ABSTRACT

Gemcitabine, 2’-2’-difluoro-2’-deoxycytidine, is an inhibitor of DNA synthesis and has been shown previously in vitro and in vivo to enhance the cytotoxic action of radiation as well as some chemotherapeutic agents. Because gemcitabine has shown clinical activity on its own in several solid tumors traditionally treated with radiotherapy, it was of interest to optimize the combination of gemcitabine and radiation. To determine the optimal gemcitabine dose to combine with irradiation and to determine the effect of gemcitabine on tumor growth, mice bearing SA-NH tumors were treated with 2.5 to 600 mg/kg gemcitabine, and subsequent tumor growth was determined. At low doses, gemcitabine induced transient growth delay, whereas higher doses showed both cytotoxic and cytostatic activity. Flow cytometric, histologic, and mitotic analyses of irradiated tumors showed that gemcitabine induced a dose-dependent inhibition of DNA synthesis and induction of apoptosis of cells in S phase. DNA synthesis recovered in cells at the G2-M boundary of the cell cycle in a dose-dependent manner, and a parasympathetic movement of cells through the cell cycle ensued. To determine the optimal schedule for gemcitabine administration in relation to irradiation, tumor-bearing mice were given a single 50 mg/kg dose of gemcitabine at various times before or after irradiation. Gemcitabine enhanced radioresponse in a time-dependent fashion. The highest enhancement factors for tumor growth delay (1.68–2.03) were observed when gemcitabine was administered 24–60 h before irradiation. Although gemcitabine reduced the radiation tumor control dose at all administration times used, the greatest enhancement of tumor radiocurability occurred when gemcitabine was administered 24 h before irradiation (dose modification factor of 1.54). Moreover, gemcitabine decreased the lung metastatic rate in mice with local tumor control from 73% in mice receiving radiation alone to 40% in mice receiving the combination (all combination times included). These results suggest that gemcitabine has strong radioenhancing properties and that the greatest interaction occurs when gemcitabine administration precedes irradiation by 24–72 h. Preliminary studies indicate that normal tissues recover more quickly than tumor tissues from gemcitabine treatment; thus, optimized scheduling of gemcitabine and irradiation may serve to improve the therapeutic ratio of the combination.

INTRODUCTION

The efficacy of radiotherapy as a single modality treatment in patients with locally or regionally advanced cancers is limited by a number of factors: (a) the tumor cells may possess radioresistance associated with hypoxia within the tumor mass or efficient DNA repair mechanisms, be unable to undergo cell death after radiation, or be able to recover from injury in the period between radiation fractions; and (b) occult tumor cells may exist outside the irradiated field and thus lead to distant recurrence. For example, for locally advanced unresectable head and neck cancers, local-regional control by radiotherapy alone is accomplished in ~30% of cases, yet many of these patients suffer recurrence outside the irradiated port (1). For these reasons, increased attention is being paid to the combined use of radiotherapy and chemotherapy (2). A variety of approaches have been investigated, including alternating chemotherapy and radiotherapy and concomitant treatment. In some cases, the results have been encouraging, with trends toward decreased rates of distant failure, increased local control, and increased organ preservation rates (3–5). At the same time, combined treatments are frequently associated with increased normal tissue toxicities, and there is considerable room for improvement of the combined treatment strategies.

Most agents have been chosen for combination with radiotherapy based on their known clinical activity in particular disease sites. For example, agents such as cisplatin (6, 7), 5-fluorouracil (8, 9), bleomycin (10), methotrexate (11), and mitomycin C (12), which have been shown previously to have activity in the treatment of head and neck cancer on their own, have more recently been used in combination with radiotherapy. Alternatively, agents that might serve to overcome resistance mechanisms associated with radiotherapy could be chosen. Our group and others previously explored the use of nucleoside analogues such as fludarabine phosphate in combination with irradiation (13, 14). The rationale for such a choice was that fludarabine is an inhibitor of DNA replication and a DNA chain terminator (15–18) and thus might poison DNA repair in radioresistant tumor cells and also slow tumor regrowth during a fractionated schedule. Indeed, in vitro and preclinical mouse tumor model studies demonstrated that fludarabine could offer a radioenhancement ratio of 1.24–2.14, depending on the timing of fludarabine administration relative to radiation, the dose of fludarabine, tumor type, and schedule of radiation (19–21).

Interestingly, whereas fludarabine enhanced radioresponse when given just before irradiation (which would be expected for a repair inhibitor), the greatest tumor radioresponse was observed when fludarabine was administered at least 24 h before radiation. Subsequent mechanistic studies demonstrated that radioenhancement was associated with fludarabine-induced apoptosis and preferential cell loss of cells in S-phase through an apoptotic pathway, delayed cell cycle progression, and subsequent parasympathetic synchronization of tumor cells into the radiosensitive G2–M phases of the cell cycle (22). In addition, on an optimized schedule, fludarabine did not significantly modify the radioresponse of a number of normal tissues (20, 21, 23). Thus, it appeared that nucleoside analogues could improve the therapeutic ratio of radiotherapy when used on an optimized schedule. A clinical Phase I trial is presently under way exploring this combination in patients with locally advanced head and neck cancer.

Because fludarabine has shown little single-agent activity in solid tumors at the schedules used (24), other nucleoside analogues with similar mechanisms of action may have more favorable characteristics for use in solid tumors. One such possibility is gemcitabine,5 a

\[ \text{Gemcitabine, 2’-2’-difluoro-2’-deoxycytidine,} \]

5 The abbreviations used are: gemcitabine, dFdC, 2’-2’-difluoro-2’-deoxycytidine; EF, enhancement factor; AGD, absolute growth delay; BrdUrd, bromodeoxyuridine; DMF, dose modification factor; fludarabine, 9-b-D-arabinofuranosyladenine-5’-monophosphate; TCD, tumor control dose.
with gemcitabine at different times before or after local tumor irradiation. Untreated tumor-bearing mice served as controls.

**Measures of Tumor Response.** The effect of radiation alone, gemcitabine alone, or the combination on tumor response was assessed by three endpoints. Tumor growth delay was assessed in serial measurements of three orthogonal tumor diameters using Vernier calipers; tumors were measured at 2–3-day intervals until the tumors grew to at least 12 mm in mean diameter. The degree of growth delay was expressed either as: (a) the AGD (defined as the time in days for tumors in the combined treatment arm to grow from 8 to 12 mm in diameter minus the time in days for the tumors in the untreated control group to reach the same size); or (b) the normalized growth delay (defined as the time for tumors in groups treated with a combined regimen to grow from 8 to 12 mm minus the time to reach the same size in mice treated with gemcitabine alone). In the tumor control dose (TCD$_{50}$) assay, mice were irradiated with single radiation doses ranging from 27 to 63 Gy in combination with or in the absence of gemcitabine and subsequently observed for tumor cure until 100 days after irradiation. The antmitotic activity of treatment was assessed by examining the lungs of mice with local tumor control that died during the observation period or that survived 100 days.

**Flow Cytometric Analysis.** To determine the effect of gemcitabine administration on DNA synthesis in tumor cells *in vivo* and on tumor kinetics, tumor-bearing mice were injected i.p. with 60 mg/kg BrdUrd (Sigma Chemical Co., St. Louis, MO) dissolved in PBS 30 min before the mice were killed by CO$_2$ inhalation and the tumor removed. The tumors were cut in small pieces and fixed in cold 70% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY) at 4°C for at least 24 h. Tumor nuclei were then extracted by pepsin digestion and prepared for dual label flow cytometry as described previously (22). Briefly, BrdUrd uptake was detected using the BR3 antibody (MD5300; Caltag Laboratories, Inc. San Francisco, CA) and a fluorescein-conjugated second antibody, and DNA content was determined after counterstaining the nuclei with 5 μg/ml propidium iodide (Aldrich Chemical Co., Milwaukee, WI). Flow cytometry was performed using a FAC-SCAN (Becton Dickinson) as described previously. For each sample, 10,000–15,000 events were collected in list mode, and cell debris and doublets were excluded from the data acquisition using a doublet discriminator (Becton Dickinson). Data acquired on a HP computer were converted to a PC format (HP Reader; Verity Software) and analyzed using PCLYSIS II software (Becton Dickinson). Effects of treatment on DNA synthesis and cell kinetics were determined on scattergrams of relative DNA content *versus* relative BrdUrd content.

**Analysis of Apoptosis.** Mice were killed by CO$_2$ inhalation at different times after treatment, and the tumors were immediately excised and placed in neutral buffered formalin. The tissues were embedded in paraffin blocks, and 4-μm sections were cut and stained with H&E. The apoptotic cells were scored on coded slides at ×400. The morphological features used to identify apoptosis in murine tumors have been described previously, illustrated, and associated with positive terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining (23). Five fields of apoptotic nuclei were counted randomly across each tumor section, and in each field apoptotic bodies were expressed as a percentage based on the scoring of 1500 nuclei (2000 nuclei for untreated controls) at each time interval after treatment.

**RESULTS**

**Antitumor Activity of Gemcitabine Alone.** Before assessing the potential of gemcitabine to enhance tumor response to radiation, the antitumor activity of gemcitabine was evaluated as a function of dose when given as a single injection. Mice bearing 8-mm SA-NH tumors were given a single i.v. injection of gemcitabine at doses ranging from 2.5 to 600 mg/kg, and tumor growth was measured until tumors grew to maximum 15 mm in diameter. As shown in Fig. 1A for three example doses (10, 50, and 400 mg/kg), gemcitabine treatment resulted in a dose-dependent delay in growth. At low doses, the growth rate appeared delayed without significant decreases in tumor size, whereas at higher doses a transient reduction in tumor size was detected. This suggested that gemcitabine had cytotoxic as well as cytostatic activity. Taking the time for the tumor to reach a 12-mm diameter as an endpoint, growth-inhibitory effects were observed with
bolus doses of gemcitabine as low as 5 mg/kg, and a steep dose-response curve was observed until about 200 mg/kg, after which the growth-inhibitory effect appeared to plateau (Fig. 1B). Perhaps high gemcitabine doses saturated the deoxycytidine kinase activation pathway or only a subfraction of the tumor cell population was sensitive to the cytotoxic mechanism of gemcitabine. None of these gemcitabine doses killed mice or achieved local tumor control when given as a single bolus.

**Effect of Gemcitabine on DNA Synthesis, Cell Cycle Progression, and Apoptosis.** To devise the best schedule for combining gemcitabine with radiation, it was important to better understand the impact of various gemcitabine doses on tumors at the cellular level. For example, if an important component of the gemcitabine-radiation interaction is to block or interfere with the fidelity of repair of radiation-induced DNA damage, it is important to achieve sufficient gemcitabine doses to inhibit DNA replication in vivo. To determine the effect of gemcitabine on DNA synthesis and tumor cell cycle progression, mice bearing 8-mm SA-NH tumors were injected with 10, 50, or 400 mg/kg gemcitabine, and tumors were harvested for immunohistochemistry or flow cytometry at 0, 1, 3, 6, 9, 12, 18, 24, 36, 48, 60, or 72 h after treatment. The mice were injected with BrdUrd 30 min before tumor harvest to determine the kinetics of DNA synthesis inhibition in these tumors.

As reported previously (22), the SA-NH tumor contains two tumor cell subpopulations with DNA indices of 1.2 and 2.2. As shown in Fig. 2, all three doses of gemcitabine inhibited DNA synthesis in the tumors within 1 h of treatment. This inhibitory effect was confirmed by immunohistochemical analysis of tumor tissue sections using an antibody to incorporated BrdUrd (Fig. 3). The duration of DNA synthesis inhibition in the tumor was dose dependent; DNA synthesis began to recover 12 h after 10 mg/kg (Fig. 2A), 18 h after 50 mg/kg (Fig. 2B), and not until 24–36 h after 400 mg/kg (Fig. 2C).

Cells that were in S phase at the initiation of treatment appeared to disappear from the tumor within 3 h of gemcitabine treatment. This effect seemed most pronounced after 400 mg/kg but was detectable even after lower doses. One possible explanation, that these cells progressed out of S phase during this period, is unlikely because DNA synthesis was profoundly inhibited (i.e., no BrdUrd uptake). Reinitiation of DNA synthesis appeared to preferentially involve cells that were originally at the G1-S transition (e.g., see the 18-h time point in Fig. 2B). Cells in S phase at the initiation of gemcitabine exposure appeared exquisitely sensitive and were not able to reinstate DNA synthesis. As a result of selective elimination of S-phase cells and a G1-S blockade during DNA synthesis inhibition, the tumor population exhibited a parasyncronous movement through the cell cycle after resumption of DNA synthesis. After 50 mg/kg, this parasyncronous population reached the more radiosensitive phase of the cell cycle (G2-M), starting around 36 h after treatment.

There was no evidence of a G2 block in these tumor populations either during the initial hours after gemcitabine administration or after the resumption of DNA synthesis. In fact, assessments of histological specimens showed no mitotic activity at 24 h, and a subsequent wave of mitoses occurred 36–60 h after 50 mg/kg gemcitabine (Fig. 4A). A similar trend was observed after the 400 mg/kg dose, but the peak of mitotic activity resumption was delayed until 48 h. After 10 mg/kg, the parasyncronous wave of kinetic movement was less pronounced, and mitotic activity gradually returned starting 24 h after gemcitabine treatment.

To better understand the mechanism of cell loss in these tumors, histological specimens obtained at various times after gemcitabine administration were examined for the presence and frequency of apoptotic cells. As shown in Fig. 4B, there was a dose- and time-dependent wave of apoptotic activity. For example, in the absence of treatment, <1% of the tumor cells appeared apoptotic. After a 50-mg/kg dose, the frequency of apoptotic cells increased within 3 h, reaching a peak of 6% at 18 h; apoptotic activity returned to normal by 60 h. After 400 mg/kg, an apoptotic peak approaching 10% was apparent between 24 and 36 h and gradually disappeared by 96 h after treatment.

**Enhancement of Tumor Radioresponse.** To determine whether gemcitabine could augment radioresponse, mice bearing 8-mm tumors were treated with a single 25-Gy dose with and without 50 mg/kg gemcitabine. To determine the optimal time of administration of gemcitabine with regard to the time of irradiation, a single gemcitabine dose was given at 1, 3, 6, 12, 24, 36, 48, 60, 72, or 96 h before or 1, 3, 6, 12, or 24 h after irradiation. The antitumor effect, as assessed by tumor growth delay, was found to be either additive or supraadditive, depending on the interval between gemcitabine administration and irradiation (Fig. 5A). A sample calculation for the degree of antitumor interaction between gemcitabine and radiation is shown in Table 1, where gemcitabine was administered 36 h before irradiation. Both gemcitabine and radiation were effective on their own in slowing tumor growth. However, when they were combined, the slowed tumor...
As shown in Fig. 5B, enhancement of radiation-induced tumor growth delay by gemcitabine was highly dependent on the interval between gemcitabine administration and tumor irradiation. Although enhancement was observed at all but one time interval (3 h before irradiation) when gemcitabine administration preceded tumor irradiation, the highest EFs were reached with the 24–60-h intervals (EFs of 1.68 to 2.03). Little modification in tumor radiosresponse was observed when gemcitabine was given within 6 h of radiation, although parallel experiments had demonstrated profound inhibition of DNA synthesis in this time period. Under conditions where gemcitabine followed irradiation, there was some evidence of enhancement of tumor radiosresponse, particularly when the drug was given 12 h after irradiation (EF, 1.36).

The original rationale for combining gemcitabine and radiation was based on the notion that gemcitabine, an inhibitor of DNA synthesis, would interfere with the repair of radiation-induced DNA damage, especially those more complex components requiring DNA resynthesis. Another rationale was based on the idea that gemcitabine induces an apoptotic signal in S-phase cells through creation of chain-terminated DNA strands. We hypothesized that radiation sensitizes cells in all phases of the cell cycle to gemcitabine by creating DNA regions that required repair synthesis and thus might generate apoptotic signals when chain terminated by gemcitabine incorporation. To explore the effect of the combination on apoptotic rates, apoptotic cells in tumors were quantified morphologically at 1, 2, 4, 6, 12, or 24 h after tumor irradiation, with or without gemcitabine pretreatment (36 h before irradiation). As shown in Fig. 6, radiation alone induced a rapid rise in apoptotic activity that peaked at <4% within 6 h of treatment. If radiation was preceded 36 h by gemcitabine treatment, a peak of apoptotic activity appeared on top of the gemcitabine-induced apoptotic curve, with similar time-dependent characteristics as that observed after radiation alone. However, the combined effect on apoptosis was only additive, suggesting that different cell populations were being affected. Thus, under these conditions, the enhancement of gemcitabine tumor radiosresponse could not be explained by increases in the sensitivity of cells for radiation-induced apoptosis.

Although the above experiments showed that gemcitabine enhanced tumor growth delay after irradiation, it was important to determine whether this interaction could lead to an improvement in SA-NH tumor cure. To address this issue, mice bearing 8-mm tumors were treated with 50 mg/kg gemcitabine and then treated with localized radiation at 1, 3, 24, 36, 48, or 72 h later. At each time point, different mice were irradiated with single doses of γ-rays ranging from 27 to 59 Gy to determine whether gemcitabine could alter the radiation dose necessary for tumor cure. Control tumor-bearing mice received local tumor radiation only. The frequency of mice with locally controlled tumors was determined at 100 days after irradiation, and the results were expressed as the radiation dose necessary to (locally) cure 50% of the tumors (TCD 50 ). As shown in Table 2, the TCD 50 of radiation alone was 51.9 Gy (95% confidence limits, 50.4–53.8 Gy). Gemcitabine reduced the TCD 50 at all time intervals, the largest effect occurring when gemcitabine was administered 1 day before irradiation, where a TCD 50 of 33.8 Gy (32.0–35.7 Gy) was observed. This effect translated into a DMF of 1.54 (i.e., 51.9/33.8 Gy). The lowest DMF was observed when gemcitabine was administered 1 h before irradiation [TCD 50, 46.0 Gy (44.4–47.8 Gy); DMF = 1.13].

Antimetastatic Activity. One goal of combined chemotherapy and radiotherapy is to provide systemic control in addition to enhancing local control. In the SA-NH tumor model system, a large proportion of mice with 8-mm tumors develop lung metastases, even if local control is accomplished with radiation treatment. To determine whether gemcitabine affected the frequency of lung metastases after
radical local radiotherapy, mice with local tumor control that died before the end of the observation period, or that survived 100 days were examined for the presence of lung metastases. The number of lung metastases could not be counted due to large size and confluency of tumor nodules. With radiation alone, 59 of 81 mice (73%) whose primary tumors were locally controlled developed lung metastases. In contrast, when gemcitabine and radiation were combined, lung metastases were detected in only 120 of 302 mice (40%; \(P, 0.001\)). Thus, in addition to enhancing local tumor radioresponse, gemcitabine reduced the incidence of distant metastases.

**DISCUSSION**

The data presented here demonstrate that gemcitabine, a nucleoside analogue and a potent chemotherapeutic agent, can significantly enhance the response of the murine sarcoma SA-NH to radiation. The improvement was evident in three types of endpoints, tumor growth delay, local tumor cure, and frequency of distant lung metastases in mice that achieved local tumor control. Additionally, the magnitude of the augmentation of tumor radioresponse was highly dependent on the time interval between gemcitabine administration and local tumor irradiation, at least in the setting of single dose treatment. Although a small degree of radioenhancement was observed when gemcitabine was given 6 h before irradiation, EFs between 1.14 and 2.03 were achieved when the drug was given 24–60 h before irradiation (Fig. 5).

The importance of the time interval and exposure duration of gemcitabine in relation to radiation exposure has been reported previously by others when studied in the *in vitro* cell culture setting (36–38). For example, longer exposures (24 h) to nontoxic gemcitabine concentrations (10 nM) before irradiation were more effective in radiosensitizing human colon carcinoma and pancreatic carcinoma cell lines than shorter exposures (4 h; EFs of 1.7–1.8 versus 1.4; Refs. 36 and 37). In these experiments, irradiation was delivered immedi-

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*Fig. 3. Visualization of DNA synthesis inhibition in SA-NH tumors (control, A) after gemcitabine administration. Mice were treated i.v. with 10 (B), 50 (C), or 400 (D) mg/kg gemcitabine and harvested at 3 h after treatment. Mice received 60 mg/kg BrdUrd i.p. 30 min before tumor harvest.*
ately after gemcitabine exposure. On the other hand, a short exposure (2 h) of colon carcinoma cells to high gemcitabine concentrations (100 nM to 3 μM) also produced large radioenhancement ratios (1.8–3.0), but only when radiation was delivered 24–48 h after gemcitabine was removed from the culture medium (38). No significant radioenhancement was observed when the cells were irradiated within 4 h after drug removal (38), a finding similar to that reported here for tumors treated in vivo.

The mechanisms involved in the radioenhancing activity of gemcitabine in vivo are not well understood. However, the studies reported here may provide some insight. Our original working hypothesis was that gemcitabine would act by inhibiting repair in vivo and so enhance the radiation effect. This hypothesis was based on the observations that gemcitabine is an effective inhibitor of DNA synthesis (27–29) and inhibits the repair of radiation-induced chromosome damage in vitro (39). If this were the only radiosensitizing mechanism, one would expect that the highest radioenhancement would occur when irradiation is administered when DNA synthesis was most inhibited in the tumor. However, we show here that, although there was a small radioenhancing effect in tumors irradiated within 6 h of gemcitabine exposure, greater enhancement occurred when gemcitabine was administered 24–96 h before radiation. It is unlikely that the small size of the enhancement observed within 6 h after gemcitabine administration was due to inadequate concentrations of the active intracellular metabolites of gemcitabine (i.e., dFdCDP and dFdCTP) because DNA synthesis was shown to be inhibited in these tumors for 12–24 h, depending upon gemcitabine dose.

A close examination of events within the tumor after gemcitabine administration may shed light on the basis for variation in radioenhancement with the timing of gemcitabine. Gemcitabine inhibited DNA synthesis in the tumor within 1 h of administration at doses between 10 and 400 mg/kg. A wave of apoptosis within the tumors

![Fig. 4. The percentage of mitotic cells (A) and of induced apoptosis (B) in SA-NH tumor at different times after gemcitabine administration. Mice were treated i.v. with 10 mg/kg (●), 50 mg/kg (▲), or 400 mg/kg (◆) gemcitabine when tumors were 8 mm in diameter. Bars, SE.](image)

![Fig. 5. Influence of the time interval between gemcitabine administration (50 mg/kg i.v.) and radiation (25 Gy) on growth delay of SA-NH tumors (A). B, enhancement factors. The hatched horizontal bars in A, shown for comparison, represent the AGD for the single agents and the theoretical AGD for a purely additive interaction. The growth delay enhancement (B) was defined as the ratio of the normalized growth delay in the combined treatment over the absolute growth delay induced by radiation alone. 0 h, time of tumor irradiation. Bars, SE.](image)

Table 1  
Effect of gemcitabine on radioresponse of SA-NH tumor: Influence of time interval between gemcitabine administration and radiation delivery

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time in days that tumors are required to grow from 8 to 12 mm</th>
<th>Growth delay</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.0 ± 0.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>9.5 ± 0.3</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td>18.3 ± 0.7</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine + radiation (36 h)</td>
<td>34.4 ± 5.3</td>
<td>2.03</td>
<td></td>
</tr>
</tbody>
</table>

* Mice bearing 8-mm tumors in the right thighs were given i.v. 50 mg/kg gemcitabine or 25 Gy local tumor irradiation. When the two agents were combined, irradiation was given 36 h after gemcitabine. Groups consisted of 8–11 mice each.
* Mean ± SE.
* AGD is defined as the time in days for tumors in the treated groups (gemcitabine or radiation) to grow from 8 to 12 mm minus the time in days for tumors in the untreated control group to reach the same size. Normalized growth delay is defined as the time for tumors in groups treated with both gemcitabine and radiation to grow from 8 to 12 mm minus the time to reach the same size in mice treated with gemcitabine alone.
* EF was calculated as the ratio of normalized growth delay in mice treated with gemcitabine plus radiation to AGD in mice treated by radiation alone.
then occurred, the timing and extent of which was also dependent on gemcitabine dose. Associated with histological evidence of apoptosis was an apparent preferential loss of cells from the S-phase component of the tumor, as detected by flow cytometric analysis of DNA content and BrdUrd uptake in vivo. It is unlikely that tumor cells moved through S phase because DNA synthesis remained inhibited during the period that S-phase cells disappeared from the tumor. When DNA synthesis resumed, tumor cells appeared to initiate DNA synthesis at the G1-S border, producing a parasynchronous movement of cells through S phase and through mitosis. An increasing enhancement ratio was observed as the parasynchronous cell population approached the G2 phase and mitosis. These combined results suggest that the radioenhancement involved the elimination of more radioresistant S-phase cells from the tumor population by gemcitabine and the redistribution of surviving cells into a more radiosensitive compartment of the cell cycle are largely consistent with our original hypothesis.

That the radioenhancing effect of gemcitabine persisted for up to 96 h after drug administration suggests that additional sensitization mechanisms must occur in the in vivo tumor setting. The magnitude and timing of the potentiation of gemcitabine of tumor radiation response was somewhat different when alternative response endpoints were used. For example, the magnitude of the potentiation of gemcitabine of local tumor cure by radiation was, in general, lower than that measured by tumor growth delay assays (compare Table 2 and Fig. 5, respectively). The reasons for this discrepancy may shed light on the mechanisms of potentiation because it is possible that these assays are most sensitive to different populations within the tumor. Tumor growth delay is most likely a manifestation of cytotoxicity in the more radiosensitive, well-oxygenated tumor cell compartment. On the other hand, the TCD_{50} assay measures the response of the more radioresistant, hypoxic tumor cell compartment, where tumor cells proliferate poorly. In addition, hypoxic areas of the tumor are farther from tumor blood vessels, which also makes them less accessible to drug. Thus, the radioresistant hypoxic fraction would be less influenced by gemcitabine both in terms of the effect of gemcitabine on cells in S phase and by its ability to achieve sufficient intracellular concentrations of active gemcitabine metabolites.

In the TCD_{50} assay, the highest DMF (1.54) was observed at 24 h after gemcitabine administration; the DMF decreased to 1.32 by 36 h. One possible explanation for this time course of sensitization is that tumor reoxygenation occurred because of the large numbers of tumor cells being lost from the tumor in a wave of apoptosis after gemcitabine treatment. Some of this increased effect at 24 h associated with reoxygenation might be compromised by 36 h because of tumor regeneration prior to irradiation. A similar radiosensitizing effect was observed after paclitaxel treatment of tumors before irradiation (42, 43). If tumor reoxygenation truly plays a role in the potentiation by gemcitabine of tumor radiocurability, gemcitabine might then be particularly effective in the setting of fractionated irradiation schedules because reoxygenation would take place between fractions. In addition, gemcitabine might also slow tumor repopulation between fractions. Our present experiments have addressed a possible role of tumor reoxygenation in gemcitabine-induced enhancement of tumor radiosensitivity. The results of initial experiments show that the magnitude of induced radioenhancement was reduced when tumors were irradiated under hypoxic conditions. This implies that reoxygenation of hypoxic tumor cells is an additional mechanism by which gemcitabine enhances tumor radiosensitivity.

One rationale for the combined use of chemotherapeutic agents and radiation is that systemic control of micrometastases may improve. We report here that gemcitabine significantly reduced the incidence of lung metastasis in mice whose primary tumor was locally controlled by radiation. Under the conditions used for these experiments, the incidence of lung metastases decreased from 73% in mice treated by radiation alone to 40% in mice treated by combined therapy. Thus, this study demonstrated that gemcitabine treatment served to reduce primary tumor cell burden, especially in combination with radiation, and this was associated with decreased systemic spread of tumor cells in a high percentage of mice. Whether gemcitabine acted on its own on systemic disease or enhanced the effect of radiation on the primary tumor and thus decreased tumor dissemination was difficult to determine because gemcitabine alone did not induce local control after single-dose administration.

The findings reported here have some similarities and some dissimilarities to those reported previously for the combination of fludarabine and radiation (19–22). Fludarabine was also found to enhance radiation effect in a dose- and schedule-related manner (19–21), and this was similarly associated with inhibited DNA synthesis in vivo. induction of a wave of apoptosis, and the generation of a parasynchronous wave of rebounding tumor cells (22). However, unlike gemcitabine, fludarabine had a stronger radioenhancing effect compared with gemcitabine when given within 6 hours of irradiation (19). This difference might reflect differences in the nature of the

![Fig. 6. Apoptosis induction by gemcitabine (50 mg/kg i.v.; ○), local tumor irradiation with 25 Gy (□), or both (△), where radiation was given 36 h after gemcitabine. The treatments were given when tumors were 8 mm in diameter. Bars, SE.](image)

| Treatment                  | TCD_{50} (Gy) | DMF  
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Radiation only</td>
<td>51.9 (50.4–53.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Gemcitabine and radiation 1 h</td>
<td>46.0 (44.4–47.8)</td>
<td>1.13</td>
</tr>
<tr>
<td>Gemcitabine and radiation 3 h</td>
<td>45.1 (43.0–48.3)</td>
<td>1.15</td>
</tr>
<tr>
<td>Gemcitabine and radiation 24 h</td>
<td>33.8 (32.0–35.7)</td>
<td>1.54</td>
</tr>
<tr>
<td>Gemcitabine and radiation 36 h</td>
<td>39.4 (37.7–41.4)</td>
<td>1.32</td>
</tr>
<tr>
<td>Gemcitabine and radiation 48 h</td>
<td>42.6 (40.6–45.7)</td>
<td>1.22</td>
</tr>
<tr>
<td>Gemcitabine and radiation 72 h</td>
<td>40.8 (38.2–44.5)</td>
<td>1.27</td>
</tr>
</tbody>
</table>

5 Mice bearing 8-mm tumors in the right thighs were given 50 mg/kg gemcitabine i.v. and/or local tumor irradiation with graded doses of γ-rays. When the two agents were combined, irradiation was given 1, 3, 24, 36, 48, and 72 h after gemcitabine. TCD_{50} assays consisted of 54–112 mice each.

6 Numbers in parentheses are 95% confidence intervals.  
7 Radiation was given 36 h after gemcitabine. The magnitude of the potentiation of gemcitabine of local tumor cure by radiation was, in general, lower than that measured by tumor growth delay assays (compare Table 2 and Fig. 5, respectively). The reasons for this discrepancy may shed light on the mechanisms of potentiation because it is possible that these assays are most sensitive to different populations within the tumor. Tumor growth delay is most likely a manifestation of cytotoxicity in the more radiosensitive, well-oxygenated tumor cell compartment. On the other hand, the TCD_{50} assay measures the response of the more radioresistant, hypoxic tumor cell compartment, where tumor cells proliferate poorly. In addition, hypoxic areas of the tumor are farther from tumor blood vessels, which also makes them less accessible to drug. Thus, the radioresistant hypoxic fraction would be less influenced by gemcitabine both in terms of the effect of gemcitabine on cells in S phase and by its ability to achieve sufficient intracellular concentrations of active gemcitabine metabolites.

In the TCD_{50} assay, the highest DMF (1.54) was observed at 24 h after gemcitabine administration; the DMF decreased to 1.32 by 36 h. One possible explanation for this time course of sensitization is that tumor reoxygenation occurred because of the large numbers of tumor cells being lost from the tumor in a wave of apoptosis after gemcitabine treatment. Some of this increased effect at 24 h associated with reoxygenation might be compromised by 36 h because of tumor regeneration prior to irradiation. A similar radiosensitizing effect was observed after paclitaxel treatment of tumors before irradiation (42, 43). If tumor reoxygenation truly plays a role in the potentiation by gemcitabine of tumor radiocurability, gemcitabine might then be particularly effective in the setting of fractionated irradiation schedules because reoxygenation would take place between fractions. In addition, gemcitabine might also slow tumor repopulation between fractions. Our present experiments have addressed a possible role of tumor reoxygenation in gemcitabine-induced enhancement of tumor radiosensitivity. The results of initial experiments show that the magnitude of induced radioenhancement was reduced when tumors were irradiated under hypoxic conditions. This implies that reoxygenation of hypoxic tumor cells is an additional mechanism by which gemcitabine enhances tumor radiosensitivity.

One rationale for the combined use of chemotherapeutic agents and radiation is that systemic control of micrometastases may improve. We report here that gemcitabine significantly reduced the incidence of lung metastasis in mice whose primary tumor was locally controlled by radiation. Under the conditions used for these experiments, the incidence of lung metastases decreased from 73% in mice treated by radiation alone to 40% in mice treated by combined therapy. Thus, this study demonstrated that gemcitabine treatment served to reduce primary tumor cell burden, especially in combination with radiation, and this was associated with decreased systemic spread of tumor cells in a high percentage of mice. Whether gemcitabine acted on its own on systemic disease or enhanced the effect of radiation on the primary tumor and thus decreased tumor dissemination was difficult to determine because gemcitabine alone did not induce local control after single-dose administration.

The findings reported here have some similarities and some dissimilarities to those reported previously for the combination of fludarabine and radiation (19–22). Fludarabine was also found to enhance radiation effect in a dose- and schedule-related manner (19–21), and this was similarly associated with inhibited DNA synthesis in vivo. induction of a wave of apoptosis, and the generation of a parasynchronous wave of rebounding tumor cells (22). However, unlike gemcitabine, fludarabine had a stronger radioenhancing effect compared with gemcitabine when given within 6 hours of irradiation (19). This difference might reflect differences in the nature of the
DNA chain-terminating event. On the other hand, the duration of the radioenhancing effect of fludarabine (i.e., around 48 h) was shorter than that observed here for gemcitabine (i.e., around 96 h). This difference may be attributable to the longer intracellular retention of active metabolites.

This longer-lasting radioenhancing effect of gemcitabine may have important clinical implications, especially with regard to the schedule of drug administration. If gemcitabine has a longer-lasting radioenhancing effect, it might be possible to administer the drug only once or perhaps twice a week to derive its full benefit in conventional radiotherapy fractionation schedules. However, it is also important to determine whether these schedules will still yield a positive therapeutic ratio. Studies are now under way to determine the effects of gemcitabine and radiation intervals on normal tissue radioreponse. Preliminary data suggest that tumor and normal tissues differ in their kinetic response to gemcitabine, and these differences may lead to the development of treatment schedules that improve the therapeutic ratio.

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Enhancement of Tumor Radioresponse in Vivo by Gemcitabine

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