Gene Therapy Targeted by Radiation Preferentially Radiosensitizes Tumor Cells

Ralph R. Weichselbaum, Dennis E. Hallahan, Michael A. Beckett, Helena J. Mauzer, Henry Lee, Vikas P. Sukhatme, and Donald W. Kufe

Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois 60637 [R. R. W., D. E. H., M. A. B., H. J. M., H. L.]; Section of Nephrology, Department of Medicine, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02114 [V. P. S.]; and Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 [D. W. K.]

Abstract

Transcriptional regulation of the promoter/enhancer region of the Egr-1 gene is activated by ionizing radiation. We linked DNA sequences from the promotor region of Egr-1 to a complementary DNA sequence which encodes human tumor necrosis factor (TNF-α), a radiosensitizing cytokine. The Egr-TNF construct was transfected into a human cell line of hematopoietic origin, HL525, which was used in an experimental animal system. HL525 (clone 2) cells containing the Egr-TNF construct which exhibits radiation induction of TNF-α were injected into human xenografts of the radioresistant human squamous cell carcinoma cell line SQ-20B. Animals treated with radiation and clone 2 demonstrated an increase in tumor cures compared with animals treated with radiation alone or unirradiated animals given injections of clone 2 alone. No increase in local or systemic toxicity was observed in the combined treatment group. The combination of gene therapy and radiation therapy enhances tumor cures without increasing normal tissue toxicity and is a new paradigm for cancer treatment.

Introduction

The cellular exposure to ionizing radiation is associated with transcriptional activation of certain immediate early genes that encode transcription factors (1). These genes include members of the jun-fos, NF-kB, and early growth response (EGR-1) gene families (2–4). The induction of these genes following X-irradiation may represent cellular responses to oxidative stress (3–5). Previous studies have demonstrated that induction of Egr-1 gene transcription is mediated by activation of CCA(A+T)richGG (CArG) motifs in the Egr-1 promoter (5, 6). Furthermore, the AP-1 binding region of the Jun promoter is sufficient and necessary for radiation-induced transcription (2). We took advantage of a radiation-inducible promoter in order to control TNF2 gene transcription within the tumor mass.

TNF-α, originally identified because of its antitumor activity (7), is a pleiotropic biological effector that regulates the activation of genes (8, 9). Cellular responses mediated by TNF-α include antiviral and immunoregulatory activity, as well as neutrophil adhesion to vascular endothelial cells. Clinical manifestations of aberrant TNF-α production include cachexia, respiratory distress syndrome, and septic shock (9). While TNF-α interacts with two specific cell surface receptors (10), many TNF-dependent events, including cytotoxicity, are mediated by the M, 55,000 receptor, TNF R, (11–14). The killing action of TNF-α is proposed to occur following receptor binding and production of superoxide and hydroxyl radicals which mediate oxidative damage (15–17). The mechanisms responsible for generation of these free radicals are unknown. TNF-α enhances the tumoricidal activity of ionizing radiation in vitro and in vivo (18–20). The recent combination of TNF-α and radiotherapy in a clinical trial has produced encouraging preliminary results (21). However, toxicity from systemic delivery of TNF-α resulted in fever, nausea, loss of appetite, fatigue, lassitude, and hypotension. We selected TNF-α as the prototype for gene therapy with radiation because this cytokine is reported to have a radiosensitizing effect in tumor cells and either no effect or a radioprotective effect on normal cells (18–22). To enhance tumor cures by radiation and to circumvent the problem of systemic toxicity associated with the combination of TNF-α and radiation therapy, we have proposed the use of gene therapy targeted by ionizing radiation (23, 24). In this concept, a construct containing a radiation-inducible promoter upstream to a cDNA encoding a toxin is transcriptionally activated within the irradiated field to enhance radiation killing. Minimal systemic toxicity is anticipated because cytokine production is localized by radiation targeting. To demonstrate the feasibility of this approach in an in vivo model, the radiation-responsive DNA sequences of the Egr-1 promoter were ligated upstream to a TNF-α cDNA. This construct was transfected into HL525 human leukemia cells so that radiation activation of an exogenous inducible promoter could be used to control TNF gene therapy in an experimental animal system. We selected the HL525 cell line because it otherwise exhibits no detectable induction of TNF-α by ionizing radiation. Also, HL525 cells transfected with pEgr-TNF are not tumorigenic in nude mice. Stable HL525 transfecteds which exhibited radiation inducibility of TNF-α in vitro were injected into human tumor xenografts of a radioresistant human squamous carcinoma cell line. We report an enhancement of tumor control following radiation treatment when combined with this cellular delivery system.

Materials and Methods

Transfection. HL525 cells, 5 × 105 cells/ml, were plated [medium 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-HEPES-buffered RPMI 1640, 20% fetal bovine serum, 1 mM sodium pyruvate, 100 μM minimal essential medium nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin] 24 h prior to transfection. On the day of transfection, the HL525 cells were resuspended at a concentration of 2 × 107 cells/ml in cold RPMI 1640 buffered with HEPES, and 0.5 ml was aliquoted into each electroporation cuvet. The following DNAs were added to the cells in the cuvet: 10 μg Xhol linearized pEgr-TNF DNA, 1.5 μg pCB6+ DNA (a neo resistance plasmid) (25); and 1.0 μg cytomegalovirus β-galactosidase DNA. The solution was gently mixed and allowed to incubate at room temperature for 5 min. The cells were transfected by electroporation at 350 V/960 μF. The cuvets were then allowed to stand for 15 min after which the cells were cultured in complete medium for 48 h. The cells were subcloned in G418 (1200 μg/ml) by plating 0.5 cell/well in 96-well plates. These subclones were grown and maintained in G418 and tested for TNF induction. The subclone HL525.Egr-TNF clone 2 gave the greatest X-ray induction of TNF and is the clone used in these experiments.

1 This research was supported by a gift from the Daniel F. and Ada L. Rice Foundation; Grants CA 58505, CA 41068, and CA 42596 from the National Cancer Institute; the Center for Radiation Therapy; and a gift from the Passis family.2 The abbreviations used are: TNF-α, tumor necrosis factor α; cDNA, complementary DNA; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase.

Received 6/2/94; accepted 7/1/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Unpublished data.
**In Vitro TNF Induction.** HL525.Egr-TNF clone 2 and HL525 cells containing pCB6+ (a neomycin resistance plasmid) were grown to late log phase and washed twice in 10 ml Opti-MEM medium. The cells were plated in Opti-MEM medium containing 0.5% fetal bovine serum at a concentration of $5 \times 10^5$ cell/10 ml. After 16 h the cells were irradiated with 20 Gy X-rays and allowed to incubate for 24 h. The cells were harvested and washed three times quickly with Ca$^{2+}$/Mg$^{2+}$ free PBS. The pellet was resuspended in 1.0 ml PBS and allowed to incubate for 24 h. The cells were harvested and washed three times with 5 X IO6 cell/10 ml. After 16 h the cells were irradiated with 20 Gy X-rays and thawing in a 37°C water bath. The samples were then assayed for TNF using a Quantikine TNF-α enzyme linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Protein content of the samples was determined using the Bio-Rad Protein Micro Assay. A 20-Gy dose was used for the following reasons: this dose produced maximal induction of transcription following irradiation (5, 6); fewer radiation treatments (2 treatments in this study) provided ease of *in vivo* experimentation; this is a standard radiation dosage used in animal tumor studies (26, 27).

**In Vivo TNF Induction.** Female nude mice bearing SQ-20B xenografts (75–320-mm$^3$ volume) were given injections of HL525.Egr-TNF clone 2 cells ($5 \times 10^6$ cells in 100 μl PBS) immediately prior to a single dose of 20 Gy. Mice were shielded with lead so that only tumors were irradiated. Nonirradiated tumors served as controls. Tumors were excised, placed in tubes, and frozen in liquid nitrogen. Tumors were then homogenized in 500 ml sodium chloride/Tris buffer, pH 7.5, containing EDTA, dithiothreitol, and protease inhibitors. Samples were homogenized on ice for 30 s using a Brinkman Polytron followed by four cycles of freezing/thawing. Samples were centrifuged for 5 min at 10,000 rpm and the supernatant was assayed for TNF-α using a Quantikine TNF-α ELISA kit. Protein content was determined using the Bio-Rad Protein Micro Assay. Animals were cared for in accordance with institutional guidelines.

**In Vivo Tumor Control Assays.** The SQ-20B squamous carcinoma cell line is radiosensitive when compared to other human tumor cells in *vivo* ($D_0 = 239$) (28). The cells were grown in Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium (3:1), 20% fetal bovine serum, 0.4 μg/ml hydrocortisone, 100 units/ml penicillin, and 100 μg/ml streptomycin. The right hind leg (thigh) was treated with a s.c. injection of 100 μl of cell suspension ($10^5$ to $5 \times 10^6$ cells) in PBS. The tumor diameters were measured twice weekly with calipers. The tumor volumes were calculated by the formula of a rotational ellipsoid

$$V = \frac{\pi}{6} \times a \times b \times c,$$

where $a$ is the longer and $b$ is the perpendicular shorter tumor axis (26, 27). Irradiation of the animals was carried out using a GE Maxitron X-ray generator operating at 150 kV and 30 mA using a 1-mm Al filter. The dose rate was 143 cGy/min. The xenografts were irradiated with 20 Gy followed 24 h later by an additional 20 Gy. HL525.Egr-TNF clone 2 cells ($5 \times 10^6$ cells) (100 μl) were injected directly into the tumors of the experimental animals using a 27-gauge needle immediately prior to the first dose of radiation. The animals were anesthetized with 0.3 μl/g Innovar Vet and 4 μg/g midazolam injected i.p. The animals were shielded with lead so that only the hind leg with the tumor was exposed to the radiation.

**Results**

**Transcriptional Regulation of the Egr-1 Promoter in HL525 Cells.** Fig. 1 shows that the radiation-inducible Egr-1 promoter fragment designated pE425, a construct containing an Egr-1 promoter sequences from -425 to +65 placed upstream of CAT (5, 6), is transcriptionally induced in HL525 cells following irradiation. Transient transfections were irradiated 24 h after electroporation and assayed for CAT activity 24 h later. We found a 2.4-fold induction of CAT activity in transient expression assays following exposure to 20 Gy. Similar studies were performed with pEgr-TNF, a construct linking Egr-1 promoter sequences upstream to the TNF-a CDNA, stably transfected into HL525 cells. A 3.2-fold induction of TNF activity was noted at 24 h after exposure to 20 Gy. In contrast, HL525 clones containing pMV-Neo demonstrated no TNF induction following irradiation. In order to extend these studies to an *in vivo* model, we injected HL525.Egr-TNF clone 2 cells into SQ-20B xenografts in nude mice. A 2.7-fold induction of TNF-α was observed in vivo at 2 h after irradiation (Fig. 1) when compared to animals given injections of clone 2 but not irradiated. No TNF-α was detected in uninjected SQ-20B tumors. There was also no detectable TNF-α in the serum of irradiated animals given injections of clone 2.

**In Vivo Use of the Egr-TNF Construct.** We then proceeded to test the significance of *in vivo* TNF-α induction by radiation in a human tumor xenograft model. The volumes of SQ-20B xenografts ranged from 270 and 290 mm$^3$ at day 0 to 546 and 1028 mm$^3$ at day 8 (Fig. 2). The mice were sacrificed when tumors reached a volume of 1953 and 2184 mm$^3$ (days 36 and 38, respectively). With two 20-Gy exposures separated by 24 h, the SQ-20B tumors increased in volume the first 5 days posttreatment, reaching a maximum volume of 377 mm$^3$, and then regressed to a minimum of 21 mm$^3$ at day 36. Tumors subsequently began to increase in size until the time of sacrifice. Animals were sacrificed on day 50 following irradiation. One of six tumors disappeared at day 15 and did not regrow by day 46.

![Fig. 1. Gene induction by ionizing radiation. HL525 cells were transfected with pE425 and β-galactosidase expression factors. The transfectants were irradiated 14 h after transfection and assayed for CAT expression 24 h later. The results are presented for the fold increase over basal expression (1-fold). *In vitro* induction was determined as follows. HL525 clones containing the Egr-TNF constructs were irradiated and cells were assayed for TNF using the TNF enzyme-linked immunosorbent. TNF levels 24 h after irradiation were compared to untreated controls. *In vivo* induction was determined as follows: Human tumor xenografts of cell line SQ-20B were grown in nude mice and injected with HL525 clones containing Egr-TNF. Tumors were excised and assayed for TNF 2 h after irradiation and compared to untreated controls.](image-url)
alone cured 1 of 6 animals. SQ-20B tumors receiving injections of HL525.Egr-TNF clone 2 (5 \times 10^6 cells) (142-mm³ initial volume) grew for the first 6 days, reached a mean volume of 375 mm³, and then began to regress. The nadir of the growth curve was observed at day 12 (178-mm³ mean volume). Clone 2-injected tumors then began to grow, reached their initial volume by day 15, and slowly continued to increase in size but remained 75 to 80% smaller than corresponding untreated tumors. SQ-20B tumors were also injected with clone 2 cells immediately prior to the first 20-Gy dose (Fig. 2). Tumor regression began at day 6 and tumors disappeared by days 16 to 19. Significant tumor regression and growth delay, compared to all other experimental groups, were demonstrated after day 20 (P < 0.0001). One tumor recurred at day 36 but was regressing at the time of sacrifice. Therefore, 6 of 7 of the animals receiving combined treatment with clone 2 and radiation were cured (6 of 7 versus 1 of 6; P < 0.0001). Animals treated with HL525 cells transfected with the neomycin resistance plasmid vector minus the TNF gene and irradiated responded in a manner similar to that of the uninjected controls (data not shown). We conclude that there is an interactive antitumor effect between radiation and TNF-α-inducible clone 2.

Normal Tissue Reaction to Combination of pEgr-TNF and Radiation. There were no observable differences in local skin desquamation or soft tissue necrosis between animals treated with radiation alone and the combination of radiation and the TNF-α-inducible clone 2. Pathological examination of representative normal tissue sections revealed no differences in fibrosis between the radiation and radiation/TNF groups (data not shown). There were no differences in weights between animals treated with radiation alone [25.33 ± 0.408 (SD) g] and radiation/TNF [25.27 ± 0.724 g]. This result is consistent with the findings that no TNF was detectable in the serum of the animals treated with radiation and clone 2 and that no systemic TNF-α toxicity was observed.

Discussion

Sersa et al. (19) reported an additive killing effect of systemic recombinant TNF-α and radiation in the MCA-K mouse xenograft system. This effect was less than that reported in the present studies with radiation-inducible TNF production. These investigators suggested that much of their observed effect in vivo was due to an enhanced immune response. However, it is unlikely that T-cell immunity contributed significantly to our antitumor results in immunodeficient nude mice. Moreover, it is noteworthy that TNF-α can lead to inhibition of tumor growth in the absence of T-cell immunity (29). Thus, the induction of genes that activate an antitumor immune response in an immunocompetent host may further enhance the utility of radiation gene targeting. We have recently conducted a phase I trial (toxicity) combining systemic TNF and ionizing radiation (21). This study demonstrates that the systemic toxicities from tumor necrosis factor limited the efficacy of treatment. We therefore linked the TNF gene to a radiation-inducible promoter to localize the TNF treatment within a tumor. The data obtained from our prototype in vivo system suggest that gene therapy can be regulated within a specific tumor volume using external stimulus.

There are several potential advantages in using radiation-inducible genetic constructs with therapeutic radiation over other gene therapy delivery systems for cancer treatment. One advantage is that multiple radiation doses (20–35 daily treatments) are usually given in a treatment course of radiotherapy. Therefore, a small increment in cell killing per treatment might increase the therapeutic index because the increase in tumor cell killing is magnified by an exponent equal to the number of treatments. Many types of localized tumors are amenable to multiple installations of radiation-inducible genetic constructs. Multiple applications of genetic constructs in combination with therapeutic radiation might overcome the potential special limitations of diffusion of cell/virus/liposome delivery. Finally, the already well-developed technology of radiation targeting might add to the localization of toxin production provided by genetic therapy.

In summary, we report that the combination of radiation treatment and a radiation-inducible promoter linked to TNF-α cDNA in an ex vivo gene delivery system produces tumor cures which are greater than those produced by treatment with either modality alone. The potential benefits of exogenous gene induction by radiotherapy include increased local tumor control as well as the potential for treatment of metastatic disease. Gene targeting by ionizing radiation provides a new conceptual basis for increasing the therapeutic ratio in cancer treatment.

Acknowledgments

We thank José S. Jimenez for technical assistance.
Radiation-targeted gene therapy radiosensitizes tumor cells

References


Gene Therapy Targeted by Radiation Preferentially Radiosensitizes Tumor Cells

Ralph R. Weichselbaum, Dennis E. Hallahan, Michael A. Beckett, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/16/4266

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/54/16/4266. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.