Modulation of Rat Mammary Carcinogenesis by Indomethacin

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ABSTRACT

Indomethacin, a nonsteroidal antiinflammatory agent which inhibits prostaglandin biosynthesis, has significant activity in inhibiting the growth and/or inducing the regression of transplantable tumors. The present study was designed to determine if, in addition to its chemotherapeutic effects, indomethacin also acts as a cancer chemopreventive agent. Fifty-day-old virgin female Sprague-Dawley rats were given a single intragastric dose of either 8 or 16 mg of 7,12-dimethylbenz(a)anthracene (time 0). Basal diet was supplemented with 25 or 50 mg of indomethacin per kg of diet by the following protocol: (a) -2 to +1 week; (b) +1 week to end; or (c) none. Administration of indomethacin by both protocols resulted in an inhibition of mammary tumorigenesis; however, the effect of -2 to +1 week indomethacin exposure was primarily on the induction of benign mammary tumors, while +1 week to end indomethacin administration inhibited the induction of both benign mammary tumors and mammary cancers. These data indicate that indomethacin has significant protective activity when administered either during the "early" stage (comprising the carcinogen-target cell interaction) or the "late" stage (postcarcinogen tumor development) of mammary carcinogenesis in rats. Possible mechanisms of indomethacin action include both local and systemic effects.

INTRODUCTION

Chemical carcinogenesis in the rat mammary gland, as in many other experimental systems, appears to be a multistage process (32). Systemic administration of a chemical carcinogen such as DMBA, followed by chronic exposure to either endogenous (e.g., prolactin) or exogenous (high-fat diet) promoter results in the rapid induction of mammary tumors in the female rat (2, 43). The multistage nature of rat mammary carcinogenesis allows for multiple periods in which pharmacological, surgical, or dietary interventions can be used in the attempt to inhibit tumor induction; in the DMBA/rat mammary cancer model, at least 2 such periods can be defined (25, 26). These periods, which are temporally and mechanistically distinct from one another, can be distinguished as: (a) an early phase, beginning at the time of carcinogen administration and ending with clearance of free carcinogen from the target tissue; and (b) a late phase, which begins following clearance of the carcinogen. Thus, the early phase of mammary carcinogenesis may be defined as consisting of the period of carcinogen metabolism and interaction with critical macromolecules in the mammary parenchymal cells, while the late phase encompasses the postcarcinogen period of tumor development and growth.

Indomethacin is a nonsteroidal antiinflammatory agent which is a potent inhibitor of the prostaglandin synthetase (cyclooxygenase) pathway of arachidonic acid metabolism (6, 7, 14). Several lines of evidence suggest that indomethacin may have inhibitory activity in both the early and late stages of DMBA-induced mammary carcinogenesis in rats. With respect to the early stage of carcinogenesis, it has been demonstrated that indomethacin can modulate carcinogen metabolism through the inhibition of prostaglandin synthetase (23, 45); prostaglandin synthetase-dependent cooxidation of PAHs and other xenobiotics has been demonstrated at several extrahepatic sites (4, 37, 38) and may be involved in target cell-mediated metabolism of procarcinogens to their ultimate carcinogenic metabolites. Suggestions of possible late-stage effects come from observations that indomethacin inhibits cell proliferation in a variety of normal and neoplastic mammalian cells in vitro (1, 9, 13) and can inhibit the growth of transplantable tumors in vivo (15, 35). Furthermore, of particular interest is the epidemiological study of Friedman and Ury (8) which noted a significant reduction in breast cancer incidence in 4867 female users of indomethacin in comparison to an age-matched control population.

The present studies were designed to determine the activity of indomethacin as an inhibitor of mammary carcinogenesis induced by DMBA. In these experiments, indomethacin was administered in the diet for defined periods in relation to carcinogen exposure, in order to determine the effects of the compound on both the early and late stages of mammary cancer induction.

MATERIALS AND METHODS

Virgin female Sprague-Dawley [Hsd:(SD) BR] rats were obtained as weanlings from Harlan/Sprague-Dawley, Indianapolis, IN. A total of 295 rats was used in the study. Animals were housed 3 to a cage in a room maintained at 22 ± 1 °C on a 14-h light, 10-h dark cycle. All animals were allowed free access to drinking water and diet throughout the study, except for a 16-h period prior to DMBA administration; during this period, animals were allowed access to drinking water only. Basal diet for the study was Wayne Laboratory Chow (Allied Mills, Chicago, IL). Basal diet was supplemented with either 50 or 25 mg of indomethacin (Sigma Chemical Co., St. Louis, MO) per kg of diet as required by the protocol. Indomethacin was administered in a sucrose carrier (10 g/kg diet); control diet contained sucrose carrier without added indomethacin.

Rats were randomized by weight into groups of 25 (for DMBA treatment) or 15 (for vehicle treatment). With day of DMBA administration taken as time 0, groups of rats received indomethacin-supplemented diet from either 2 weeks prior to until 1 week after DMBA administration (~2 to +1 week), or beginning 1 week post-DMBA and continuing until the end of the study (+1 week to end). When not receiving the indomethacin supplement, animals received the control diet containing sucrose carrier only. Control groups received diet supplemented with sucrose carrier throughout the study.

At age 50 days, animals received a single intragastric administration of 16 or 8 mg of DMBA (Sigma) dissolved in 1 ml of sesame oil, or
sesame oil only. Beginning 4 weeks after DMBA administration, rats were palpated twice weekly to monitor mammary tumor appearance and were weighed weekly. When moribund, animals were killed by CO₂ asphyxiation. Otherwise, groups receiving 16 mg of DMBA were killed 150 days after DMBA administration, and groups receiving 8 mg of DMBA were killed 200 days post-DMBA. Animals killed or found dead were necropsied promptly. Mammary tumors were removed and coded as to location, and any other grossly abnormal tissues were taken for histological study. At necropsy, particular attention was paid to the gastrointestinal tract, the major target for indomethacin toxicity (44). Tissues were fixed with 10% buffered formalin, stained with hematoxylin and eosin, and classified histopathologically. Only histologically confirmed mammary tumors were used in the data analysis.

At the termination of the study, animals receiving sesame oil only were killed, and the abdominal-inguinal mammary glands were excised, fixed in formalin, stained with alum carmine, and prepared as wholemounts. Wholemount preparations were examined with a dissecting microscope in order to determine possible effects of indomethacin treatment on mammary gland development and differentiation.

Values for tumor incidence and multiplicity were calculated using the life table method (31). Intergroup comparisons for number of tumors per rat were made by analysis of variance, using square-root transformed data, as suggested by Snedecor and Cochran (39). Tumor incidence curves were compared by the logrank test (31), and mean group weights were compared by analysis of variance.

RESULTS

High DMBA Dose. Indomethacin had significant anticarcinogenic activity when given as a dietary supplement to rats receiving 16 mg of DMBA. When administered by the -2 to +1 week protocol, the 2 doses of indomethacin had similar chemopreventive activity, reducing tumor multiplicity by approximately one-third compared to control (Table 1). This inhibition of mammary tumor induction was effected primarily by a significant reduction in number of benign tumors; although carcinoma multiplicity was reduced by 15 to 20% in indomethacin-treated groups, this inhibition was not statistically significant. However, indomethacin administered from Weeks -2 to +1 did delay mammary carcinoma appearance, resulting in a 10- to 20-day shift in the tumor latency curve compared to control (Chart 1).

Chronic, postcarcinogen (+1 week to end) administration of indomethacin also resulted in a significant inhibition of mammary tumorigenesis in rats treated with 16 mg of DMBA. In contrast to the -2 to +1 week protocol, however, administration of indomethacin by the +1 week to end protocol showed a marked dose effect. Administration of the low indomethacin dose from Week +1 to end had no effect on mammary tumor induction by 16 mg of DMBA; neither tumor incidence, nor multiplicity, nor latency was statistically different from control. However, administration of the high indomethacin dose by this protocol did result in a significant inhibition of mammary carcinogenesis: total tumor multiplicity was reduced by over 50% from control levels, with significant reductions in numbers of both mammary cancers and...
benign mammary tumors (Table 1). In addition, administration of the high dose of indomethacin caused a shift to the right in the mammary cancer latency curve, indicating a delay in mammary carcinoma appearance. This delay was 10 to 20 days during the first 100 days of the study, and increased to approximately 60 days by the end of the experiment (Chart 1).

**Low DMBA Dose.** Indomethacin was also effective in inhibiting mammary tumorigenesis induced by the 8-mg dose of DMBA. Although some differences in indomethacin dose-response relationships were noted, the overall patterns of indomethacin modulation of mammary tumor induction by 8 mg of DMBA were similar to those reported for the higher DMBA dose.

As was seen in animals treated with 16 mg of DMBA, exposure to the high indomethacin dose by the −2 to +1 week protocol resulted in a significant inhibition of mammary tumor induction (Table 2). Again, however, the reduction in mammary tumor multiplicity observed in animals administered indomethacin from Weeks −2 to +1 was due almost entirely to a reduction in benign tumor number; administration of indomethacin by this protocol had no statistically significant effect on mammary cancer multiplicity. In contrast to the efficacy of the high dose of indomethacin administered from Weeks −2 to +1, the low dose of indomethacin given by this protocol had no effect on mammary tumor induction by the low dose of DMBA. This differential dose response of −2 to +1 week indomethacin administration was not seen in animals treated with the high dose of carcinogen.

Administration of the indomethacin dietary supplements from Week +1 to end was relatively more effective in inhibiting mammary carcinogenesis induced by 8 mg of DMBA than were the same supplements administered by the −2 to +1 week protocol (Table 2). Both the high and low indomethacin doses resulted in significant reductions in mammary cancer multiplicity and total tumor multiplicity; the greater reduction in mammary tumor number seen in the groups receiving the high indomethacin dose was attributable to greater efficacy in reducing the number of induced fibroadenomas.

**Indomethacin Toxicity.** The anticarcinogenic activity of indomethacin observed in the present studies was not a function of general toxicity. As indicated in Tables 1 and 2, dietary administration of indomethacin at levels of 50 or 25 mg/kg diet had no influence on animal body weight gain. Terminal body weights in all indomethacin-treated groups were within 5% of the relevant control group, and no statistically significant reductions in group body weights were observed at any time during the experiment. Similarly, no significant differences in animal survival were attributable to indomethacin administration; survival ranged from 76 to 84% in rats treated with 16 mg of DMBA, and from 88 to 100% in groups receiving the 8-mg DMBA dose.

The general health status of animals fed indomethacin was good throughout the experiment. Previous studies have reported that the gastrointestinal tract is the most sensitive target organ for indomethacin toxicity (18, 44). In the present study, no gastrointestinal toxicity was noted in any animals fed indomethacin from Weeks −2 to +1, nor was toxicity observed in rats fed the low indomethacin dose from Weeks +1 to end. However, a low (12%) incidence of gastrointestinal adhesions was noted at sacrifice in animals fed the high indomethacin dose by the +1 week to end protocol. It appears unlikely that this gastrointestinal toxicity was responsible for the inhibition of tumor induction reported above; all animals with grossly observable adhesions were tumor bearers, with tumor burdens ranging between 1 and 10 tumors/animal. No accumulation of ascites fluid was noted in indomethacin-treated or control rats.

**Indomethacin and Mammary Gland Development.** Observation of mammary gland wholemounts taken at sacrifice from solvent control groups revealed a marked influence of chronic indomethacin exposure on mammary gland differentiation. Mammary glands taken from rats in the dietary control group showed significant lobuloalveolar differentiation, a degree of structural development characteristic of old virgin rats (Fig. 1). A similar degree of structural development was observed in mammary glands taken from rats administered indomethacin from Weeks −2 to +1, indicating that a brief exposure to indomethacin had little effect on mammary gland morphology. By contrast, however, indomethacin administration from Week +1 to end resulted in a dramatic inhibition of normal mammary differentiation. Glands from many indomethacin-treated rats showed little parenchymal development and were composed primarily of naked ducts with limited lobuloalveolar differentiation (Fig. 2).

It should be noted that this inhibition of mammary development was seen in glands from approximately 60% of the rats exposed to indomethacin; remaining animals in the +1 week to end indomethacin group showed mammary development similar to that of controls. This differential effect suggests that other, as yet undetermined, factors may modulate indomethacin activity in mammary differentiation and carcinogenesis. Animal variability in response to indomethacin may also be a factor in the less than total inhibition of carcinogenesis observed in the present studies.

**DISCUSSION**

Dietary supplementation with indomethacin resulted in a significant inhibition of DMBA-induced mammary carcinogenesis in the present study. Indomethacin was an effective inhibitor of mammary tumor induction when administered from either 2 weeks prior to until 1 week after DMBA administration, or from 1 week postcarcinogen until the end of the experiment. Although indomethacin had significant inhibitory influences on both the early and late stages of mammary tumorigenesis, a differential effect of the 2 treatment protocols was noted with respect to the histological type of tumors sensitive to inhibition; while administration of indomethacin beginning 1 week after DMBA inhibited the induction of both benign and malignant mammary neoplasms, treatment with indomethacin by the −2 to +1 week protocol resulted in a statistically significant inhibition of the induction of benign tumors only. This differential effect suggests that indomethacin may act via different mechanisms and/or on different targets during the early and late stages of mammary tumor
induction.

The mechanism or mechanisms by which indomethacin modulates mammary tumorigenesis are unknown. However, the broad spectrum of biological activities of this compound suggests a variety of possible mechanisms. These putative mechanisms for indomethacin inhibition of cancer induction may be either local or systemic in nature.

Local, mammary gland-mediated effects could be the mechanistic basis for the inhibition of carcinogenesis achieved by both indomethacin administration protocols. The inhibition of prostaglandin synthetase-dependent cooxidation of PAHs has not been reported, such alterations have been demonstrated in a variety of extrahepatic tissues, including target tissues for several chemical carcinogens. It has been proposed that the ability of the mammary gland to metabolize PAH carcinogens is important in carcinogenesis in this organ site. Should prostaglandin synthetase cooxidation of PAHs be involved in such mammary cell-mediated activation, inhibition of this pathway by indomethacin could result in a significant reduction in tumorigenesis.

Because DMBA binding to mammary parenchymal DNA is complete by 1 week after its administration, no interference with carcinogen metabolism or binding can be implied as a mechanism for the chemopreventive action of the +1 week end indomethacin protocol. However, other mammary gland-mediated effects may provide the basis for the inhibition of carcinogenesis seen with postcarcinogen indomethacin exposure. In the present study, it was noted that chronic administration of indomethacin had a major influence on the structural development of the rat mammary gland. This alteration in mammary gland differentiation has been reported with several other inhibitors of mammary cancer induction, most notably the retinoids, retinyl acetate and N-(4-hydroxyphenyl)retinamide. The biochemical basis for the effect of indomethacin on mammary gland differentiation is unknown at the present time. However, it should be noted that indomethacin has been reported to decrease the prolactin receptor content of mouse liver membranes in vivo; such activity in the mammary gland could result in a significant inhibition of prolactin-induced structural differentiation in this tissue.

Indomethacin may also serve to modulate mammary tumor induction through systemic effects on the carcinogen-treated rat. A variety of indomethacin influences which are not limited to the mammary gland may be involved in the modulation of tumor induction in this tissue. Indomethacin reversibly inhibits cell proliferation in a variety of mammalian cells in vitro and can inhibit the growth of transplanted tumors in vivo. This growth-regulatory activity has been demonstrated for cells and tumors of both mammary and nonmammary origin. Indomethacin also has a number of immunopotentiating activities, including enhancement of natural and antibody-dependent cell-mediated cytotoxicity and the modulation of suppressor cell activity. Biological response modifiers such as Bacillus Calmette-Guérin and maleic anhydride-divinyl ether copolymer both have significant activity as inhibitors of mammary carcinogenesis in rats, suggesting that modulation of the immune response can be a mechanism through which tumor induction may be suppressed. Furthermore, indomethacin inhibits the induction of ornithine decarboxylase activity by tumor promoters; a correlation between chemopreventive activity and modulation of ornithine decarboxylase activity has been demonstrated for numerous inhibitors of skin carcinogenesis, and recent evidence suggests that alterations in polyamine synthesis may be a mechanism for inhibition of mammary carcinogenesis. Finally, recent evidence suggests that angiogenesis may be enhanced by prostaglandins and inhibited by indomethacin; such inhibitory activity could result in an inhibition of tumorigenesis.

The data from the present study demonstrating an inhibition of carcinogenesis by indomethacin are consistent with previous studies reporting anticarcinogenic activity of this compound in other animal tumor models. Several groups have reported that dietary or drinking water administration of indomethacin can inhibit tumor induction in rat colon and mouse esophagus, while topical application of indomethacin inhibits tumorigenesis induced in mouse skin by either the DMBA/12-O-tetradecanoylphorbol-13-acetate 2-stage system or exposure to UV light.

The influence of indomethacin on chemical carcinogenesis in the mammary gland appears to be somewhat more complex. The data from the present study are at variance with those of Carter et al. who found that, while postcarcinogen administration of indomethacin significantly inhibited mammary tumor induction in DMBA-treated rats fed a high-fat diet, the compound did not reduce tumor incidence or multiplicity in rats maintained on a basal, low-fat regimen. Several differences in protocol between the work of Carter et al. and the present study may explain the apparent discrepancy in findings. In their experiment, Carter et al. used a DMBA dose of 5 mg to induce a 1.03 mammary tumors/rat in the control group at the end of the experiment (154 days). By contrast, in the present studies, DMBA doses of 16 and 8 mg were used, which induced 8.66 tumors/rat in the control group at 150 days (16-mg dose) and 9.64 tumors/rat in controls at 200 days (8-mg dose). It is possible that indomethacin is less effective in the inhibition of mammary tumorigenesis in a low carcinogen dose, low tumor response model than in a high dose, high response model. In such a situation, anticarcinogenic efficacy could be seen in our experiment and not under the experimental protocol used by Carter et al. Possible support for this idea comes from the data of Carter et al., demonstrating that, while indomethacin had no chemopreventive activity in the low response model (low carcinogen dose alone), the compound did possess significant anticarcinogenic activity against mammary tumorigenesis induced in a higher response model (the same carcinogen dose promoted by a high-fat diet).

A second difference in protocol concerns the experimental diets used in the 2 experiments. While the present study was conducted using a chow diet exclusively, Carter et al. switched their animals from a chow diet to a semipurified, casein-based diet at 3 days post-DMBA. Chow diets contain a variety of components which are theoretically not present in semipurified diets; the possible enhancement of indomethacin anticarcinogenicity by a component of the chow diet which is absent in the casein diet is a possible explanation for the differential activity of indomethacin as observed in the 2 experiments.

In support of the data from the present study, it should be noted that these data are consistent with our previous report of...
the activity of the prostaglandin synthesis inhibitor, flurbiprofen, against chemical carcinogenesis in the rat mammary gland (27). It should also be noted that, although Carter et al. (3) saw no influence of indomethacin on mammary tumor incidence or multiplicity in rats fed a low-fat diet, mean tumor size in animals fed indomethacin and the low-fat ration was only one-third of that seen in animals fed the same diet without indomethacin (3).

These data indicate that indomethacin has significant protective activity against the chemical induction of tumors in the rat mammary gland. Although a statistically significant reduction in mammary tumor response was found in the present study, consideration of the present results together with the data of Carter et al. (3) indicate a need for further study of the anticarcinogenic activity of this class of compounds. More specifically, the question of the interaction between indomethacin activity and carcinogen dose remains unanswered at the present time. Furthermore, although an inhibition of carcinogenesis was observed in the present study, this chemoprevention was accompanied by the induction of treatment-related toxicity in certain groups. The toxicity seen in the present study was mild and is apparently unrelated to anticarcinogenic efficacy. However, the presence of any toxicity as the result of administration of a chemopreventive drug is an unacceptable situation; modalities to eliminate such toxicity, or the design of more active and less toxic drug congeners, would be required prior to the consideration of this class of compounds for possible use in clinical chemoprevention trials.

ACKNOWLEDGMENTS

The authors thank Candido Leilo and Patricia Moser for assistance in preparation of the manuscript.

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CANCER RESEARCH VOL. 45 APRIL 1985

1807

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Fig. 1. Photomicrograph of abdominal-inguinal mammary gland from rat in dietary control group. Note degree of lobuloalveolar differentiation. Alum carmine, ×30.

Fig. 2. Photomicrograph of abdominal-inguinal mammary gland from rat in group receiving +1 week to end indomethacin exposure. Gland consists primarily of naked ducts, with little lobuloalveolar differentiation. Alum carmine, ×30.
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