The Mechanism of Local Tumor Irradiation Combined with Interleukin 2 Therapy in Murine Renal Carcinoma: Histological Evaluation of Pulmonary Metastases

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ABSTRACT

We have demonstrated that tumor irradiation enhanced the therapeutic effect of interleukin 2 (IL-2) on pulmonary metastases from a murine renal adenocarcinoma, Renca. To investigate the mechanism of interaction between tumor irradiation and IL-2 therapy, we have histologically evaluated the effects of each therapy alone or in combination on Renca pulmonary metastases. Following treatment of established lung metastases with irradiation and IL-2 therapy, lung sections were processed for H&E or immunohistochemical staining. We found that tumor irradiation or IL-2 therapy locally induced vascular damage, resulting in multifocal hemorrhages and mononuclear cell mobilization in the lung tissue. This effect was amplified in lungs treated with the combined therapy. Immunohistochemistry showed that irradiation produced a macrophage influx into irradiated tumor nodules, and systemic IL-2 therapy induced T-cell infiltration in tumor nodules. Lungs treated with the combined therapy exhibited massive macrophage, T-cell, and natural killer cell mobilization in disintegrating tumor nodules and in the lung tissue. This combined therapy caused a decrease in the number of proliferating tumor cells and an increase in the number of apoptotic cells, which were more marked than with either therapy alone. We suggest that the macrophages mobilized by radiation-induced tissue injury could play a role in phagocytosis of apoptotic tumor cells, processing and presenting of tumor antigens for a systemic immune response activated by IL-2. Tumor destruction may result from the concomitant action of activated T cells, natural killer cells, and macrophages infiltrating the tumor nodules.

INTRODUCTION

The treatment of metastatic disease remains a clinical challenge in several types of cancer, although researchers have made considerable progress in the knowledge of tumor biology and host antitumor immune response. Metastatic RCC is refractory to conventional treatments, including hormones, chemotherapy, and radiation therapy, and results in approximately 10,000 deaths annually in the United States (1). Immunotherapy with IL-2 has been approved by the Food and Drug Administration for use in the treatment of patients with metastatic RCC, yet its application is limited by toxicity and restricted to highly selected patients who are expected to benefit from treatment (2, 3).

In an effort to decrease the toxicity and increase the therapeutic efficacy of IL-2, we have tested the administration of local ionizing irradiation (X-rays) to part of the tumor prior to therapy with low doses of IL-2 in a metastatic murine renal adenocarcinoma model (Renca; Refs. 4–6). A Renca pulmonary metastasis model was established by inducing multiple lung metastases following i.v. injection of Renca cells (5, 6). Irradiation at 300 cGy was selectively administered to the left lung, followed by systemic IL-2 therapy. Although the Renca model, like RCC in humans, is relatively resistant to radiation therapy (5, 6), the combination of local tumor irradiation with IL-2 proved to be more effective than either treatment modality alone (5, 6). The combination therapy produced an increased tumor reduction in both lungs, although only the left lung was irradiated, suggesting that radiation enhanced the systemic effect of immunotherapy (6). We and others have demonstrated that tumor irradiation can also enhance the effectiveness of immunotherapy in other tumor models, provided that the tumor is sensitive to immunotherapy (7–10). Local tumor irradiation augmented the effect of IL-2 therapy in the MCA-205 murine sarcoma model, which is responsive to IL-2, but not in the IL-2-resistant B16 murine melanoma model (7). The mechanisms of enhancement of IL-2 therapy by local tumor irradiation remain to be elucidated and represent the goal of this study. To determine the effects of each treatment and the combined treatment on Renca lung metastases, lung sections obtained following therapy were analyzed by means of histopathology and immunohistochemistry. The data presented in this study show the alterations induced in situ by radiation and IL-2 therapy on the pulmonary tumor nodules and their microenvironment and may help our understanding of the interaction between the two modalities.
MATERIALS AND METHODS

Tumor Model. Renca, a murine RCC line of spontaneous origin in a BALB/c mouse (kindly provided by Dr. Robert Wiltrout, National Cancer Institute, Frederick, MD), was maintained in vivo by serial i.p. passages (4, 6). Renca cells were also cultured in vitro prior to in vivo implantation, as described previously (6). To induce pulmonary metastases, Renca cells were injected in HBSS and injected i.v. at 10^5 cells in 0.5 ml HBSS, via a tail vein, in 4–6-week-old female BALB/c mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN; Ref. 6).

Experimental Protocol. Five days following Renca cell injection, local tumor irradiation was administered either at a dose of 300 cGy (300 rads) or 800 cGy to the left lung only in vivo. To induce pulmonary metastases, Renca cells were also cultured in vitro and injected i.v. at 10^5 cells in 0.5 ml HBSS, via a tail vein, in 4–6-week-old female BALB/c mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN; Ref. 6).

RESULTS

Kinetics of Renca Tumor Growth in Lungs. i.v. injection of Renca cells in BALB/c mice can consistently induce numerous pulmonary metastases (150–250/lung), which are detectable by india ink staining of the lungs at 15–25 days after injection (5, 6). To follow the tumor metastases development in min at room temperature in 100% methanol containing 3% H_2O_2 prior to staining with peroxidase-conjugated stains to block endogenous peroxidases. The peroxidase-conjugated avidin-biotin complex (ABC Elite Kit; Vector Laboratories) method was then applied using diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as a chromogenic substrate, according to the manufacturer’s instructions. Before mounting, the sections were lightly counterstained with hematoxylin. Negative controls were included by incubating sections with appropriate IgG isotypes for each antibody.

Immunohistological staining for PCNA and p53 protein was carried out on paraffin-embedded sections. After digesting the deparaffinized sections with 1% pepsin (pH 1.5) for 15 min, they were incubated for 1 h at room temperature with either the mouse monoclonal antibody Ab-1 specific to PCNA (1:200 dilution) or the sheep anti-p53 Ab-7 polyclonal antibody, recognizing both wild-type and mutated p53, used at a 1:500 dilution (both antibodies were purchased from Oncogene Science, Inc., Cambridge, MA). Antibodies were diluted in antibody diluent with background-reducing components (DAKO Corp., Carpinteria, CA). Biotinylated goat F(ab')_2, antirabbit IgG (1:100; Caltag Laboratories) for anti-PCNA or biotinylated rabbit antiship IgG for anti-p53 (1:8000; Oncogene Science) were then applied for 45 min, followed by extensive washes in PBS and endogenous peroxidase blocking as described above. The avidin-biotin complex technique was used as described above. To control for staining specificity, negative controls were included by incubating sections with isotype control IgG for PCNA or with normal isoserum for p53. Positive controls for p53 were also included using a paraffin-embedded cutaneous xeroderma pigmentosum tumor known to overexpress p53.

Staining of Apoptotic Cells. To detect apoptotic cells, paraffin-embedded sections were pretreated with proteinase K (20 μg/ml) for 10 min and stained using an ApopTag in situ apoptosis detection kit peroxidase (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. This kit uses an in situ DNA end-labeling method based on detection of digoxigenin-labeled genomic DNA due to DNA fragmentation in apoptotic cells (11). In a first step, the method uses the enzyme terminal deoxynucleotidyl transferase to add residues of digoxigenin nucleotides to the 3’-OH ends of double- or single-stranded DNA generated by DNA fragmentation. Digoxigenin DNA is then detected by peroxidase-labeled antidigoxigenin followed by diaminobenzidine development. To control for the specificity of apoptosis staining, distilled water was used instead of the enzyme terminal deoxynucleotidyl transferase in the staining procedure on lung tissue sections. As a positive control for apoptotic cells, an actinomycin D-treated MCF-10 human mammary carcinoma cell line or Renca cells treated for 24 h with actinomycin D were stained using the ApopTag kit and showed intensive staining in the majority of the cells.
the lungs, a histological analysis of serial sections of Renca-bearing lungs was performed at various time points following Renca cell injection. Mice were injected i.v. with $10^5$ Renca cells and on days 1, 3, 5, 12, and 20 were randomly sacrificed. Lungs were processed and stained with H&E as described in “Materials and Methods.” One day after injection, tumor cells were detectable inside the capillaries of the lungs (Fig. 1A). By day 3, some of the tumor cells appeared extravascularly along the interalveolar spaces (not shown). On day 5, tumor cells could be found in small clusters forming small tumor nodules scattered in lung tissue (Fig. 1B). On day 12, the tumor nodules were larger (0.5–1.5 mm diameter), well demarcated, and easily detectable in the lung sections (Fig. 1C). By day 20, tumor nodules were seen by naked eyes on the lungs and histologically appeared as well-demarcated and vascularized large nodules of 3–5 mm diameter (Fig. 1D). Morphologically, the Renca tumor nodules contained undifferentiated pleiomorphic cells with granular cytoplasms and did not show a tendency to form glands or trabecular patterns (Fig. 1D). Careful microscopic examination of the tumor nodules showed a minimal level of leukocytic infiltration, little necrosis, and few apoptotic cells in sections obtained on days 5–20 after Renca cell injection.

**Histological Evaluation of Renca Lung Metastases following Therapy.** We have previously demonstrated that local tumor irradiation effectively enhanced the therapeutic effect of IL-2 therapy on Renca pulmonary metastases (5, 6). To evaluate histologically the effects of either therapy alone or combined on the pulmonary tumor nodules in situ, Renca tumor-bearing mice were treated either on day 5 after Renca cell injection with LLX at 300 cGy, or on days 6–10 with IL-2 alone, or with LLX on day 5 followed by IL-2 on days 6–10, as described in “Materials and Methods.” Additional groups of mice were treated with 800 cGy LLX alone or combined with IL-2. On days 12 and 20 after Renca cell injection (2 and 10 days after LLX and IL-2 therapy), lungs were isolated from duplicate mice for each therapy, processed, and stained with H&E for morphological examination. The antitumor effect was assessed by India ink staining of metastases of lungs isolated from the remaining mice on day 25,
Histological studies showed that IL-2 produced diffuse capillary dilation with subsequent multifocal hemorrhages and mononuclear cell mobilization at the vicinity of the tumor nodules and in the lung tissue (Fig. 2, A and B). This effect was observed in both the left and right lungs and was pronounced 2 days following the end of IL-2 therapy (day 12; Fig. 2A) and milder 10 days later (day 20; Fig. 2B). We observed a reduction in the size of some of the tumor nodules following IL-2 therapy compared with control tumors treated with 5% dextrose. When local tumor irradiation was administered alone at 300 cGy, multifocal hemorrhages were also observed in the irradiated lung tissue on day 12, as well as the presence of many macrophage-like cells with wide and clear cytoplasm appearing mainly at the vicinity of the tumor nodules (Fig. 2C). By day 20 (15 days after LLX) the lung showed less congestion, but the tumor nodules still contained macrophage-like cells (Fig. 2D). Capillary damage and macrophage mobilization were not observed in sections of the nonirradiated right lung (not shown), indicating a direct local effect of ionizing radiation on the tumor and its microenvironment. The histological changes observed following LLX alone or IL-2 alone were accentuated and persistent in sections of lungs treated with LLX and IL-2 therapy. Multifocal hemorrhages, lymphocytes, and macrophages were found associated with tumor cells in disintegrated tumor nodules or residual small tumor nodules (Fig. 2, E and F), in contrast to the larger nodules observed in untreated lungs (Fig. 1D). This effect was observed both on irradiated and nonirradiated lungs. Cryostat sections of lungs treated with LLX and IL-2 showed extensive hemorrhages in pulmonary tissue and inside tumor nodules following staining with H&E (not shown) and corroborated our observations on paraffin-embedded sections.

Identification of Infiltrating Mononuclear Cells in Renca Tumor Nodules Following Therapy. To identify the nature of the infiltrating mononuclear cells observed by H&E staining of treated tumor nodules, immunohistochemistry for macrophage, T-cell, and NK cell staining was performed on cryostat sections of lungs isolated on days 12 and 20 after Renca cell injection. LLX administered at 300 cGy produced a macrophage influx into tumor nodules. They already appeared around and inside the nodules 2 days after LLX (day 7 after Renca cell injection, not shown) and could be readily detected in high numbers at 7 days after treatment (day 12; Fig. 3A). Some macrophages persisted in irradiated tumors and were still present at 2 weeks after irradiation (day 20; Fig. 3B). The number of tumor-infiltrating macrophages was considerably increased following LLX at a higher dose of 800 cGy (Fig. 3C). These data obtained with MAC-1 staining were confirmed by F4/80 staining of macrophages. Following IL-2 treatment alone, the degree of macrophage infiltration varied in separate tumor nodules but was not as extensive as following irradiation. The presence of several macrophages was noted at the periphery and inside some of the tumor nodules, whereas other nodules showed only a few macrophages (not shown). However, sections of lungs treated with LLX and IL-2 showed a marked mobilization of macrophages in the vicinity of the tumor nodules by day 12 (Fig. 3D). This effect was pronounced by day 20 both in the pulmonary tissue and in disintegrating tumor nodules (Fig. 3E). Interestingly, both irradiated and nonirradiated lungs were invaded by macrophages, although the relative number of macrophages remained low in less-responding residual metastases (not shown). Control mice lungs treated with 5% dextrose showed only a few macrophages inside the tumor nodules on days 12 and 20 (Fig. 3F).

Similarly to the low macrophage infiltration in untreated tumors, only a few CD3+ T cells were observed at the periphery of tumor nodules in control mice lungs (Fig. 4A). T-cell mobilization by therapy showed a different pattern than that of macrophages. Systemic IL-2 treatment alone induced a rapid increase in the number of tumor-infiltrating CD3+ T cells in several lung metastases, which remained elevated even at 10 days after termination of the therapy (day 20; Fig. 4B). This IL-2-induced T-cell influx was noted in tumor nodules in both the left and right lungs. In contrast to IL-2 therapy, we did not observe notable T-cell infiltration (not shown) following irradiation of lung tumors with either 300 or 800 cGy. However, lungs treated with LLX and IL2 exhibited a massive infiltration of CD3+ T cells in the tumor nodules, and scattered T cells were seen throughout the lung tissue (Fig. 4C). The T cells seemed to reside in regressing and/or disintegrating tumors even at 10 days after the end of therapy in both irradiated and nonirradiated lungs. Immunohistochemistry for T-cell subsets revealed a large number of CD8+ T cells (Fig. 4D) and a few CD4+ T cells (not shown) in tumors treated with LLX and IL-2.

Staining of lung sections for NK cells also showed the presence of NK cells in residual or regressing tumors following treatment with LLX and IL-2 (Fig. 4E). However, the relative number of cells did not seem to differ in the different treatment modalities.

Effect of Radiation on Cell Division. Irradiation inhibits the growth of Renca cells in a dose-dependent manner (6). Cell growth inhibition may be the result of cell cycle arrests caused by radiation-induced DNA damage (12). The tumor suppressor gene p53 seems to have a role in this process, because DNA damage can cause an increase in p53 protein levels, resulting in a cascade of events leading to G1 arrest in the cell cycle or apoptosis (programmed cell death) by self-destruction of damaged cells (13, 14). To test for radiation damage in vivo, tumor-bearing lung sections were stained for p53, apoptosis, and PCNA. Renca tumor metastases failed to express detectable amounts of p53 in both untreated and irradiated lungs obtained at either day 12 (7 days after irradiation) or day 20 (15 days after irradiation). However, when p53 staining was performed at an early time point of 2 days after irradiation with 800 cGy, a few tumor cells in the 7-day small tumor nodules exhibited intensive staining for p53 (not shown).

To evaluate whether the effects of radiation and IL-2 treatments could result in tumor cell apoptosis, lung sections were stained for apoptosis using an in situ DNA end-labeling technique (ApopTag kit), as described in "Materials and
Fig. 2. Effect of radiation and IL-2 therapy on Renca lung metastases. Lungs obtained on days 12 and 20 after Renca cell injection were processed for H&E staining. A. day-12 subpleural metastasis obtained 2 days after ending IL-2 therapy. Note the multifocal hemorrhages (arrows) and mononuclear cells in lung tissue and the tumor (arrowheads), as emphasized at this low magnification. B. residual large tumor nodule (arrowheads) observed on day 10 after IL-2 treatment (day 20 after Renca cell injection) showing mononuclear infiltrates in the tumor. C. day 12 left lung section showing hemorrhages (arrow) and macrophage-like mononuclear cells around and inside a small tumor nodule (arrowheads) 7 days after LLX. D. day-20 left lung section treated with LLX. The lung shows almost complete recovery from irradiation, but a few macrophage-like cells can be found in the periphery of the subpleural tumor (arrowheads). E. day-12 lung section treated with LLX and IL-2 therapy. Note multifocal hemorrhages (arrow) and lymphocyte- and macrophage-like cells in association with tumor cells (arrowheads). F. day-20 lung section treated with LLX and IL-2 therapy; residual regressing tumor (arrowhead) surrounded and infiltrated by mononuclear cells detectable at a high magnification. All H&E: A and B, ×125; C–F, ×325. The two different magnifications were selected to emphasize the major histological findings. In all figures either the left or right lung sections are shown following IL-2 or LLX and IL-2, because the alterations induced by these treatments were observed in both the left and right lungs, whereas left lung sections are shown for radiation treatment, due to localized changes in the left lung only.
Fig. 3 Tumor macrophage infiltration induced by radiation and IL-2 therapy. MAC-1 staining of cryostat sections of lungs obtained on day 12 or 20 after Renca cell injection. A, day-12 left lung 7 days after irradiation with 300 cGy. Note that the tumor nodule (arrowheads) is infiltrated by many macrophages (arrows). B, day-20 left lung; 15 days after irradiation (300 cGy), the number of macrophages (arrows) is decreased in the tumor nodule (arrowheads). C, day-20 left lung irradiated with 800 cGy showing large numbers of infiltrating macrophages (arrows) in tumor nodules (arrowheads). D, day-12 lung treated with LLX and IL-2 showing macrophages (arrows) around and inside the tumor nodule (arrowheads). E, day-20 lung treated with LLX and IL-2. Several macrophages (arrows) are associated with a disintegrating tumor nodule (arrowheads). F, day-20 untreated tumor (arrowheads) showing only a few macrophages (arrow). All of the sections were slightly counterstained with hematoxylin; ×125. No reactivity was observed in the negative controls used in immunostainings.

Methods.” Untreated tumors showed minimal staining for apoptosis (Fig. 5A), whereas irradiation alone induced an increase in the number of apoptotic cells in the tumor nodules observed on day 12 (7 days after radiation), which was more pronounced with a dose of 800 cGy (Fig. 5B). IL-2 therapy alone also induced an increase in the number of apoptotic cells (not shown). Lungs treated with the combined therapy of LLX and IL-2 showed tumor nodules with numerous apoptotic cells on day 12 (Fig. 5C).

To estimate the ability of the cells to divide 10 days
Fig. 4 Tumor T-cell or NK cell infiltration following radiation and IL-2 therapy. Immunohistochemical staining on day 20 Renca lung metastases using antibodies to CD3 (A–C) or CD8 (D) for T cells and NK 1.1 for NK cells (E). A, untreated tumor (arrowheads) with only a few T cells at the periphery. B, tumor nodule (arrowheads) from a lung treated with IL-2 showing increased T-cell infiltration (arrows). C–E, lungs treated with LLX and IL-2 showing disintegrating or regressing tumor nodules (arrowheads) containing many CD3+ T cells (C, arrows), numerous CD8+ T cells (D, arrows), and NK cells (E, arrows). Sections shown are slightly counterstained with hematoxylin; ×125. No specific stainings were found in negative controls of sections in which the primary antibody was replaced by isotype control IgG (not shown).

following therapy (day 20 after Renca cell injection), PCNA was used as a marker for dividing cells. PCNA is a protein involved in DNA replication and repair, cell cycle progression, and cellular proliferation (15). The majority of the cells in the control tumor nodules showed positive staining for PCNA (Fig. 5D). IL-2 treatment seemed to cause no change or a slight decrease in the number of PCNA+ cells (not shown). The number of PCNA+ cells was decreased following irradiation.
Fig. 5 Immunohistochemical staining for apoptotic cells and PCNA on lung sections treated with radiation and IL-2 therapy. A–C. Staining with the ApopTag peroxidase kit for apoptotic cells in lung sections obtained on day 12 after Renca cell injection. A, untreated tumor showing few apoptotic cells. B, subpleural tumor from left lung irradiated with 800 cGy with an increased number of apoptotic cells. C, Numerous apoptotic cells are seen in tumor nodule treated with LLX (800 cGy) and IL-2. D–F, immunostaining for PCNA of left lung sections obtained on day 20 after Renca cell injection. D, untreated tumor nodules exhibiting numerous PCNA+ tumor cells. E, number of PCNA+ tumor cells is reduced by irradiation alone. F, only a few PCNA+ cells remain in a small tumor nodule following LLX and IL-2 therapy. No immunoreactivity was found in negative controls (not shown), which were incubated with IgG isotype antibody. Arrowheads, tumor nodules. Sections shown are slightly counterstained with hematoxylin (A–C) or methyl green (D–F); ×250.

(Fig. 5E). This decrease was observed only on tumor nodules from the irradiated left lung and not in the ones of the nonirradiated right lung. Following LLX and IL-2 therapy, the tumor nodules in the lungs were smaller or disintegrated compared with control lungs, and in the remaining tumor nodules, only a few PCNA+ cells were detectable (Fig. 5F). Quantitation of the number of stained cells and a comparison between the percentage of positive cells in the tumor nodules is not accurate, due to large variations in tumor nodule size and the number of tumor cells they contain, in untreated and treated lungs. Analysis of multiple sections showed that not all the tumor nodules evenly responded to IL-2 or radiation, but the presence of both small tumor nodules and large tumor nodules following IL-2 or radiation was observed. Following the combined therapy, the tumor...
nODULES WERE positive for p53 2 days after irradiation but not at later cell cycle arrest or apoptosis (13). Tumors irradiated with 800 cGy were positive for p53 proteinkvels, initiating a cascade of events leading to G1 role in this process, because DNA damage can cause an increase to apoptosis (I 3). The tumor suppressor gene p53 may have a variety of genes associated with growth control, and may lead observations in situ an increase in apoptotic cells and a decrease in the number of

HISTOPATHOLOGY. A histological analysis of lung sections, obtained at different time points following i.v. Renca cell injection, showed that small tumor nodules were established in the lung tissue by day 5 and grew rapidly into large tumor nodules by day 20. The tumor nodules consisted of undifferentiated pleomorphic cells with granular cytoplasms characteristic of Renca tumor morphology (16) and showed little necrosis and a few apoptotic cells. Leukocytic infiltration was minimal in untreated tumors, and only a few macrophages and T cells were seen by immunohistochemistry.

Local tumor irradiation administered on day 5 to established Renca metastases (as seen by histology; Fig. 1B) produced transient vascular damage, with multifocal hemorrhages and subsequent mononuclear cell mobilization in the lung tissue and tumor nodules. Immunohistochemistry demonstrated that the inflammatory cells infiltrating the tumor nodules consisted of macrophages, which could be detected early at 2 or 7 days after irradiation. The magnitude of cellular infiltration was dose dependent and restricted to the irradiated sites of the lungs. Our data confirmed previous findings in irradiated rat lungs showing that irradiation can increase vascular permeability by causing DNA damage in endothelial cells, which leads to an inflammatory response (17). In addition, we found that irradiation caused an increase in apoptotic cells and a decrease in the number of PCNA+ dividing cells in the tumor nodules. These effects were more pronounced at a higher dose of irradiation. These observations in situ corroborate our previous findings on Renca cell growth inhibition by radiation in a dose-dependent manner both in vitro and in vivo (6). The radiation effect may be due to DNA damage, resulting in growth rate alterations and the induction of a variety of genes associated with growth control, and may lead to apoptosis (13). The tumor suppressor gene p53 may have a role in this process, because DNA damage can cause an increase in p53 protein levels, initiating a cascade of events leading to G1 cell cycle arrest or apoptosis (13). Tumors irradiated with 800 cGy were positive for p53 2 days after irradiation but not at later time points. These data suggest that p53 is up-regulated early in Renca cells following radiation and corroborate recent data showing a time-dependent increase in the intensity of p53 staining on Renca cells in vitro following irradiation.4 Additional studies are needed to determine the nature of the p53 molecule in Renca cells and its involvement in the radiation-induced alterations of tumor cells.

Treatment with IL-2 alone, on days 6–10 after Renca cell injection, also produced vascular changes in the microenvironment that resulted in transient multifocal microscopic hemorrhages and mononuclear cell mobilization. These findings were present in both the left and right lungs. IL-2-induced vascular damage is well documented and is responsible for the toxic effects of IL-2 therapy, including the vascular leak syndrome and the accumulation of extracellular fluids (18). IL-2 therapy was found to be associated with endothelial cell activation, leading to vascular leakiness of macromolecules (18). Following therapy with high doses of IL-2 in melanoma patients, Rubin et al. (19) observed a marked infiltration of macrophages and T cells, including both CD4 and CD8 subsets, in regressing melanoma lesions. In our study, murine lung sections obtained following IL-2 therapy showed increased numbers of tumor-infiltrating T lymphocytes and variable degrees of macrophage infiltration. An increase in the number of apoptotic cells was also observed in tumor nodules treated with IL-2 compared with untreated tumors and may be due to the effect of cytotoxic cytokines produced by IL-2-activated immune cells.

The alterations induced in the tumor nodules and their microenvironment by irradiation or IL-2 were amplified by the combined treatment. Extensive hemorrhages associated with mononuclear cell infiltrates were observed throughout the pulmonary tissue in both lungs. The cellular infiltrates were particularly numerous at the sites of disintegrating tumors and consisted of macrophages; T lymphocytes, including CD8 cytotoxic and CD4 helper T-cell subsets; and NK cells. These cells may play an active role in tumor destruction, as confirmed by our previous findings that depletion of either CD8 or CD4 T-cell subsets or NK cells abrogated the antitumor effect mediated by the combined therapy in the Renca pulmonary metastases model (6). The histological studies of lung metastases treated with the combined therapy revealed a substantial disintegration of numerous tumor nodules and a striking decrease in the sizes of other remaining tumor nodules. These observations were noted in both the left and right lungs, suggesting a systemic antitumor effect of the combined therapy, and confirmed our previous findings on induction of greater tumor reduction by the combined therapy than either irradiation or IL-2 alone (5, 6). The combined therapy caused an increase in the number of apoptotic cells inside the tumor nodules observed 2 days after the end of therapy. At a later time point, 10 days after therapy, only a few PCNA+ dividing cells were detectable. These data correlate with the tumor reduction and demonstrate the effects of both IL-2 and radiation on inhibiting tumor cell proliferation by causing cell death.

We have documented alterations induced by tumor irradiation that may enhance the activation of the immune system by IL-2, resulting in systemic elimination of cancer. Macrophages mobilized by radiation-induced tissue damage, a well-known

4 Manuscript in preparation.
phenomenon of tissue injury, may play a key role in the interaction between both modalities. We have shown that ionizing radiation causes apoptosis of selected tumor cells, probably through DNA damage. The macrophages mobilized by tissue injury can act as scavengers by ingesting the apoptotic tumor cells or apoptotic bodies resulting from their fragmentation (20) and may process tumor peptides for antigenic presentation to lymphocytes. Some of the macrophages may circulate in the blood or lymph to reach the bronchopulmonary and hilar lymph nodes and cause a systemic sensitization of lymphocytes by presentation of tumor peptides. Subsequent IL-2 therapy induces vascular alterations, leading to increased infiltration of activated immune cells, including lymphocytes and macrophages in the tumor nodules, which can kill tumor cells directly or indirectly via cytokine release. Activated macrophages are themselves capable of causing apoptosis in tumor cells by release of cytokines or cytolytic molecules, a phenomenon that could also contribute to a more powerful antitumor response. We have previously demonstrated that irradiation up-regulates H-2K\(^a\) class I MHC antigens on Renca cells (6); this effect may also render the tumor more susceptible to recognition by the immune system activated by IL-2 therapy. Additional studies are ongoing to further clarify the mechanism of action of tumor irradiation and immunotherapy.

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