Dependence of Indomethacin-induced Potentiation of Murine Tumor Radiosensitivity on Tumor Host Immunocompetence

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ABSTRACT

In a previous study (Furuta, Y., Hunter, N., Barkley, H. T., Jr., Hall, E., and Milas, L., Cancer Res., 48: 3008–3013, 1988), we demonstrated that inhibition of prostaglandins in murine tumors by indomethacin results in the augmentation of tumor response to single doses of ionizing radiation. The results of the present study show that indomethacin augmented tumor response to fractionated irradiation as well, the enhancement factor being more than 2. The effect of indomethacin on tumor growth and on tumor radiosensitivity was assayed in normal mice, mice deficient in T-cells (nu/nu mice), and mice whose general immunocompetence was suppressed by whole-body irradiation. The antitumor activity of indomethacin was not significantly influenced by the immunocompetence of the tumor host. Since indomethacin inhibited tumor neoangiogenesis, we postulated that this inhibition is a major mechanism responsible for the antitumor activity of indomethacin. In contrast, potentiation of tumor radiosensitivity by indomethacin was greatly dependent on immunocompetence of tumor host: it was significantly reduced, or even abolished, when tumor grew in nude and whole-body irradiation mice. Thus, while immunocompetence of the tumor host has no significant effect on antitumor action by indomethacin, it plays a decisive role in the potentiation of tumor radiosensitivity by indomethacin.

INTRODUCTION

We recently reported (1) that murine tumors differ greatly in their ability to produce PGs.2 While some tumors produce PGs in scarcely measurable traces, if at all, other tumors produce them in large quantities. Only the PG-producing tumors responded to treatments with the PG inhibitor indomethacin by slowing their growth. Furthermore, indomethacin treatment significantly potentiated tumor radioresponsiveness (2). In contrast, indomethacin was either ineffectual in influencing radioreponse of a number of normal tissues or it protected one of them (hematopoietic system) (2, 3). These data imply that inhibition of PGs in tumors could be used to improve radiotherapy of malignant tumors.

The mechanisms by which indomethacin potentiates tumor radiosensitivity are not known. Based on recent findings that PGs are radioprotective agents (4, 5), we presumed (2) that indomethacin most likely increased tumor radiosensitivity by lowering PGs in the tumor, thus abrogating the radioprotective actions of PGs. Some other mechanisms have also been considered (2). The experiments described in this article consider additional aspects of the indomethacin-induced potentiation of tumor radiosensitivity. They show that the effect of indomethacin is greatly dependent on immunological competence of tumor hosts, suggesting that the indomethacin-induced potentiation of tumor radioresponsiveness is mainly an immunological phenomenon.

MATERIALS AND METHODS

Mice. Inbred male C3H/Kam mice and the athymic NCr/Sed-nu/nu nude male mice bred and maintained in our own specific-pathogen-free mouse colony were used. They were 11–14 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.

Tumors. Experiments were performed using two sarcomas syngeneic to C3H/Kam mice: the immunogenic methylcholanthrene-induced fibrosarcoma FSA (6) and the nonimmunogenic spontaneous fibrosarcoma NFSA (7). Single-cell suspensions were prepared by trypsin digestion of nonnecrotic tumor tissue (6). Viability of cells was more than 95% as assessed by phase-contrast microscopy and trypan blue exclusion. Both tumors are good producers of PGs; FSA produces mainly PGE2 and NFSA produces mainly 6-kPGL2α (1).

Assays of Tumor Response to Indomethacin, Radiation, or Both.

Tumors were generated by injecting 5 × 106 viable FSA or NFSA cells into the right thighs of mice. The mice were either normal, nude mice or C3H/Kam mice that had been exposed to 6 Gy WBI 1 day before being given injections of tumor cells. When tumors grew to 6 or 8 mm in diameter, the mice were treated with indomethacin or vehicle daily for 3–10 consecutive days. Tumor growth before, during, and after treatments with indomethacin or vehicle was determined by measuring three mutually orthogonal diameters of tumors at 1, 2, or 3-day intervals with a vernier caliper and calculating the mean values. This measurement was performed until tumors reached 16–18 mm in diameter. The effect of indomethacin was assessed by the extent of tumor growth retardation caused by indomethacin treatment.

When tumors grew to 6 mm or, in most experiments, to 8 mm they were exposed to either single or fractionated doses of γ-radiation. The individual doses of radiation are listed in the description of experiments in “Results.” The mice that were exposed to local tumor irradiation also received indomethacin or vehicle before, or after, or before and after local tumor irradiation. The regression and regrowth of tumors was followed until tumors reached 16–18 mm in diameter as described above. The effect of radiation with or without indomethacin was expressed by the tumor growth delay or by the TCD50 value (radiation dose yielding local tumor control in 50% of animals). In experiments in which tumors were exposed to single doses of irradiation, tumor growth delay was taken 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 groups to reach the same size. However, in the experiment in which tumors were exposed to fractionated irradiation tumor growth delay was taken as the time in days for tumors in the treated groups to grow from 8 to 16 mm in diameter minus the time in days for tumors in the control groups to reach the same size. The reason for the latter was that tumors exposed to fractionated

Received 1/8/90; revised 4/12/90.

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1This investigation was supported by NIH Research Grant CA-06294. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture and Department of Health and Human Services.

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5The abbreviations used are: PG, prostaglandin; TCD50, dose of irradiation yielding local tumor control in 50% of animals; WBI, whole-body irradiation (irradiated); FSA, fibrosarcoma; NFSA, nonimmunogenic spontaneous fibrosarcoma.
irradiation started to slow their growth only after they reached 11-13 mm in diameter in size. Groups consisted of 7 to 10 mice each. In the TCD50 assays mice were checked for the presence of tumor at the irradiated site at 2-7-day intervals for up to 120 days after irradiation. TCD50 values were computed by the logit method of analysis (8). TCD50 assays contained 48-68 mice each.

WBI and Local Tumor Irradiation. For WBI, mice were exposed to a single dose of 6-Gy X-rays in groups of seven in a Lucite container. The radiation was delivered with a Phillips 250-kVp X-ray unit, at a dose rate of 1.79 Gy/min. Irradiation to the tumor was delivered from a dual-source 157Cs irradiator at a dose rate of 8 Gy/min. During irradiation, unanesthetized mice were immobilized on a jig and the tumor centered in a circular radiation field 3 cm in diameter.

Intradermal Assay for Tumor Angiogenesis. A triangular skin flap was constructed at the right abdominal region of mice anesthetized with Nembutal (0.06 mg/body weight) by making a skin incision along the midline of the abdomen and extending it to the right groin. The skin flap was separated from the s.c. tissue by a gentle pull laterally and then was searched for an area with the fewest tiny blood vessels possible using a dissecting microscope with a magnification of x20. After the number of blood vessels was recorded at the tumor cell injection site, 104 NFSA cells were injected intradermally in a volume of 0.03 ml of phosphate-buffered saline using a 30-gauge needle. The skin flap was then brought back to the midline and closed using surgical clips. One day after the injection of tumor cells the mice began receiving treatments with indomethacin or vehicle, which continued daily for 9 consecutive days. The number of blood vessels as well as tumor size was determined at 2-day intervals, starting 2 days after tumor cell injection and continuing until 8 or 10 days after tumor cell injection. This was performed under a dissecting microscope (x20) in anesthetized animals in which the skin flap at each of the above days was reopened by removing surgical clips and pulling the flap laterally. Tumor volume was calculated using the formula for calculating ellipsoidal mass (\(\frac{4}{3}\pi abc\)).

RESULTS

Fractionated Irradiation. It was established earlier that indomethacin augments tumor radiocurability when combined with large single doses of radiation delivered to the tumor (2). This experiment was performed to determine whether indomethacin also augments tumor radiocurability when combined with fractionated radiotherapy. Eight-mm FSA tumor transplants growing in the leg were treated with 2, 2.5, or 3 Gy given 10 times, at 6-h intervals between individual irradiations, to achieve a total dose of 20, 25, or 30 Gy. Indomethacin treatment was initiated before radiotherapy was begun, at a time when tumors were 6 mm in diameter, and it was continued daily for 10 consecutive days. Because indomethacin slowed the growth of tumors, local tumor radiation in mice receiving indomethacin was begun 3-4 days later than local tumor radiation in mice receiving no indomethacin. Tumor growth delay was used to assess the effects of these treatments.

A significant radiation dose-dependent delay in tumor growth was observed after local tumor irradiation. This fractionation irradiation schedule slowed the growth of tumors only after they reached 11-13 mm in diameter. The effect of radiation was much more pronounced in mice that received indomethacin than in the control mice (Fig. 1). For clarity the effect of indomethacin plus radiation on tumor growth was plotted only for groups in which 2 Gy was used. Also, the radiation-caused tumor growth delay in both indomethacin-treated and indomethacin-untreated mice as a function of radiation dose was plotted in Fig. 1, inset. Indomethacin increased the radioreponse of FSA by a factor of more than 2, which is a higher value than we reported when indomethacin was combined with single radiation doses (enhancement factor of 1.55) (2).
and similarly augmented tumor radioreponse.

In a separate experiment, using the FSA tumor and TCD50 as the end point of treatment response, administration of indomethacin 3–4 days before local tumor irradiation reduced the TCD50 value, but not significantly. In contrast TCD50 was significantly lowered when indomethacin was given both before and after irradiation (data not presented).

Antitumor Effects of Indomethacin, Given as the Only Treatment or in Combination with Local Tumor Irradiation, in WBI and Nude Mice. The above observation (Table 1), that administration of indomethacin after tumor irradiation augments tumor radioreponse, implies that the level of PGs in tumors need not be reduced at the time of irradiation in order to potentiate radiation effect. This observation further implies that indomethacin did not potentiate radiotherapy by reducing the radioprotective effect of PGs, in contrast to our major working hypothesis from earlier studies (1, 2). Consequently, we explored the possibility that indomethacin potentiated tumor radioreponse through modification of the tumor host immune system. Although the data from Table 1 show that indomethacin augmented the radioreponse of both immunogenic FSA and nonimmunogenic NFSA, and some other evidence from our earlier studies (1, 2) did not implicate modulation of the immune system as a significant mechanism, we were unable to ascribe the potentiating effects of indomethacin to other, more likely causes.

To determine whether a change in immunocompetence of the tumor host affects the effect of indomethacin, we investigated the antitumor efficacy of indomethacin given either as the only treatment or as a combination with local tumor irradiation, in normal, WBI, and nude mice. Both immunogenic FSA and nonimmunogenic NFSA were used. The mice were exposed to 7-day treatments with indomethacin starting when tumors grew to 8 mm in diameter. The results presented in Fig. 2 show that both tumors responded to indomethacin by slowing their growth regardless of the host immune competence. In fact, both FSA and NFSA responded to the drug slightly, but not significantly, better in WBI and nude mice than in normal mice. This implies that indomethacin given as the only treatment did not retard tumor growth through the involvement of the immune system. A similar conclusion was reached in an earlier communication by us (1).

In the experiments in which indomethacin was combined with tumor irradiation, FSA or NFSA tumors, growing in normal, WBI, or nude mice, were exposed to single doses of local tumor irradiation once tumors grew to 8 mm in diameter. Indomethacin treatment was initiated 3 h after tumor irradiation and was continued daily for 7 consecutive days. Radiation doses for FSA ranged from 18 to 40 Gy and those for NFSA ranged from 25 to 50 Gy. The effects of local tumor irradiation alone or combined with indomethacin were evident by a delay in tumor growth. Indomethacin greatly increased the radioreponse of both tumors in normal mice (Fig. 3). However, its affect on the radioreponse of FSA was totally abolished and its affect on radioreponse of NFSA greatly reduced in WBI and nude mice (Fig. 4).

The influence of indomethacin upon tumor radioreponse was also assessed using the radiocurability TCD50 assay. Control or WBI mice bearing 8-mm FSA or NFSA tumors were exposed to local tumor irradiation. In addition to irradiation, mice were treated with indomethacin. In the case of the mice bearing FSA tumors, indomethacin treatment was initiated when tumors were 6 mm in diameter and was continued daily for 10 consecutive days. Here, local tumor irradiation was performed 3–4 days after the initiation of indomethacin treatment. In the case of the mice bearing NFSA, indomethacin treatment was started 3 h after local tumor irradiation and was continued daily for 7 consecutive days. The TCD50 values at 120 days after irradiation (Table 2) show that whereas indomethacin significantly augmented tumor radiocurability of both tumors in normal mice, it did not influence tumor radiocurability in WBI mice. WBI by itself significantly increased the TCD50 value (reduced radiocurability) of immunogenic FSA but
Fig. 4. Growth delay in days for FSA (circles) and NFSA (squares) as a function of radiation dose in whole body irradiated C3H/Kam mice (4) and in athymic Ncr/Sed-ns nu nude mice (8) treated (solid symbols) or untreated (open symbols) with indomethacin. Bars, SE of the mean values. Irradiation with single doses of γ-rays was delivered when tumors grew to 8 mm in diameter. Treatment with indomethacin, 35 μg/ml, in drinking water, was started when FSA tumors grew to 6 mm in diameter and was continued daily for 7 consecutive days. WBI with 6 Gy was delivered 1 day before transplantation of tumor cells.

Table 2: Effect of indomethacin on radiocurability of FSA and NFSA tumors in normal or WBI mice

<table>
<thead>
<tr>
<th>Status of tumor-bearing hosts*</th>
<th>TCD50 (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>36.9 (35.7–38.0)</td>
</tr>
<tr>
<td>Normal + indomethacin</td>
<td>27.9 (25.6–30.5)</td>
</tr>
<tr>
<td>WBI</td>
<td>54.4 (51.1–57.7)</td>
</tr>
<tr>
<td>WBI + indomethacin</td>
<td>54.4 (51.1–57.7)</td>
</tr>
</tbody>
</table>

*Normal or WBI mice bearing 8-mm FSA or NFSA tumors were exposed to local tumor irradiation with single doses of γ-rays. Treatment with indomethacin, 35 μg/ml, in drinking water, was started when FSA tumors grew to 6 mm in diameter and was continued daily for 10 days. Indomethacin treatment of mice bearing FSA tumors was started 3 h after local tumor irradiation and was continued daily for 7 consecutive days. WBI with 6 Gy was delivered 1 day before transplantation of tumor cells.

INDOMETHACIN AND TUMOR RADIORESPONSE

Fig. 5. Effect of indomethacin on tumor angiogenesis. The mice were given intradermal inoculations of 10⁶ NFSA cells, and the number of vessels at the injection site was determined on the indicated days thereafter in indomethacin-treated (●) and control (○) mice. Tumor volume in indomethacin-treated (●) and indomethacin untreated (○) mice was also plotted. Bars, SE of the mean values. Treatment with indomethacin, 35 μg/ml, in drinking water, was started 1 day after tumor cell inoculation and was continued daily for 9 days.

DISCUSSION

The results presented here show that indomethacin potentiates response of murine tumors to ionizing radiation administered either in large single doses or as fractionated irradiation. The magnitude of response to fractionated irradiation was higher than that observed for single doses of local tumor irradiation. These data, and our additional findings that indomethacin protects some normal tissues against radiation damage, such as the lung (10) and hematopoietic tissue (2, 3), and that it does not influence the radioresponse of some other normal tissues (2, 3), suggest that indomethacin has significant potential in the therapy of malignant tumors when combined with radiotherapy.

A major thrust of the present study has been to search out mechanisms by which indomethacin augments tumor radioresponse. Initially (2) our working hypothesis was that indomethacin potentiates tumor radioresponse by lowering PGs in tumors, which have been reported to act as radioprotectors (4, 5). Other mechanisms were also considered including perturbation in the cell cycle, abolition of PG stimulation of cell growth, and immunopotentiation (2). A radioprotection mechanism was examined by the experiment in which indomethacin was given to mice before or after completion of radiotherapy, since true radioprotective agents usually must be present in tissues at the time of radiation. In our study potentiation of tumor radioresponse was in fact more evident when indomethacin was given after than when it was given before tumor irradiation. This implies that the reduced PG levels in tumors, one consequence of indomethacin (1), at the time of irradiation was not a prerequisite for indomethacin's potentiation effect, and it further suggests that in the tumor systems used here PGs did not act by protecting tumor cells against radiation injury.

That indomethacin was more effective when given after, than before, tumor irradiation also negates the possibility that indomethacin potentiates tumor radioresponse by causing tumor cells to accumulate in cell cycle phases more sensitive to ionizing radiation. A measurable tumor growth, and that indomethacin significantly reduced the number of newly formed vessels. This reduction in neovascularization was associated with tumor growth retardation.
ing radiation. This possibility was advanced in our earlier publication (2) after we observed that indomethacin-treated tumors had higher percentages of cells in G2M and G1 cell cycles than tumors in untreated mice (1).

In a previous publication (2) we also considered a possibility that indomethacin augmented tumor radioresponse through augmentation of antitumor immunological resistance mechanisms. At the time this possibility appeared remote because (a) antitumor activity of indomethacin was dependent not on tumor immunogenicity, but rather only on the ability of tumors to produce PGs and (b) indomethacin was equally effective in retarding tumor growth in normal and immunosuppressed mice (1). However, we reconsidered the possible role of immunological resistance in the present study after we failed to produce evidence that the indomethacin-induced augmentation of tumor radiocurability was mediated by the abolition of radioprotection or by cell cycle perturbation (see the above discussion).

As in an earlier study (1), indomethacin, given as the only treatment, had antitumor effects on both immunogenic (FSA) and nonimmunogenic (NFSA) tumors in both normal mice and mice whose immune system was suppressed by WBI. In addition, we observed that indomethacin was effective against the two tumors in nude mice (mice deficient in T-cells). Thus, we were unable to detect any participation of immunological mechanisms in the antitumor activity of indomethacin. It is likely, however, that indomethacin did augment antitumor immune responses, but the number of additional tumor cells killed by these responses must have been too low to be readily demonstrated by changes in tumor size. This point will be elaborated in more detail later in this text.

We postulate that the retardation in tumor growth caused by indomethacin is primarily due to inhibition of tumor angiogenesis. It has already been shown that PGs possess angiogenic properties and that neovascularization caused by PGs can be inhibited by indomethacin (9). In the present study tumor cells injected intradermally caused neovascularization before measurable tumor growth. Indomethacin inhibited this neovascularization, and subsequently tumor growth was delayed (Fig. 5). This mechanism easily explains the observed independence of antitumor activity of indomethacin from tumor immunogenicity and tumor host immunocompetence, as well as the dependence of indomethacin's antitumor activity on the ability of tumors to secrete PGs. As we reported earlier indomethacin retards the growth of tumors that secrete PGs, but not of tumors that do not secrete these substances (1).

In contrast to the antitumor effect of indomethacin alone the antitumor effect of local tumor irradiation plus indomethacin depended on tumor host immunocompetence. The indomethacin-induced increase in tumor radioresponse, readily observed in normal mice using both tumor growth delay and TCD50, was either greatly reduced or completely abolished in WBI and nude mice. This shows that indomethacin did augment antitumor immunological mechanisms, which in turn improved tumor radiotherapy. It is important to note that the radiation doses used here greatly reduced the total number of clonogenic tumor cells in tumors. For example, at the TCD50 radiation dose level, less than an average of one cell per tumor remains viable. With so few tumor cells remaining in tumors for several days after irradiation, immunological mechanisms stimulated by indomethacin could either completely eradicate the tumor cells, leading to increased tumor radiocurability or could further reduce the number of tumor cells (without eradicating them), thus delaying tumor appearance. This explanation is well supported by the evidence that indomethacin greatly decreased TCD50 values and delayed appearance of postirradiation recurrences in immunocompetent mice. However, once tumors regrow to a palpable size their further growth is unlikely to be significantly affected by the immune response, especially if it is not very strong. At this later phase of tumor regrowth, weeks to months after irradiation, the animals were no longer under indomethacin treatment. As discussed earlier (1) PGs can inhibit many immunological reactions; however, which inhibitory effects are counteracted by indomethacin to result in augmentation of tumor radioresponse is not known, and is being currently investigated in our laboratory. However, it remains unclear why an action of indomethacin to suppress angiogenesis in unirradiated tumors was not seen in tumors exposed to irradiation, as evident by the failure of indomethacin to affect the growth of irradiated tumors in immunocompromised mice. A possible explanation could be that a small number of tumor cells that survived radiation treatment (see above discussion) started to proliferate at some time after the suppressive effect of indomethacin on tumor angiogenesis was already ceased. This aspect of indomethacin treatment warrants further study.

In conclusion, indomethacin can markedly potentiate the response of solid tumors to both single or fractionated irradiation. The antitumor activity of indomethacin against well-established tumors is mediated primarily through suppression of tumor angiogenesis. However, augmentation of antitumor immune responses is the dominant mechanism in the increase in tumor radioresponse following indomethacin and radiotherapy.

REFERENCES
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