of evidence strongly support our contention that the anticarcinogenic effect of concurrent treatment with 4-HPR and tamoxifen was not mediated through a generalized toxic response to the treatment regimen. Several lines of evidence strongly support our contention that the anticarcinogenic effect of concurrent treatment with 4-HPR and tamoxifen was not mediated through a generalized toxic response to the higher dose of tamoxifen was more efficacious than the other single treatments in reducing the percentage of rats bearing four or five additional cancers. In contrast, it is obvious that compared to the single treatments, the coadministration of 4-HPR and tamoxifen at either dose was immediately and increasingly more effective in suppressing the appearance of additional lesions.

The data summarized in Table 1 show that upon termination of the study, the mean body weight (±SEM) calculated for Group 7 (279 ± 6 g) was significantly greater than those of Group 4 (245 ± 3 g) or Group 5 (239 ± 3 g). However, when each final group mean weight is normalized to that calculated upon starting chemopreventive treatment, the following percentage increases result: Group 1, 18%; Group 2, 18%; Group 3, 18%; Group 4, 14%; Group 5, 12%; Group 6, 4%; Group 7, 24%; Group 8, 14%; Group 9, 20%. The percentage weight increase of Group 8 was only 70% of that shown by Group 9, a slightly smaller difference than observed between rats in Groups 4 and 5 compared to Group 7. However, it appears likely that the relatively larger percentage increase in mean body weight of Group 7 relative to the other tumor-bearing groups was at least partially a function of the total tumor burden per group, which were as follows: Group 1, 393 g; Group 2, 621 g; Group 3, 616 g; Group 4, 146 g; Group 5, 253 g; Group 6, 381 g; Group 7, 791 g. While the difference in the tumor burden of Group 5 compared to Group 7 comprises a major proportion of the mean body weight differences at the end of the study, it should be noted that, on average, rats in Group 5 utilized 10-13 g of food daily, which is about 13-33% less than normal, and could account for some of the weight difference between rats in this group compared to Group 7.

DISCUSSION

The present results show that, when compared to treatment with either 4-HPR or tamoxifen alone, the combination of the retinoid and high dose of tamoxifen was also more effective in suppressing the development of palpable carcinomas which appear at an earlier point following the institution of treatment. These data are compatible with other results showing that dietary administration of either retinyl acetate (4) or 4-HPR (16) alone had little effect on the appearance of frank mammary tumors during the late stages of promotion or progression.

The present results with the low dose of tamoxifen alone are at variance with previous data showing an immediate inhibitory effect of bilateral ovariectomy toward the appearance of palpable lesions early in the study (4). In the present study, the rate of appearance of new cancers during the first 40 days of tamoxifen treatment (30 µg/wk) was identical to that observed in the groups which received placebo diet. However, after 40 days of treatment with tamoxifen at 30 µg/wk, the rate of appearance of new cancers slowed compared to that observed in the full-fed control group. It is obvious, however, that ovariectomy and tamoxifen treatment cannot be considered to be equivalent in vivo, due to the partial estrogen agonist activity of tamoxifen in certain tissues of the rat (15), and the direct and indirect effects of ovariectomy on the levels of a host of other hormones (i.e., progesterone, prolactin) or growth factors which may interact in mammary carcinogenesis (20). Although we have no direct evidence to support the idea, it is possible that as a consequence of its administration via s.c. injection, the systemic accumulation of a full estrogen antagonistic level of tamoxifen did not occur during the first several weeks of treatment at 30 µg/wk. Since tamoxifen exhibits suboptimal activity when administered at low doses that are insufficient to achieve complete estrogen receptor blockade (15), it is possible that under the conditions of the present study, the antiestrogen was unable to immediately inhibit tumor development. This interpretation is supported by the observation that the higher dose of tamoxifen (60 µg/wk) was effective sooner than the lower dose, as demonstrated by a distinct reduction in the rate of appearance of palpable tumors within 30 days of commencing antiestrogen treatment. These observations parallel those of Jordan and Allen (17), who showed that the latency of 7,12-dimethylbenz(a)anthracene-induced mammary tumors was directly related to the dosage of tamoxifen (1-4000 µg/wk, s.c.) when it was administered to rats for the period encompassing 30 to 60 days after carcinogen treatment.

The validity and interpretation of the data gleaned from any cancer chemoprevention study are predicated upon observations made in the absence of any significant negative responses (e.g., reduced weight gain) to the treatment regimen. Several lines of evidence strongly support our contention that the anticarcinogenic effect of concurrent treatment with 4-HPR and tamoxifen was not mediated through a generalized toxic response to the high dose of tamoxifen was also more effective in suppressing the development of palpable carcinomas which appear at an
chemopreventive regimen, nor to suppression of body weight gain as a function of reduced caloric intake. First, in female Sprague-Dawley rats, the 2 weeks encompassing the day of carcinogen administration are considered to represent a critical period for mammary tumor induction (18). The latter point is relevant to the present results, since all of the rats in the study began receiving adjunct chemopreventive treatment at a time well past the documented critical period for induction of mammary tumors (17). Second, with the exception of a 13% suppression of weight gain compared to the appropriate controls, rats in Group 8, which received 4-HPR plus 60 μg/wk Tx, exhibited no overt signs of agent-induced toxicity upon postmortem examination. Third, in the carcinogen-exposed groups, those animals which received combination adjunct treatment exhibited parameters of tumorigenesis which were consistently inhibited to a greater extent than those in the group which received restricted rations of placebo diet. This demonstrates that mere suppression of body weight gain, as a function of lower caloric intake, was not a significant factor in the experimental results. Fourth, Thompson and his colleagues have rigorously shown that restricting the caloric intake of MNU-treated rats to yield an effect on body weight greater than that observed in the present study (i.e., 80% of full-fed controls) is insufficient to significantly inhibit mammary tumorigenesis when compared to the full-fed controls (19). Finally, the substantial difference in total tumor burden of rats in Group 7 compared to those in Groups 4 and 5 makes it difficult to reliably separate potential agent-induced inhibition of weight gain from apparent differences possibly related to tumor weight.

The data described in this paper are compatible with earlier results from our laboratory showing an apparently specific effect of 4-HPR against the progression and growth of mammary tumors which are presumably independent of the requirement for estrogen stimulation to grow (8). This is especially evident with regard to the data showing a significant reduction in the tumor burden of rats which received the retinoid with tamoxifen, an effect that probably is related to the decreased rate of tumor-related mortality observed most prominently in Group 4. Given the fact that tamoxifen can only be considered to be tumoricstatic (20), with tumor recurrence or relapse from remission occurring after withdrawal of therapy, the inclusion of 4-HPR in a clinical treatment regimen may provide a substantial degree of protection against therapy failure by specifically eradicating or blocking the proliferation of cells which are or become independent of the need for estrogen stimulation.

In conclusion, the data herein reported show that the coadministration of 4-HPR and tamoxifen, commencing immediately following the removal of the first cancer from MNU-induced female Sprague-Dawley rats, was significantly more efficacious in preventing the development of subsequent tumors than was the administration of either agent alone. Enhanced inhibition of mammary carcinogenesis by combination treatment with 4-HPR/Tx was manifested by significant reduction of cancer incidence, multiplicity and burden, with increased survival at termination of the study. In contrast to the inhibitory effect of either 4-HPR or tamoxifen only at early stages of promotion or progression, treatment with a combination of those agents was apparently more efficacious in blocking the appearance of lesions at all stages of promotion or progression. Finally, it appears that the increased anticarcinogenic efficacy of 4-HPR and tamoxifen when administered in combination is a function of the dose of antiestrogen.

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