Combined Antitumor Effect of Radiation and Ibuprofen in Human Prostate Carcinoma Cells

Sanjeeewani T. Palayoor, Edward A. Bump, Stuart K. Calderwood, Suzanne Bartol, and C. Norman Coleman

Joint Center for Radiation Therapy, Harvard Medical School, Boston, Massachusetts 02215

ABSTRACT
Recent clinical observations indicate that ibuprofen may alleviate the radiation-induced dysuria that almost invariably occurs during radiation therapy for prostate cancer. Because the use of ibuprofen could consequently become common during radiation therapy for prostate cancer, we have been interested in the potential interactions between ibuprofen and ionizing radiation on prostate tumor cells. The effects of γ-irradiation and/or ibuprofen on PC3 and DU-145 human prostate carcinoma cells were evaluated in vitro using three model systems. Clonogenic survival was determined by plating cells 24 h after treatment of nearly confluent monolayers. Analysis of cell growth, cell detachment, and apoptotic cell death was carried out over a period of up to 9 days after treatment of PC3 and DU-145 monolayers. The effect of ibuprofen and/or radiation was also probed by observing the inhibition of growth of established PC3 and DU-145 colonies that were treated on the 14th day of colony growth. Ibuprofen enhanced the radiation response of prostate cancer cells in all three in vitro models. Both the cytotoxic and radiosensitizing effects of ibuprofen seem to require concentrations that are higher than those reported to inhibit prostaglandin synthesis, suggesting that other molecular mechanisms may be responsible for ibuprofen cytotoxicity.

INTRODUCTION
Recent studies have suggested that NSAIDs may be useful in cancer treatment, either alone or in combination with radiation therapy. It has been observed in the clinic that ibuprofen seems to alleviate the radiation-induced dysuria that almost invariably occurs during radiation therapy for prostate cancer, and a randomized clinical trial is now in progress to determine whether this is the case. Whereas this suggests a potential radioprotective activity of NSAIDs against the acute effects of treatment, several studies have indicated that anti-inflammatory agents may have antitumor activity or may enhance the antitumor effectiveness of ionizing radiation. The in vitro growth of HT-29 human colon carcinoma cells is inhibited by the anti-inflammatory agents sulindac sulfide and sulindac sulfone (1-3) and other NSAIDs (4). Milas et al. and Furuta et al. (5-7) have shown that the anti-inflammatory agent indomethacin can improve the antitumor efficacy of ionizing radiation in PG-producing transplanted murine sarcomas. Recently, we showed that ibuprofen enhances the effect of radiation treatment of human prostate carcinoma xenografts in immunodeficient mice (8). The radiation dose-modifying factor for DU-145 tumors was 1.8 and 1.7 for a 1-week and a 2-week fractionated regimen, respectively. Administration of ibuprofen along with radiation therapy to animals bearing LNCaP tumors resulted in a 2-fold increase in tumor growth delay compared with radiation alone. Cyclooxygenase and lipoxygenase inhibitors have been tested as modulators of cancer chemotherapy as well (9, 10).

The primary mechanism of action of NSAIDs is generally considered to be inhibition of PG synthesis by inhibition of cyclooxygenases COX-1 and COX-2 (11). PGs have been implicated in the regulation of growth and in the spread of tumors (6, 12). Indomethacin, an inhibitor of PG synthesis, inhibited tumor growth and augmented the radioreponse of certain transplanted murine tumors that produce PGs (7, 13). However, the role of PGs in modulation of tumor growth seems to be controversial, because others have demonstrated that the PGs PGD2, Δ12-PGJ2, a metabolite of PGD2, and PAG2 have antiproliferative and cytotoxic effects on a variety of tumors in vitro as well as in vivo (14, 15).

It seems that mechanisms other than COX inhibition could be responsible for growth inhibition and cytotoxicity of NSAIDs. Whereas cyclooxygenases are inhibited by NSAIDs at concentrations in the micromolar range (16), NSAIDs produce anti-inflammatory effects, inhibit growth, and induce cytotoxicity at much higher doses (1-4, 17-19). Moreover, chronic NSAID treatment of transformed CEFs results in increased rather than decreased COX-1 and COX-2 mRNAs and COX-2 protein (20). Hanif et al. (21) reported that NSAIDs inhibit cell proliferation and induce apoptosis in HCT-15 colon carcinoma cells that lack cyclooxygenase transcripts as effectively as in HT-29 cells that produce PGE2, PGF2α, and PGI2. Finally, HT-29 colon carcinoma cell growth is inhibited by both sulindac sulfide, a PG synthesis inhibitor, and sulindac sulfone, a deriv-
ative that essentially lacks PG synthesis-inhibitory activity, suggesting that reduction of PG levels is not necessary for the antineoplastic activity of this class of drugs (1).

One of the potential mechanisms of antineoplastic action of NSAIDs seems to be induction of apoptosis. NSAIDs inhibit cell growth and induce apoptosis in a variety of cell types (1-4, 20-22). Because the expected effects of NSAIDs, such as apoptosis or inhibition of cell growth, involve altered cellular regulation, the effect of NSAIDs could be dependent on protracted perturbation of cellular signaling, a process that could be sensitive to the microenvironment of the cell during the post-treatment period that precedes the onset of observable end points. To allow these protracted processes to occur, we used in vitro models that incorporate a posttreatment incubation as part of the experimental protocol. Clonogenic survival was determined by plating PC3 cells 24 h after treatment of nearly confluent monolayers. Analysis of cell growth, cell detachment, and apoptotic cell death was carried out over a period of up to 9 days after treatment of PC3 and DU-145 monolayers. The effect of ibuprofen and/or radiation was also probed by observing the inhibition of growth of established PC3 and DU-145 colonies that were treated on the 14th day of colony growth. This assay is referred to in the text as the in vitro growth delay assay.

MATERIALS AND METHODS

PC3 and DU-145 human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin and subcultured weekly. All tissue culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY). Ibuprofen was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as a 100-mg stock in distilled water and filter sterilized. Cells were irradiated using a Cesium irradiator at 96 kGy/mm (Gamma Cell 40; Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada).

Experimental Design. Approximately 1 X 10⁶ cells were seeded per 25 cm² flask in 6 ml of medium. After 48 h, cells were treated with ibuprofen for 2 h in HGLP buffer consisting of 20 mM HEPES (pH 6.8), 5.5 mM glucose, 1.8 mM CaCl₂, 5.4 mM KCl, 1 mM MgSO₄, and 120 mM NaCl. Although the flasks were near confluence at the time of treatment, the cells were still in exponential growth. Irradiation was carried out 1 h after addition of HGLP buffer, with or without ibuprofen. Thus, for combined treatment, ibuprofen was present 1 h before, during, and after irradiation. At the end of treatment, the drug was removed, the monolayer was washed once with 3 ml of media, and flasks were incubated further in drug-free media. Control and radiation alone treatment groups were similarly treated with HGLP buffer for 2 h. The vast majority of NSAIDs are weakly acidic with ionization constants (pKₐ) ranging from 3 to 5. The proportion of a NSAID that is not ionized at a particular pH influences the distribution of NSAIDs in cells and tissues (23).

Cell proliferation and apoptotic DNA fragmentation were analyzed in detached and attached cells separately on days 2, 4 or 5, 7, and 9 using four parallel flasks. Forty-eight h after treatment, media with detached cells were collected from all four flasks and pooled. One flask was used to analyze the attached cells. Fresh media were added to the remaining three flasks, and they were further incubated. On day 4, media with detached cells were collected from the three flasks, and attached cells from one flask were analyzed. The same procedure was repeated on day 7. On day 9, detached and attached cells from the remaining flasks were counted and analyzed. Because the media were replenished every 2 days, the cells collected at different time points represent a population of cells that detached during a specific 2-day interval (for example, days 2–4 or days 7–9 and so forth) and are not a sum total of cells detached over a 9-day period. Because the media were changed every 2 days and dying cells were removed for analysis, the possibility of extensive breakdown of cells and accumulation of debris was reduced.

The total number of cells (detached + attached) per flask, the percentage of detached cells in the flask, and the percentage of trypan blue-positive cells in the detached cell population were determined for each treatment on days 2, 4, and 7 or 9.

Biochemical Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. DNA fragmentation was analyzed separately in the detached and the attached cells as described previously (24). Briefly, cells were lysed in 20 μl of 10 mM EDTA and 50 mM Tris-HCl (pH 8.0) buffer containing 0.5% sodium lauryl sarcosinate and 0.5 mg/ml proteinase K and incubated at 50°C. After 1 h, 10 μl of 0.5 mg/ml RNase A were added, and incubation was continued for another hour. Samples were heated to 70°C, and 10 μl of 10 mM EDTA (pH 8.0) containing 1% (w/v) low gelling temperature agarose (Life Technologies, Inc.), 0.25% (w/v) bromphenol blue (Bio-Rad, Hercules, CA), and 40% (w/v) sucrose were added. Samples were loaded into dry wells of 2% agarose gel. Electrophoresis was carried out in Tris-borate buffer (pH 8.0) at 70 V. Within each experiment, an equal number of detached cells was used for analysis of DNA fragmentation in each lane. One 10⁶ attached cells were used for analysis of DNA fragmentation in all experiments. Low molecular weight DNA was quantitated in some experiments by the diphenylamine assay as described previously (25). All reagents were obtained from Sigma unless otherwise specified.

Clonogenic Cell Survival. Cells were irradiated in HGLP buffer with or without ibuprofen as described above. Cells that remained attached 24 h later were trypsinized, counted, and plated for clonogenic survival in 60-mm dishes in drug-free media for 13 days. Colonies of 50 or more cells were counted after staining with 0.4% crystal violet in 95% ethanol.

In Vitro Growth Delay Assay. Single cell suspensions of PC3 and DU-145 cells (100 cells/dish) were added to 60-mm dishes, and dishes were incubated without any treatment as described for the clonogenic assay. After 2 weeks, the well-established colonies were treated with ibuprofen and radiation in HGLP buffer, as described earlier. At the end of a 2-h treatment, drug-free medium was added to the dishes, and they were incubated further for another week. During this time, the medium was changed twice. After 1 week, colonies were stained as described for the clonogenic survival assay. Colony size and morphological features of the colonies were scored using a ZOOM stereo 2000 Leica microscope.
RESULTS

Cell Growth, Cell Detachment, and Induction of Apoptosis in PC3 Cells Treated with 8 Gy of γ-Irradiation. Total cell counts were lower 48 h after treatment of PC3 cells with 8 Gy of γ-irradiation than in untreated control flasks. The proportion of cells that had detached and the percentage of detached cells that were trypan blue positive were higher in the treated flasks (Table 1). We have previously characterized the apoptotic internucleosomal DNA fragmentation response of irradiated PC3 cells (24). We noted in those studies that PC3 cells that detach from the monolayer exhibit a low level of spontaneous internucleosomal DNA fragmentation, and that there is an increase in DNA fragmentation in detached cells from irradiated PC3 monolayers 48 h after irradiation. Cells that remain attached do not exhibit internucleosomal DNA fragmentation. We report here that internucleosomal DNA fragmentation occurs in PC3 cells over an extended period of time after irradiation, based on biochemical analysis of detached cells on day 5 or even as late as day 9 (Fig. 1). Quantitative analysis by diphenylamine assay also indicates that the percentage of low molecular weight DNA increases in the irradiated cells, compared to the unirradiated controls (Fig. 1). Agarose electrophoresis and diphenylamine assay indicate that the DNA from attached cells is intact, even as late as day 9 after irradiation (Fig. 1, last two lanes).

Cell Growth, Cell Detachment, and Induction of Apoptosis in PC3 Cells Treated with Ibuprofen. Ibuprofen inhibited PC3 cell growth (IC50 = 1.5–2 mM), based on cell counts 48 h after treatment of monolayers for 2 h in HGLP buffer (Table 1). Treatment with 2 mM ibuprofen resulted in an increase in the proportion of detached cells, an increase in the number of trypan blue-positive cells, and induction of apoptosis. Induction of apoptosis by ibuprofen was dependent on the dose and time of treatment with ibuprofen and was only evident in the detached cells. Fig. 2 shows DNA fragmentation patterns in detached cells 48 h after treatment with 3 mM ibuprofen for 1 or 2 h (Fig. 2, Lanes 1–3). Cells that remained attached to the dishes did not exhibit DNA fragmentation (Fig. 2, Lanes 4 and 5). At 48 h, the percentage of the detached cells that are trypan blue positive increased from 8% in controls to 60% in cells treated with 3 mM ibuprofen for 1 h (data not shown). Typical apoptotic DNA ladders were observed by agarose gel electrophoresis for cells that were treated for 1 h with 3 mM ibuprofen (Fig. 2, Lane 2). However, the majority of the DNA seemed to be intact in cells treated for 2 h with 3 mM ibuprofen (Fig. 2, Lane 3), although 79% of cells were trypan blue positive, suggesting that other modes of cell death (e.g., necrosis) may be involved.

Effect of Combined Treatment of PC3 Cells with Radiation and Ibuprofen. Low concentrations of ibuprofen (0.5–1.0 mM) did not alter cell growth, cell detachment, or viability assessed by trypan blue staining when combined with 8 Gy of irradiation. Combined treatment with 2 mM ibuprofen and 8 Gy of irradiation resulted in an increase in the proportion of detached cells relative to that observed with radiation or ibuprofen alone (Table 1). The total number of cells/flask 48 h after treatment was 46% of that in untreated controls for cells exposed to 2 mM ibuprofen, 54% for cells treated with radiation, and 38% for cells exposed to the combined treatment. Fig. 3 shows the percentage of cells (detached + attached) that became

### Table 1 Effect of ibuprofen and radiation on PC3 cells (48 h)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. of cells (% of control)</th>
<th>Percentage of detached cells that were trypan blue positive</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 11a</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>0.5 mM Ibuprofen</td>
<td>94 ± 3</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>1.0 mM Ibuprofen</td>
<td>87 ± 7</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>2.0 mM Ibuprofen</td>
<td>46 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>8 Gy</td>
<td>54 ± 7</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>0.5 mM Ibuprofen + 8 Gy</td>
<td>60 ± 7</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>1.0 mM Ibuprofen + 8 Gy</td>
<td>50 ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>2.0 mM Ibuprofen + 8 Gy</td>
<td>38 ± 2</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

* Nearly confluent PC3 cells were treated with ibuprofen and 8 Gy of γ-irradiation as described in "Materials and Methods." At 48 h, the detached cells and the attached cells were counted separately. Less than 5% of the attached cells were trypan blue positive.

* Data represent means ± SE of three separate experiments.

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Fig. 1 Time course of DNA fragmentation in PC3 cells treated with 8 Gy of γ-irradiation. DNA from 0.5 × 10^6 (day 2), 0.8 × 10^6 (days 5 and 7), and 0.6 × 10^6 (day 9) detached cells and 1.0 × 10^6 attached cells (day 9) was analyzed by agarose gel electrophoresis. The percentage of low molecular weight DNA in supernatant was quantitated by diphenylamine assay as described previously (25) on days 5, 7, and 9 and is shown below each lane.

Fig. 2 Effects of ibuprofen on PC3 cell growth, cell detachment, and induction of apoptosis. Total no. of cells, percentage of detached cells that were trypan blue positive, and percentage of attached cells are indicated for each lane.
Apoptosis and Clonogenic Cell Death by Radiation and Ibuprofen

Fig. 2 Agarose gel electrophoretic analysis of DNA from PC3 cells treated with 3 mM ibuprofen. Lanes 1–3, detached cells; Lanes 4 and 5, attached cells. Lanes 1 and 4, control; Lanes 2 and 5, 3 mM ibuprofen for 1 h; Lane 3, 3 mM ibuprofen for 2 h. DNA fragmentation was analyzed 48 h after ibuprofen treatment. DNA from 0.8 × 10⁶ cells was loaded into each well.

Trypan blue positive after various treatments. Treatment with 2 mM ibuprofen led to a sustained elevation in the percentage of cells that became trypan blue positive [6.7 versus 1.1% for controls (in both cases the average for days 2, 4, 7, and 9)]. Radiation (8 Gy) treatment led to a steadily increasing percentage of trypan blue-positive cells that reached a maximum (23%) on day 7. Combined treatment with 8 Gy of irradiation and 2 mM ibuprofen resulted in similar kinetics of cell death as observed with radiation alone, although the magnitude of the response was greater (41% trypan blue-positive cells on day 7).

Fig. 4 shows that cell cultures treated with 2 mM ibuprofen gradually recovered by day 7 and repopulated the flask by days 7–9. Cells exposed to the combined ibuprofen and radiation treatment, however, did not recover and continued to detach in high proportions and exhibited apoptotic DNA ladders even on day 9 (Table 2; Fig. 5). Combined treatment with 2 mM ibuprofen and lower doses of radiation also resulted in greater cytotoxicity than observed with radiation alone (data not shown).

Effect of Radiation and Ibuprofen on DU-145 Cells. DU-145 cells did not exhibit apoptotic DNA fragmentation after treatment with radiation or ibuprofen by 48 h. Faint DNA ladders appeared in detached control and treated cells on days 5–7 after treatment. Nevertheless, as in the case of PC3 cells, 2 mM ibuprofen inhibited DU-145 cell growth (Fig. 4) and increased cell detachment and cell loss (data not shown).

Effect of Radiation and Ibuprofen on the Clonogenic Cell Survival. Fig. 6 shows the clonogenic cell survival of PC3 cells treated with radiation and/or ibuprofen. Monolayers were treated for 2 h in HGLP buffer with or without ibuprofen and plated for clonogenic assay 24 h later. Although a concentration of 2 mM ibuprofen was required to induce apoptosis and to increase the proportion of trypan blue-positive cells, ibuprofen was effective at lower concentrations in enhancing radiation cytotoxicity by clonogenic assay. At a surviving fraction of 0.1, the radiation enhancement ratios (corrected for the plating efficiency for treatment with ibuprofen only) for 1 and 1.5 mM ibuprofen were 1.2 and 1.6, respectively. The plating efficiencies for cells treated with ibuprofen alone, relative to untreated controls, were 1.11 ± 0.1 for 1 mM and 0.53 ± 0.1 for 1.5 mM. The radiation enhancement ratio is the ratio of radiation doses required to achieve an equal effect on cell survival. The radiation enhancement ratio for 1 mM ibuprofen in the case of DU-145 cells was also 1.2 at a surviving fraction of 0.1 (data not shown).

Effect of Radiation and Ibuprofen in an in Vitro Growth Delay Assay. Fig. 7 shows the photomicrographs of DU-145 colonies that were treated with ibuprofen and radiation when the colonies were approximately of 2–3 mm in diameter. Untreated colonies continued to expand in size and occupied a considerable area of the dish (Fig. 7A). Growth was significantly inhibited in dishes where colonies were treated with 2 mM ibuprofen (Fig. 7D). Dramatic reduction in growth was seen in dishes that were treated with 2 mM ibuprofen and radiation (Fig. 7, E and F) compared to dishes treated with radiation alone (Fig. 7, B and C).

DISCUSSION

We report here that ibuprofen is cytotoxic to prostate cancer cells in vitro and enhances the effect of ionizing radiation on these cells. Clonogenic survival was used as a definitive end point for cytotoxicity. However, ibuprofen is not a typical...
Fig. 4  Effect of ibuprofen and radiation on PC3 and DU-145 cell growth. Nearly confluent monolayers were treated with 2 mM ibuprofen, 8 Gy of \( \gamma \)-radiation, and ibuprofen + radiation as described in "Materials and Methods." Control and radiation groups were treated with HGLP buffer alone for 2 h. The total number of cells (detached + attached) was determined on the days indicated. Each data point represents the average ± SD of two to three separate experiments.

**Table 2**  PC3 cell growth on day 2 and day 9 after exposure to ibuprofen and radiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 9</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of cells per flask (( \times 10^6 ))</td>
<td>% of detached cells</td>
</tr>
<tr>
<td>Control</td>
<td>8.6</td>
<td>2</td>
</tr>
<tr>
<td>2 mM ibuprofen</td>
<td>3.0</td>
<td>13</td>
</tr>
<tr>
<td>8 Gy</td>
<td>4.1</td>
<td>4</td>
</tr>
<tr>
<td>2 mM ibuprofen + 8 Gy</td>
<td>3.5</td>
<td>7</td>
</tr>
</tbody>
</table>

*Nearly confluent PC3 cells were treated with ibuprofen and 8 Gy of \( \gamma \)-radiation as described in "Materials and Methods." On days 2 and 9, the detached cells and the attached cells were counted separately and were also used for analysis of DNA fragmentation shown in Fig. 5.*

cytotoxic agent in that it is not known to act by directly producing molecular lesions. We were therefore also interested in effects that ibuprofen (with or without radiation) might have on cell growth and apoptotic response, using model systems that would allow the expression of latent regulatory effects in a more physiological context than is possible in the traditional clonogenic assay.

A model in which PC3 monolayers were exposed to ibuprofen and/or radiation and then allowed to remain as monolayers for several days after treatment was used to observe the kinetics
Detached cells Attached cells

Fig. 5 Agarose gel electrophoretic analysis of DNA from PC3 cells on days 2 and 9 after treatment with 2 mM ibuprofen and 8 Gy of γ-irradiation. Control and radiation groups were treated with HGLP buffer alone for 2 h. Lanes 1–4, DNA from detached cells; Lanes 5–8, DNA from attached cells. Lanes 1 and 5, control; Lanes 2 and 6, 2 mM ibuprofen; Lanes 3 and 7, 8 Gy of γ-irradiation; Lanes 4 and 8, 2 mM ibuprofen + 8 Gy of γ-irradiation. DNA from \(0.7 \times 10^6\) detached cells (day 2) and from \(0.6 \times 10^6\) detached cells (day 9) was loaded into each well.

Fig. 6 Clonogenic cell survival of PC3 cells treated with ibuprofen and radiation. Cells were treated with ibuprofen for 2 h as described in “Materials and Methods.” Control and radiation groups were treated with HGLP buffer alone for 2 h. After 24 h, cells were harvested by trypsinization, counted, and plated for clonogenic assay. The plating efficiency of the control cells was 0.7 ± 0.01. Net survival was calculated by correcting for the plating efficiency for treatment with ibuprofen alone. The data represent the means ± SE of six (1 mM ibuprofen) and three (1.5 mM ibuprofen) separate experiments.

of cell death and the delayed induction of apoptosis by analysis of DNA degradation patterns by agarose gel electrophoresis. Radiation treatment led to an increasing proportion of trypan blue-positive detached cells, which was accentuated by combined treatment with ibuprofen (Table 2). DNA fragmentation ladders were observed in the irradiated PC3 cells even on days 4–9. (Figs. 1 and 5).

Growth patterns of irradiated mammalian cells in vitro were described in the 1960s (26, 27). Radiation induces cell cycle perturbations with a prominent dose-dependent G2-M-phase block. Some cells die before the first postirradiation mitosis (28, 29). The average number of postirradiation divisions completed by cells that are destined to die is dose dependent. Chinese hamster V79 cells exposed to 10 Gy of irradiation complete approximately one cell division before they die (26).

Some of the irradiated cells that escape immediate cell death nonetheless sustain dose-dependent persistent damage, and these cells eventually die after additional cell divisions (30). There is increasing evidence that many cell types undergo apoptosis after the first postirradiation mitosis or even later (31–33). We reported previously that PC3 cells recover from the radiation-induced G2-M-phase block by 48 h after exposure to 8 Gy of γ-irradiation (24). Exposure to 8 Gy of γ-irradiation resulted in detachment of approximately 10–12% of cells, and approximately 5–6% of the irradiated cells were apoptotic 48 h after treatment (24). The experimental design of the present study allows monitoring of cell death over an extended period and demonstrates that, in agreement with the fundamental radiobiological observations discussed above, irradiated PC3 cells are eliminated continuously, and that one of the modes of cell death seems to be apoptosis. Recently, in a 6-day time-lapse study of rat embryonic cells irradiated with 9.5 Gy, Vidair et al. (34) found that apoptotic death occurred well beyond the first postirradiation mitotic division. At least 96% of the apoptotic episodes in that study were postmitotic and took place one to four cell divisions and 2–97 h after a given division.

Although treatment with ibuprofen increased the proportion of detached cells and increased the proportion of trypan blue-positive cells, this did not always result in an increase in DNA fragmentation, suggesting that another mode of cell death could be involved. Continuous exposure to NSAIDs has been reported to induce apoptosis as well as necrosis in various cell types (1, 20). Treatment of HT-29 colon cancer cells with high concentrations of sulindac sulfide increased the percentage of cells undergoing necrotic cell death (1). NSAIDs induced apoptosis in v-src-transformed CEFs, but nontransformed CEFs reportedly exhibited necrosis (20). These cells took up trypan blue but did not show DNA fragmentation.

It is likely that the mode of death or cellular response will depend on several factors, such as dose, acute versus chronic
exposure to NSAIDs, and microenvironmental conditions during treatment. A possible consequence of this could be that multiple (possibly antagonistic) biological effects could result from NSAID administration, and that dose and schedule could be of critical importance in achieving a therapeutic gain. It should be noted that the clonogenic survival of PC3 cells that are treated with radiation in media is generally somewhat lower (24, 35–37) than what we have observed in HGLP buffer, suggesting that this treatment condition may allow some PLD repair to occur.

The anti-inflammatory activity of NSAIDs raises a possibility that the in vivo effects of these drugs are mediated by the immune system. The in vitro cytotoxicity of ibuprofen observed in the present study along with with other reports where NSAIDs were shown to induce apoptosis in various cell types in vitro (1–4, 20–22) clearly indicates a direct cytotoxic potential of NSAIDs. This is also true in some in vivo studies where indomethacin seemed to act through nonimmunological mechanisms, because the antitumor effect of indomethacin was not reduced by immunosuppression of the tumor host (6). Indomethacin showed antitumor activity in normal mice, mice deficient in T cells (nude mice), and mice whose general immunocompetence was suppressed by whole-body irradiation. On the other hand, potentiation of tumor radioresponse by indomethacin was greatly dependent on the immunocompetence of the tumor host (5). Radiosensitization was significantly reduced or even abolished when tumors were grown in nude mice or in mice that had received whole-body irradiation. In contrast to that study, we have shown that ibuprofen is a good radiosensitizer of DU-145 and LNCaP human prostate cancer xenografts in nude mice and SCID mice, respectively (8).

Recent studies suggest that the effects of NSAIDs could be mediated through their effects on transcription factors. One of the targets for NSAIDs is HSF-1, the transcriptional regulator of the heat shock genes. The NSAID family of drugs all activate HSF-1 to a form that localizes to the nucleus and binds to DNA (19, 38, 39). HSF-1 activation is not mediated through PG endoperoxide H synthase and occurs at NSAID concentrations above those required for PG endoperoxide H synthase inhibition (39). Significantly, aspirin and indomethacin enhance HSP70 gene transcription with prolonged binding of HSF-1 to HSE in K562 human erythroblastic cells treated with hyperthermia at concentrations that are much lower than those required to inhibit cell proliferation (19). These NSAIDs thus modulate the heat shock response and enable thermostability in human erythroblastic cells in vitro. Activation of HSF-1 by NSAIDs in human monocytes is associated with repression of genes for the cytokines IL-1β, tumor necrosis factor α, IL-6, IL-8, IL-10, and the cell adhesion factor intercellular adhesion molecule.5 HSF-1 directly represses cytokine promoters (IL-1β) and activates the HSP70B promoter in THP-1 human monocytes (40).

The transcription factor NFκB also seems to be a target for the NSAIDs. Aspirin and other NSAIDs have been reported to inhibit NFκB activation (41, 42). However, the role of NFκB in apoptosis seems to be controversial. NFκB is activated by various apoptotic stimuli including ionizing radiation and certain chemotherapeutic agents and tumor necrosis factor α (43–45). However, the activation of NFκB apparently provides sig-

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significant protection against apoptosis (44). Blocking NFκB activation by overexpression of super-repressor IkBα enhances apoptosis (cell kill) in many cell systems (44–46). These studies suggest that activation of NFκB could be one of the mechanisms of cellular resistance to killing by some apoptotic agents, and that blocking NFκB activation could be one way to make tumor cells respond better to radiation and chemotherapy. Various agents, including NSAIDs, inhibit the activation of NFκB and concomitantly induce HSF, suggesting a link between these two transcription factors (47).

Ibuprofen has several effects on tumor cells that suggest it may be useful in cancer therapy, although the mechanism of action of ibuprofen in this regard is not known. Treatment of cancer cells with ibuprofen results in growth inhibition and cytotoxicity. Ibuprofen had a radiosensitizing effect in the present in vitro study, which is consistent with our earlier results using xenografts (8). NSAIDs may also prove to be beneficial in cancer therapy by inhibition of tumor angiogenesis (5). Our preliminary data show that ibuprofen inhibits constitutive and hypoxia-induced expression of the angiogenic factor vascular endothelial growth factor in PC3 cells.6

NSAIDs reportedly protect normal tissues from radiation injury. Ibuprofen seems to alleviate the radiation-induced dysuria that almost invariably occurs during radiation therapy of prostate cancer.4 Indomethacin improved the therapeutic ratio for radiotherapy in C3Hf/Kam mice with transplanted fibrosarcomas (13). In that study, indomethacin was found to be a radioprotector to the hematopoietic system. It did not enhance the normal tissue radioreponse of two other normal tissues, hair follicles and tissue responsible for development of leg contractures. However, a slight radiosensitization was observed in the case of the jejunum with an enhancement factor of 1.12. Ace-
tyloxyacetic acid has been demonstrated to protect mice against radiation-induced nephropathy (48). It would seem that a therapeutic gain can be achieved using NSAIDs and radiation therapy; however, additional studies will be necessary to determine the clinical applicability of these findings.

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Combined antitumor effect of radiation and ibuprofen in human prostate carcinoma cells.


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