ABSTRACT

Indomethacin, an inhibitor of prostaglandin (PG) synthesis, was investigated for its ability to increase radiorestraint of two fibrosarcomas, FSA and NFSA, in C3H/Kam mice. In addition, the effect of indomethacin on radiosensitivity of hematopoietic tissue, jejunum, hair follicles, and tissues involved in the development of radiation-induced leg contractures was determined. Indomethacin greatly increased radiorestraint of 8-mm tumors, as assessed by both tumor growth delay and TCD50 assays. Enhancement factors for tumor growth delay and tumor radiocurability (TCD50) were 1.55 and 1.39, respectively, for FSA, and 1.4 and 1.26, respectively, for NFSA tumors. Of four normal tissues assessed, two (hair follicles and tissues responsible for development of leg contractures) showed no change in radiosensitivity after treatment with indomethacin, one (hematopoietic tissue) exhibited radioprotection, and one (jejenum) exhibited slight radiosensitization (enhancement factor, 1.12). Therefore, indomethacin significantly augmented tumor radiocurability but had minimal effect on radiosensitivity of some normal tissues.

INTRODUCTION

Indomethacin, a potent inhibitor of prostaglandins (PGs) synthesis, exhibits antitumor activity against many experimental and human tumors (reviewed in Ref. 1). We have recently shown that the response to indomethacin treatment is dependent on the ability of tumors to produce PGs (2). Because indomethacin usually manifests its effect in a slight or moderate tumor growth retardation, its therapeutic potential is likely to lie only in its combination with other treatment modalities. There have already been a few reports showing that inhibitors of PG synthesis, including indomethacin, can improve the therapeutic effect of some other treatments (3–7).

In relation to the combination of PG inhibitors with tumor radiotherapy flurbiprofen was reported to increase radiorestraint of NC carcinomas (5) and S-180 sarcoma (6) in mice to single-dose local tumor irradiation. The effect was determined by the reduction in tumor weight at a given time after the treatment. On the other hand, PG inhibitors either did not influence radiosensitivity of certain normal tissues (8) or they caused radioprotection (9, 10).

However, a number of recent studies have shown that PGs can also act as potent radioprotectors of some normal tissues, such as jejunum (11, 12) and bone marrow (12, 13). If PGs act as radioprotectors of both normal tissues and tumors, their inhibition would result in radiosensitization of both types of tissues. The magnitude in the response between the two tissues could, however, differ considerably if the response is dependent on the tissue level of PGs. As we reported earlier (2) indomethacin was effective against tumors only if they were producing PGs. The present study was designed to determine whether indomethacin augments radiocurability of PG-producing tumors and if so, to what extent. The effect of indomethacin on radiosensitivity of a number of normal tissues was also measured to determine whether the treatment would result in therapeutic gain.

MATERIALS AND METHODS

Mice

Inbred C3H/Kam mice of both sexes bred and maintained in our own specific pathogen-free mouse colony were used. They were 11 to 14 weeks old at the beginning of the experiments. Within each experiment, mice of the same sex were used and were housed four to seven per cage.

Indomethacin

Mice were given indomethacin (Sigma Chemical Co., St. Louis, MO) or vehicle (0.5% ethanol and 5% phosphate buffered saline) in the drinking water. Indomethacin was dissolved in absolute ethanol and diluted in distilled water containing 5% phosphate buffered saline to achieve a final indomethacin concentration of 35 μg/mL. Water bottles were changed every 3 days.

Tumor Growth Delay

Tumors were generated by injecting 4.6 × 105 viable FSA cells or 4.5 × 105 viable NFSA cells into the right thighs of mice. When tumors grew to 6 mm in diameter, mice were treated with indomethacin or vehicle for 10 consecutive days. When tumors grew to 8 mm in diameter, which occurred 1 to 4 days after the treatment with vehicle or 2 to 6 after the treatment with indomethacin was started, they were exposed to single doses of γ-radiation. The irradiation doses were 20, 25, and 30 Gy for FSA and 20, 30, 40, and 50 Gy for NFSA. Irradiation to the tumor was delivered from a dual-source 125I irradiator at a dose rate of 8 Gy per min. During irradiation, unanesthetized mice were immobilized on a jig and the tumor centered in a circular radiation field 3 cm in diameter. To obtain tumor growth curves, three mutually orthogonal diameters of tumors were measured at 1-, 2-, or 3-day intervals with a vernier caliper and the mean values were calculated. Regression and growth of tumors was followed until tumor diameter reached approximately 17 mm. Tumor growth delay was expressed as the time in days for tumors in the treated groups to grow from 8 to 12 mm in diameter minus the time in days for tumors in the control group to reach the same size. Groups consisted of seven to 10 mice each.

Assays for Tumor Response to Radiation

Experiments were performed using two sarcomas syngeneic to C3H/Kam mice: the immunogenic methylcholanthrene-induced fibrosarcoma FSA (14) and the nonimmunogenic spontaneous fibrosarcoma NFSA (15). Single-cell suspensions were prepared by trypsin digestion of nonneoplastic tumor tissue (14). Viability of cells was more than 95% as assessed by phase-contrast microscopy and trypan blue exclusion.

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3 The abbreviations used are: PG, prostaglandin; TCD50, dose of irradiation yielding local tumor control in 50% of animals; LD50, dose of irradiation causing death in 50% of animals within 30 days of irradiation; WBI, whole-body irradiation (irradiated); EF, enhancement factor; PF, protection factor; PGE2, prostaglandin E2; PGF2α, prostacyclin.

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TCD₅₀ Assay

The entire procedure for generating FSA and NFSA tumors, treatment with indomethacin or vehicle, and local tumor irradiation was the same as described above. Here, the single doses of γ-radiation delivered to FSA ranged from 18 to 45 Gy and those delivered to NFSA ranged from 35 to 85 Gy. Mice were checked for the presence of tumors at the irradiated site at 2- to 7-day intervals for up to 120 days. TCD₅₀ values were computed by the logit method of analysis (16). TCD₅₀ assays contained 48 to 68 mice each.

Normal Tissue Response

Hematopoietic Tissue. Mouse lethality (LD₅₀/₃₀) and endogenous spleen colony formation were used to evaluate the response of hematopoietic tissue to combinations of radiation and indomethacin. In the LD₅₀/₃₀ assay, mice were exposed to single X-ray doses of WBI ranging from 6.5 to 11 Gy. Indomethacin (35 µg/ml) was given for 6 days before WBI. Mouse mortality was determined daily for 30 days after irradiation. In the endogenous spleen colony assay, mice were exposed to WBI ranging from 6.6 to 9.5 Gy. Indomethacin (35 µg/ml) was given in drinking water for 6 days before WBI. Mice were killed 8 days after WBI, and the number of colonies on the surface of the spleen were counted by eye or with a dissection scope.

Gut. The microcolony assay introduced by Withers and Elkind (17) was used to determine the survival of crypt epithelial cells in the jejunum of mice exposed to ionizing radiation. Mice were exposed to WBI with single doses of X-rays ranging from 11.8 to 15 Gy given at a dose rate of 1.85 Gy/min. The mice were given indomethacin (35 µg/ml) in drinking water for 6 consecutive days starting with 3 days before WBI and were killed 3.5 days after WBI. The jejunum was prepared for histological examination, and the number of regenerating crypts in the jejunal cross-section was counted. To construct radiation survival curves, the number of regenerating crypts was converted to the number of surviving cells by applying a poisson correction for crypts regenerating from more than one stem cell. Lines were fitted to data points by least-squares regression analysis.

Hair Loss. Hair loss (hair epilation) was determined on irradiated legs of mice in the FSA TCD₅₀ experiment (see above, TCD₅₀ assay) 32 days after irradiation. Only mice with no recurrent tumors were used for the determination of radiation-induced hair loss. At each radiation dose point, the number of mice having 100% hair epilation was scored. The ED₅₀ was then determined by the logit method of analysis (16).

Reduction in Leg Extension. Radiation-induced leg contraction (reduction in leg extension) was determined on mice in the TCD₅₀ assays for FSA and NFSA that had no recurrent tumors present. The measurement was performed at 116 days after irradiation, when the leg contraction was at a plateau (18). The method used for the assessment of the leg contraction was described in detail previously (19). Briefly, mice in a Lucite jig with tails between the vertical posts of the jig had both the nonirradiated and irradiated legs extended over a scale measuring millimeters. Readings were made at the ankle. The leg extension reduction values were obtained by subtracting the length of the irradiated leg from that of the nonirradiated leg.

RESULTS

Tumor Response. Tumor growth delay and tumor cure were used as the endpoints in the assessment of the effect of indomethacin on tumor radiosensitivity. Tumors in the right legs were generated by intramuscular injections of 4.6 × 10⁵ FSA or 4.5 × 10⁵ NFSA cells. When tumors grew to 6 mm in diameter the mice were given indomethacin or vehicle in the drinking water, a treatment that was continued for 10 days. When these tumors reached 8 mm in diameter, which occurred several days after the initiation of this treatment, they were exposed to local tumor irradiation in single doses of 20, 25, or 30 Gy in the case of FSA, and of 20, 30, 40, or 50 Gy in the case of NFSA tumors.

Fig. 1 shows the effect of indomethacin on the growth of the two tumors. Both tumors exhibited similar but significant reduction in their growth.

Fig. 2 shows the effect of indomethacin on FSA (Fig. 2A) and NFSA (Fig. 2B) growth in C3Hf/Kam mice. The treatment with indomethacin, 35 µg/ml in drinking water, was started when tumors were 6 mm in diameter and was continued daily for 10 days. Open symbols, tumor growth in control mice; bars, SE.
and NFSA (Fig. 2B) radioresponse. Radiation alone caused a dose-dependent tumor growth delay. The growth delay after the combined treatment was more than the sum of growth delays caused by either irradiation or indomethacin. To express this obvious augmentation of tumor radioresponses by indomethacin quantitatively the enhancement factors were determined from the plot of radiation-caused tumor growth delays in the indomethacin-treated and control mice (Fig. 3). Indomethacin increased the radioresponse of FSA by a factor of 1.55 and radioresponse of NFSA by a factor of 1.4. In addition to its ability to increase tumor growth delay, indomethacin combined with higher doses of radiation was curative to a significant proportion of mice. For example, while 30 Gy cured zero of seven mice bearing FSA, it cured five of six mice when combined with indomethacin. Similarly, 40 Gy alone cured none of seven mice bearing NFSA; it cured four of 10 mice when combined with indomethacin.

To quantitate the indomethacin-induced augmentation of tumor radioresponse, TCD50 assays were performed. The experimental procedure was the same as that described above for the tumor growth delay experiments. Single doses of local tumor irradiation ranged from 18 to 45 Gy for FSA and from 35 to 85 Gy for NFSA tumors. The dose-response curves for tumor control are shown in Fig. 4. Indomethacin greatly reduced the TCD50 value for FSA from 38.9 (37.5–40.6) Gy to 27.9 (21.7–33.8) Gy, and for NFSA from 63.1 (57.3–67.8) to 50.2 (44.6–55.6). In parentheses are 95% confidence limits. Here, the enhancement factors were 1.39 for FSA and 1.26 for NFSA.

Normal Tissue Radioresponse. The effect of indomethacin on radiation response of normal tissues was studied using hematopoietic tissue, jejunum, hair follicles, and tissues responsible for the development of radiation-induced leg contractures. While the first three tissues respond with early radiation injury, leg contractures are a manifestation of late radiation damage.

The effect of indomethacin on radiation damage of the hematopoietic tissue was determined by LD50/30 and endogenous spleen colony assays. Indomethacin, given in the drinking water for 6 days before WBI, did not influence the LD50/30 value: LD50/30 was 7.9 (7.8–7.9) Gy in mice treated with indomethacin and WBI and 8.0 (7.9–8.1) Gy in mice that received WBI only. In parentheses are 95% confidence limits. In contrast, indomethacin given for 6 days before irradiation resulted in a significantly higher number of endogenous spleen colonies compared to that in mice that received WBI only (Fig. 5). The slope of the radiation response curves were similar for both the experimental and the control groups.

The effect of indomethacin on the radioresponse of jejunal crypt cells is shown in Fig. 6. Indomethacin was given in the drinking water for 6 consecutive days starting with 3 days before a range of single doses of WBI. The radiation response curves show that indomethacin only slightly increased the radioreponse of jejunum: the enhancement factor was 1.12. Here two additional groups were included. In one group the mice were treated with indomethacin for 3 days before irradiation, and in the other group the mice were given indomethacin for the first 3 days after irradiation (Fig. 6). In the former group indomethacin increased the radioreponse of crypt cells to nearly the same extent as indomethacin given both before and after irradiation. In contrast, indomethacin given after WBI had no effect on the radioreponse of crypt cells.

The effect of indomethacin on radiation-induced hair loss was determined on the same mice that were used in the TCD50 experiment with FSA (see above). At 32 days after irradiation, mice free of macroscopic tumor (recurrence) were checked for...
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Results of our study show that indomethacin treatment significantly increased response of two murine tumors to local tumor irradiation, as evidenced by the increase in tumor growth delay and the augmentation in tumor curability. Enhancement factors for tumor growth delay and tumor radiocurability were 1.55 and 1.39, respectively, for FSA, and 1.4 and 1.26, respectively, for NFSA tumors. A few earlier studies available on the combination of PG inhibitors and local tumor irradiation have suggested that such agents can increase tumor radiocurability (5, 6), but they did not provide information sufficient to allow calculation of enhancement factors.

A number of mechanisms could have contributed to the increased tumor radioresponse and, although they were not studied here, they will be briefly considered. In light of considerable recent evidence that PGs act as radioprotectors of some normal tissues (11–13), it is reasonable to assume that indomethacin increased tumor radioresponse by lowering the level of PGs in the tumor. Both FSA and NFSA produce large amounts of different types of PGs, which are greatly inhibited by the treatment with indomethacin (2). FSA produces mainly PGE2 and NFSA produces mainly PG12 (2). Both PGE2 and PG12 have been demonstrated to radioprotect jejunal crypt cells (12, 20); in addition, PG12 was recently reported to protect B16 melanoma cells from radiation damage (20).

Both PG (2) and tumor-associated macrophages4 in tumors are reduced by indomethacin, which creates a condition within a tumor that may be unfavorable to cell proliferation because macrophages from many malignant tumors (21–23), including NFSA (24), stimulate proliferation of tumor cells. Although the mechanism by which this stimulation is achieved is unknown, in some cases tumor cell growth stimulation is mediated by macrophage-secreted PGs (23). Macrophage-stimulated tumor cell proliferation has been implicated as a cause for poor tumor response to cyclophosphamide (22) or local tumor irradiation (24). This mechanism of inhibition of tumor cell proliferation by reducing tumor-associated macrophages and PGs could explain, at least partly, the indomethacin-induced increase in radioresponse of NFSA tumor, but not that of FSA tumor because macrophages from the FSA tumor are cytotoxic for FSA cells (25).

Perturbations in the cell cycle of tumor cells can be responsible for the indomethacin-induced increase in tumor radioresponse, if indomethacin caused accumulation of cells in the cell cycle phases more sensitive to radiation damage. In an earlier report we showed that indomethacin caused the accumulation of cells in the G2 + M phases of cell cycle (2), phases generally considered to be the most sensitive to ionizing radiation (26).

Immunological modulation by indomethacin that would increase tumor cell kill by antitumor immune mechanisms does not appear to be a significant mechanism. We base this conclusion on the observation that the effects of indomethacin alone as well as of its combination with local tumor irradiation were similar against immunogenic FSA and nonimmunogenic NFSA (Figs. 1, 2, and 4) and on other evidence discussed in our earlier report (2). This includes the inability of whole body irradiation to influence the antitumor effect of indomethacin and the inability of indomethacin to significantly change the s.c. tumor take of the immunogenic FSA either in normal or whole body irradiated mice.

Finally, PGs are vasoactive agents, and as such are likely to

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4 Furuta, Serra, Willingham, and Milas, Effect of WBI and indomethacin on tumor macrophage content, unpublished manuscript.
participate in regulation of tumor blood flow and perfusion. Both PGE₂ and PGI₁ have vasodilating activities (27, 28), while thromboxane is a potent vasoconstrictor and, in addition, causes platelet aggregation and thrombus formation (28). It is reasonable to anticipate that both qualitative and quantitative changes in these activities of PGs will modify tumor blood supply, tumor oxygenation status, and through these, tumor cell radiosensitivity. A report by Rankin and Pernetton (29) that administration of PGE₂ lowers tumor blood flow supports the vasoactive mechanism as a likely participant in alteration of tumor radiocurability after treatment with indomethacin.

To be therapeutically beneficial, agents that increase radiation damage to tumors, including indomethacin, must be less effective in increasing normal tissue damage by irradiation. Using hematopoietic tissue, jejunal crypt cells, hair follicles, and tissues involved in the development of radiation-induced leg contractures, we have clearly demonstrated that the radiation response of these tissues was modified much less than that of the two tumors studied here, implying that indomethacin provided a significant therapeutic benefit.

Radiation damage of hematopoietic tissue was assessed using the endogenous spleen colony and LD₅₀/₃₀ assays. WBI reduced the number of hematopoietic spleen colonies more in mice treated with the vehicle than in mice treated with indomethacin. The PF was 1.46. However, this protection did not manifest in preventing or reducing mouse mortality from WBI. Both PGs and their inhibitors have been found to regulate proliferation and maturation of hematopoietic cells (3, 4, 12, 13, 30, 31), and to modify radiation response of hematopoietic cells (12, 13), but the results are quite inconsistent. Indomethacin (30, 31) and flurbiprofen (3, 4) stimulate repopulation of hematopoietic cells in WBI mice, but this can be achieved by administering PGE₂ as well (13). PGE₂ also increased radioresistance of bone marrow stem cells (12), whereas flurbiprofen had no effect on hematopoietic stem cell radioreponse (8). Whether the observed higher number of hematopoietic cells in WBI mice that had been treated with indomethacin was the result of stimulated stem cell proliferation or their protection against radiation damage is not clear, and is a subject of our current investigations.

Different PGs (11, 12, 20, 32) and leukotriens (32) are moderate to potent radioprotectors of jejunum crypts in mice. The protection was observed using both clonogenic crypt cell survival and LD₅₀/₃₀ assays. The mechanisms of the radioprotection are complex but they appear to involve interference with radiation-produced free radicals that kill cells and inhibition of sloughing of radiation-damaged cells from the surface of villi. In the present study, indomethacin increased radiation response of crypt cells when given for 3 days before irradiation. The radiosensitization was, however, modest (by a factor of 1.12), and did not increase further by continuation of indomethacin administration for additional 3 days after WBI. Indomethacin given after radiation had no influence on jejunum radioreponse. Based on the observations by Hanson and associates (11, 12, 20, 32, 33) the most plausible explanation for the indomethacin-induced increase in radioreponse of jejunum is that the drug lowered the PG level in crypt cells and thus removed its radioprotective effect. In other studies (8), flurbiprofen given 1 day before irradiation did not change radioreponse of crypt cells, but it is possible that one day's drug administration is insufficient to reduce PGs to the level low enough to cause radiosensitization.

Indomethacin did not modify radioreponse of either hair follicles or tissues responsible for leg contractures. Thus, of four tissues examined here two showed no change in radiation response, one (jejenum) was mildly radiosensitized, and one (hematopoietic tissue) was slightly protected. It should be noted that there have been reports on the effect of PG inhibitors on radioreponse of other tissues. Indomethacin afforded a significant radioprotection to esophagus in opossums (9) and to parotid glands in rats (10). Thus, most studies reported by others, including our results presented here, have shown that inhibitors of PGs act as radioprotectors of some normal tissues and cause no change in radioreponse of some other tissues; only exceptionally do they cause a modest radiosensitization.

There have been a number of studies that assessed the effect of nonsteroidal antiinflammatory agents in combination with radiotherapy of human tumors. Weppelmann and Monkmeyer (34) observed that both 5- and 10-year survival of patients with the FIGO Stages I-IV carcinoma of the cervix was significantly increased when in addition to radiotherapy the patients were treated with oxyphephobutazine daily during the period of radiation treatment. Another study (35) that combined indomethacin and radiotherapy in the treatment of advanced cancer of the head and neck showed no influence of indomethacin on patient survival during the 2-year observation period after radiotherapy, but it showed a significant protection by indomethacin against radiation mucositis. This study, however, had a small number of patients and was very heterogeneous in the extent of disease and tumor anatomic localizations. It should be emphasized that intertumor heterogeneity has a negative impact on clinical trials by reducing or abolishing the ability to demonstrate a real therapeutic benefit for a subset of the population (36). In this regard our earlier observation that the antitumor effect of indomethacin is dependent on PG production by tumors (2) could have an important predictive value. Pretreatment determinations of PG-secreting and -nonsecreting tumors should enable the identification of patients likely to benefit from indomethacin treatment.

Overall, our data clearly demonstrate that indomethacin can increase therapeutic gain when combined with tumor radiotherapy. While a significant increase in tumor radioreponse was observed, of four normal tissues examined two showed no change in radioreponse, one exhibited slight radioprotection, and one showed slight radiosensitization. Further studies on the combination of tumor irradiation and indomethacin or other PG inhibitory agents are warranted, especially those designed to elucidate how they increase tumor radioreponse and those aimed to define settings under which the greatest therapeutic gain can be achieved.

REFERENCES


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INCREASED TUMOR RADIORESPONSE BY INDOMETHACIN


Increase in Radioresponse of Murine Tumors by Treatment with Indomethacin

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