Featured Article

Ionizing Radiation Causes a Dose-Dependent Release of Transforming Growth Factor α *In vitro* from Irradiated Xenografts and during Palliative Treatment of Hormone-Refractory Prostate Carcinoma

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ABSTRACT

Purpose: **Characterize the radiation response for transforming growth factor (TGF) α shedding *in vitro* and *in vivo*. We also report the shedding of TGF α by patients undergoing irradiation for hormone-refractory prostate cancer.**

Experimental Design: **TGF α levels were determined by ELISA. DU145 xenografts were established on the flanks of athymic nu/nu mice. Expression of phospho-extracellular signal-regulated kinase (ERK)1/2 and phospho-epidermal growth factor receptor (EGFR) and the DNA repair proteins XRCC1 and ERCC1 were determined by Western analyses.**

Results: **Exposure to ionizing radiation results in a dose-dependent release of TGF α. Once released, TGF α stimulates EGFR-ERK1/2 signaling in unirradiated cells. Blockade of the EGFR with the tyrphostin AG1478 eliminates the up-regulation XRCC1 and ERCC1 by TGF α or irradiation. After irradiation, cells are refractory to additional transactivation of EGFR by additional irradiation for 8 to 12 hours. Irradiation during this refractory period does not increase the expression of XRCC1 or ERCC1. Ligand activation of EGFR is maintained during the refractory period. Irradiation of DU145 xenografts also results in the activation of ERK1/2, release of TGF α, and a similar refractory period. Ionizing irradiation also results in the**

release of TGF α for patients undergoing radiation therapy for hormone-refractory prostate cancer.

Conclusions: **Irradiation results in a dose-dependent increase in TGF α capable of enhancing the growth of DU145 xenografts. TGF α is also shed following radiation therapy of patients treated for hormone-refractory prostate cancer. Radiation transactivation of the EGFR produces a radio-refractory period, which lasts for several hours. During this period, additional irradiation fails to induce XRCC1, ERCC1, or additional TGF α release.**

INTRODUCTION

Genotoxic stress initiates mitogen-activated protein kinase [extracellular signal-regulated kinase (ERK)1/2] signaling to organize molecular pathways involved in proliferation, DNA repair, and apoptosis (recent reviews in refs. 1–3). Others and we (4–6) have demonstrated that exposure to ionizing radiation leads to prompt transactivation of the epidermal growth factor receptor (EGFR) followed by a secondary delayed activation, which is growth factor dependent.

After exposure to ionizing radiation, both DU145 prostate carcinoma cells and A431 vulvar squamous cell carcinoma cell lines display biphasic patterns of ERK1/2 activation (5, 7). Both cell lines depend on transforming growth factor (TGF) α/EGFR/ERK1/2 signaling for autocrine growth stimulation (8–10), and both shed TGF α during the delayed or second phase of ERK1/2 activation (5, 7, 10). The prompt phase of ERK1/2 activation, characterized by its rapid activation and deactivation, results in a brief pulse of increased ERK1/2 activity. This phase of ERK1/2 activation is largely independent of exogenous TGF α (5). A delayed phase of activation, however, initiated some 1 to 2 hours later, lasts for several hours, and can be abolished by neutralizing antibody to TGF α (4, 5). Unlike the initial, prompt phase of ERK1/2 activation, which can only be partially blocked by targeting signaling events upstream from Ras activation, delayed ERK1/2 activation by ionizing radiation appears to occur exclusively through ligand activation of EGFR (5).

In the present work, we characterize the radiation response for the release of TGF α from DU145 prostate carcinoma cells, *in vitro* and *in vivo*, and report for the first time that ionizing radiation also appears to produce TGF α release in patients treated for metastatic prostate cancer. We further show that after exposure to ionizing radiation, DU145 cells are refractory for several hours to further radiation-induced TGF α release. This refractory period can be initiated by a radiation exposure producing a limited release of TGF α, indicating that exhausting

available TGF α does not cause the refractory state. Reirradiation during the refractory period also fails to activate ERK1/2 or up-regulate the DNA repair proteins XRCC1 and ERCC1. These events are discussed with respect to their importance for cellular response to radiation-induced genotoxic stress.

MATERIALS AND METHODS

Human Controls. Male volunteers were recruited from the staff of the Massey Cancer Center, Virginia Commonwealth University.

Radiation Therapy Patients. Patients were recruited as a part of an Institutional Review Board-approved institutional protocol examining cytokine production in irradiated cancer patients. Patients were recommended to receive palliative irradiation for hormone-refractory prostate cancer before being informed of the protocol and seeking informed consent. All subjects completed informed consent interview and documentation before venipuncture.

Animals. Nu/nu athymic mice, purchased from National Cancer Institute Laboratories, were maintained according the NIH guidelines for handling. Upon arrival, mice were quarantined for 14 days and observed for evidence of murine pneumonia complex or *pseudomonas oropharyngeal*. Thereafter, mice were released into the vivarium, maintained on 6 a.m. to 6 p.m. light-dark cycle, and fed lab blox (Purina Corp.) and chlorinated water *ad libitum*.

Materials. All cell culture reagents were obtained from Life Technologies, Inc. Mouse monoclonal anti-β-actin, phospho-ERK1/2(1 μg/mL E-4, anti-phosphotyrosine; 1 μg/mL PY20), and secondary antibodies antirabbit and antimouse IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnology (Palo Alto, CA), whereas XRCC1 and ERCC1 mouse monoclonal antibodies were from Neomarkers (Fremont, CA). Human recombinant EGF was purchased from Collaborative Biomedical Products (Bedford, MA). PD098059 and tyrphostin AG1478 were from Calbiochem (San Diego, CA). The anti-phospho-ERK1/2 antibody was from Cell Signaling, and the anti-ERK1/2 antibody was from Upstate Biotechnology, (Charlottesville, VA).

Cell Cultures and Protein Extraction. Cells of the human prostate carcinoma cell line DU145 were obtained from the American Type Culture Collection (Manassas, VA). Western analysis confirmed the RB and TP53 status of this line. Cells were cultured in RPMI 1640 supplemented with 5% FBS, 10 units/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L L-glutamine and were maintained at 378°C in 95% air/5% CO_2 throughout the experiment, except during the irradiation. Cells were plated at a density of 3.2×10^4 cells/cm^2 plate area unless otherwise stated. The mitogen-activated protein kinase kinase (MEK)1 inhibitor PD98059 in DMSO was routinely added 1 to 3 hours before irradiation of exponentially growing cultures unless otherwise indicated. In the indicated experiments, anti-TGF α antibody (1 mg antibody/mL medium) was added 60 minutes before irradiation. A control antibody, 1 mg/mL, to the transcriptional regulator TF$_{IID}$ was of the same antibody subtype, IgG2. After the indicated period, cell metabolism was blocked on ice. Cells were resuspended in ice-cold extraction buffer containing 25 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 5 mmol/L EGTA, 5 mmol/L benzamidine, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and 20 μg/mL each of leupeptin, aprotinin, and pepstatin. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA).

Xenografts. DU145 cell xenografts were produced as flank tumors in nu/nu athymic female mice. Cell suspensions (5×10^6 cells in 100 μL) were implanted s.c. under ketamine anesthesia. Mice were observed every third day until onset of tumor development. Thereafter, tumors were scored for size with caliper measurements made in orthogonal directions. Tumor volumes were estimated using the formula, $4/3\pi[(a+b)/4]^3$, where a and b represent the measured orthogonal tumor diameters.

Immunoprecipitation and Western Immunoblotting. Cell cultures were grown in medium alone or supplemented with AG1478, PD 98059 or EGF (5 ng/mL). Cells washed with cold PBS were harvested in cold lysis buffer [0.5% NP40, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl with protease inhibitors freshly added, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L leupeptin, and 1.54 μmol/L aprotinin]. Crude cell lysates were passed through a 21-gauge needle four times, incubated on ice for 30 minutes and then centrifuged for 20 min at 4°C. Equal protein samples were incubated overnight at 48°C with anti-EGFR antibody or anti-activated EGFR antibody (Transduction Laboratories, Lexington, KY). Immunoprecipitates were incubated with 50% protein A-agarose suspension (Life Technologies, Inc.) for 1 hour at 4°C, washed with cold lysis buffer, and centrifuged. Pellets were resuspended in sample buffer [0.125 mol/L Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, and 4% β-mercaptoethanol], heated for 5 min at 95°C, resolved by 8% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Membranes were blocked with TTBS buffer [20 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, and 0.05% Tween 20] containing 5% nonfat dried milk for 2 hours at 4°C with continuous rotation. The immunoprecipitates with anti-EGFR antibody were then detected with phosphotyrosine monoclonal antibody (PY99) from Santa Cruz Biotechnology. The blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature and then washed three times for 15 minutes with TTBS buffer. The membrane was immersed in enhanced chemiluminescence for 1 minute and exposed to X-ray film.

ELISA. ELISA kits for mammalian TGF α from Oncogene. Assays were done according to the manufacture protocol with slight modifications. Briefly, cells were grown to ∼60% confluence in RPMI medium alone or supplemented with AG1478, PD 98059 60 minutes before the addition of 5 ng/mL EGF. Cells were exposed to ^{60}Co γ-radiation, and the media sampled after various intervals of incubation at 37°C. Medium sample and TGF α standard in triplicate were added to the TGF α–precoated wells and incubated 4 hours at room temperature. Samples then were processed according to the manufacture protocols. Media without cells, and media incubated with EGF were used as a negative control in each assay. All reactions were measured using a spectrophotometric plate reader at a wavelength of 490 nm (BioTek Instruments, Inc., Highland Park, VT).

ERK1/2 Activity. ERK1/2 was assayed from immune-precipitated cell lysates as described previously (5). Briefly, 50 μL of Protein A-agarose (slurry 25 μL bead volume) was washed twice with 1 mL of PBS containing 0.1% (v/v) Tween 20 and resuspended in 0.1 mL of the same buffer. Antibodies (2 μg, 20 μL) and serum (20 μL) were added to each tube, and the tubes were incubated (3 hours, 4°C). For preconjugated antibodies, 10 μL of slurry (4 μg of antibody) were used. Clarified aliquots of lysate (0.25 mL;100 mg of total protein) were mixed with Protein A-agarose–conjugated antibodies in duplicate using gentle agitation (2.5 hours, 4°C). Protein A-agarose antibody–antigen complexes were recovered by centrifugation, the supernatant was discarded, and the complexes were washed (10 minutes) sequentially with 0.5 mL of buffer A (twice), PBS, and buffer B [25 mmol/L HEPES (pH 7.4), 0.1 mmol/L Na_3VO_4]. Immunoprecipitates were incubated (final volume, 50 μL) with buffer B (50 μL) containing 0.2 mmol/L [γ-32P]ATP (5000 cpm/pmol), 1 mmol/L Microcystin-LR, and 0.5 mg/mL myelin basic protein, which initiates reactions at time 0. After 20 minutes, 40 μL of the reaction mixture were spotted onto a 2-cm circle of P81 paper (Whatman, Maidstone, United Kingdom) and immediately placed into 180 mmol/L phosphoric acid. Papers were washed four times (10 minutes each) with phosphoric acid and once with acetone, and incorporation of ^{32}P into myelin basic protein was quantified by liquid scintillation spectroscopy.

Irradiation. The irradiation technique has been described previously (5, 6). Tumor-bearing mice were irradiated under ketamine anesthesia. For irradiation, tumors were isolated by cerrobend blocking. Cells attached as monolayers were exposed at room temperature or ice temperatures. In each case ^{60}Co γ-radiation was administered at a dose rate of 1.1 Gy/minute. Dosimetry was provided by the Medical Physics Section, Department of Radiation Oncology, MCV Hospitals.

RESULTS

Radiation-Induced TGF α Release Increases ERK1/2 Activity. Previously, we reported that ionizing irradiation produces a biphasic increase in ERK1/2 activity as measured by the phosphorylation of myelin basic protein by ERK1/2 immunoprecipitates from several cell lines (5–7). The data shown in Fig. 1 confirm our previous study. Here, the late phase of ERK1/2 activation in DU145 cells is shown to result from a second round of EGFR/ERK1/2 signaling, which has been initiated by the radiation-induced release of TGF α (5, 6). These data show that the increase in ERK1/2 activity produced 3 hours after irradiation was blocked through interference with EGFR-ERK1/2 signaling, either at the receptor level (tyrphostin AG1478) or by inhibiting MEK1/2 (PD98059). For these experiments, ERK1/2 activity was measured in cell homogenates, as described in Materials and Methods, 3 hours after irradiation of exponentially growing DU145 cells attached as monolayers. The tyrphostin AG1478 (5 ng/mL) and PD98059 (10 μmol/L) were added 30 minutes after irradiation. The elimination of late phase ERK1/2 activation by neutralizing antibody to TGF α added after irradiation confirms the importance of ligand activation of the EGFR after irradiation.

Also shown in Fig. 1 are experiments using media transferred from irradiated cultures to control cultures. This maneu-
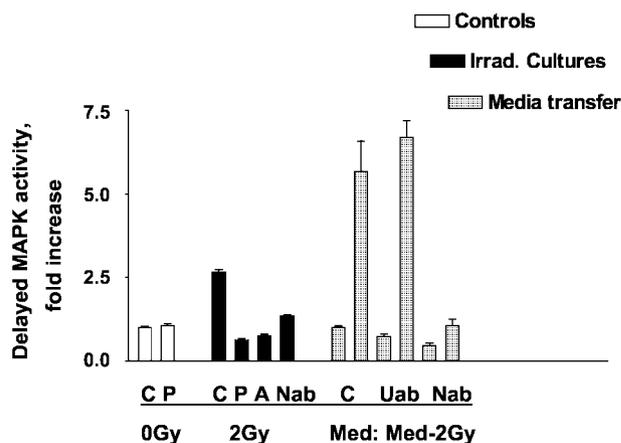

Fig. 1 Radiation induced TGF α results in ERK1/2 activation 3 hours after irradiation. ERK1/2 activity was determined as described in Materials and Methods 3 hours after irradiation (■) or sham irradiation (□). *Dotted bars* show ERK1/2 activity in cultures 20 minutes after the transfer of media from paired samples, either unirradiated (Med) or irradiated (Med-2Gy). Additional treatments shown include P: PD98059 (10 μmol/L), A: AG1478 (5 ng), Nab: neutralizing antibody to TGF α; and Uab: an unrelated antibody. Experiments were performed in triplicate *Error bars* represent 2SD. MAPK, mitogen-activated protein kinase.

ver confirmed that a soluble activity released after irradiation (2 Gy) was responsible for the late activation of ERK1/2. For these experiments, paired cultures were irradiated or sham-irradiated. Three hours after irradiation, media were aspirated from the sham-irradiated dishes and replaced with media from the irradiated cultures. Cells were harvested and ERK1/2 activity determined after 10 minutes at 37°C. A 60-minute preincubation of the media from irradiated cells with neutralizing antibody for TGF α, as described in Materials and Methods, but not an unrelated antibody (anti-TF$_{IID}$), abolished the media transfer effect.

Time Course of Radiation-Induced TGF α Shedding. After demonstration of the involvement of TGF α, we sought to quantify the radiation-induced shedding response. Using an ELISA specific for human TGF α, we examined the time course of TGF α shedding from DU145 cells during the first 24 hours after irradiation. For these measurements, exponentially growing DU145 cells (1 × 10^6 per sample) were exposed to ^{60}Co γ-radiation, and the media sampled after various intervals of incubation at 37°C. The data in Fig. 2*A* show that exposure to either 7 or 14Gy resulted in similar kinetics of TGF α release. No human TGF α was detected in the original culture media. Unirradiated cultures produced ~46 (+/− 18 SD) pg/10^6 cells. After 7 Gy, a maximum TGF α value of 1256 (+/− 420 SD) pg/10^6 cells occurred at ~2 hours. The concentration of TGF α returned to control values after ~6 hours. Maximum TGF α release after 14 Gy was 2170 (+/− 750 SD) pg/10^6 cells. The decrease in TGF α for times >4 hours is dependent on the presence of DU145 cells (data not shown).

TGF α Is Released in a Radiation Dose-Dependent Fashion. After examining the kinetics of TGFα release, we measured the radiation dose-response over the dose range of 0–14Gy. Sub-confluent cultures of exponentially growing
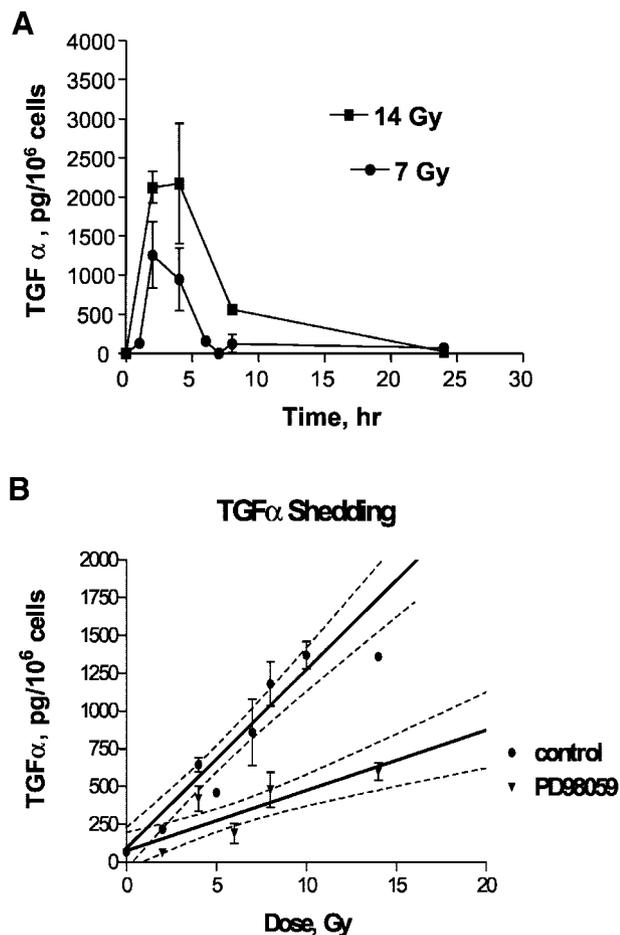
Fig. 2 A depicts the time course of TGF α release after IR. Fifty μL samples of culture media were removed for ELISA as described in Materials and Methods. Data points represent the mean TGF α levels (+/− 2SD) for triplicate samples. *B* shows the dose-response generated by irradiation as described for *A*. Samples were removed for TGF α assay 3 hours after irradiation. PD98059 [10 μmol/L in DMSO, 1:333 (v:v)] was added 3 hours before irradiation. DMSO [1:333 (v:v)] control produced no detectable TGF α shedding above background levels (46 pg/ml/1 × 10^{6} cells). Data are otherwise as described for *A*.

DU145 cells were exposed to graded doses of ^{60}Co γ-radiation. Following 3 h incubation at 37°C, 50 μL samples of the medium were removed for the ELISA assay. The resulting data are shown in Fig. 2B. TGFα release was an approximately linear function of the radiation dose over the range 0–10 Gy with $r^2 = 0.96$ (goodness of fit). Elimination of the 14 Gy data point from the analysis did not significantly improve the goodness of fit statistic. The value of the slope was 118 (+/− 11 SD) pg/Gy/10^6 cells. Confidence intervals (95%) for the slope are shown on the figure.

MEK1/2 inhibition confirmed the requirement for ERK1/2 signaling for the radiation-induced release of TGF α. As shown in Fig. 2*B*, addition of the MEK1/2 inhibitor PD98059 (10 μmol/L) 3 hours before irradiation markedly reduced TGF α shedding. With the addition of PD98059, the curve slope decreased to 39 (+/− 8 SD) pg/Gy/10^6 cells. This level of

MEK1/2 inhibition reduces the radiation up-regulation of ERK1/2 (4, 5) without halting proliferation of DU145 cells. Ten μmol/L PD98059 alone decreased TGF α level from 69 (+/− 14 SD) to 58 (+/− 12 SD) pg/10^6 cells. However, increasing the MEK1/2 inhibitor to 40 μmol/L resulted in undetectable TGF α in unirradiated cultures, and only 60 (+/− 5 SD) pg/10^6 cells after 8 Gy, which is <4% of the TGF α shed after an 8-Gy exposure of control cells. Forty μmol/L PD98059 completely arrested proliferation of DU145 cells.

TGF α Shedding after a Second Radiation Exposure. Absence of a second pulse of TGF α shed after radiation exposure implies that the late activation of ERK1/2 produces little or no re-release of TGF α. Because this result could be due either to an altered sensitivity to the growth factor after irradiation or the inability of irradiated cells to release additional TGF α, we examined both possibilities. We first tested this notion by examining TGF α–shedding after a second radiation exposure. For these experiments, exponentially growing DU145 cells were irradiated and the TGF α concentration determined as a function of incubation time. Four hours after the initial radiation exposure, parallel irradiated cultures were exposed to a second dose. The culture media was replaced and the release of additional TGF α measured as a function of time. The results are shown in Fig. 3*A*. Cells irradiated once demonstrated the usual peaked response at ~2 to 3 hours, returning to control values of TGF α by 8 hours. Cultures were refractory to the second irradiation, releasing a much smaller amount of TGF α. Only the value determined at 8 hours (4 hours after the second exposure) differed significantly from the control values. Two-dose irradiation was similarly performed using 2X3 and 2X6 Gy. In each case, the TGF α released 3 hours after the second irradiation was <10% of that released 3 hours after the first.

The duration of this nonresponsive or refractory period was measured by increasing the time between radiation doses. For these experiments, TGF α levels were determined either after a single exposure or 3 hours after a second dose. The 3-hour incubation after the second exposure allowed maximal TGF α release. For these experiments, the culture media was not changed throughout the experiment. Shown in Fig. 3*B*, these data indicate that the capacity to shed TGF α in response to irradiation slowly returned as the two radiation exposures were separated by longer intervals. TGF α release after 15 hours was not significantly less than that observed after a single exposure.

After Irradiation Induced EGFR/ERK1/2 Signaling, EGFR/ERK1/2 Signaling Remains Intact to Ligand Activation but not to Further Transactivation by Irradiation. Radiation-induced transactivation of EGFR/ERK1/2 signaling in DU145 cells results in ERK1/2 activation and up-regulation of several proteins involved in DNA repair. The data in Fig. 4*A* show that the addition of either ligand or a single radiation exposure (2 Gy) results in up-regulation of XRCC1, ERCC1, and phosphorylation of ERK1/2. Combined ligand activation and transactivation by irradiation increase further these responses. When the EGFR inhibiting tyrphostin AG1478 is administered before the addition of EGFR ligand and irradiation, however, up-regulation of XRCC1, ERCC1 and ERK1/2 phosphorylation are largely blocked. The data depicted in Fig. 4*B* show that tyrosine 1173 of EGFR is phosphorylated following a single exposure of 7 Gy but not after a second 7-Gy
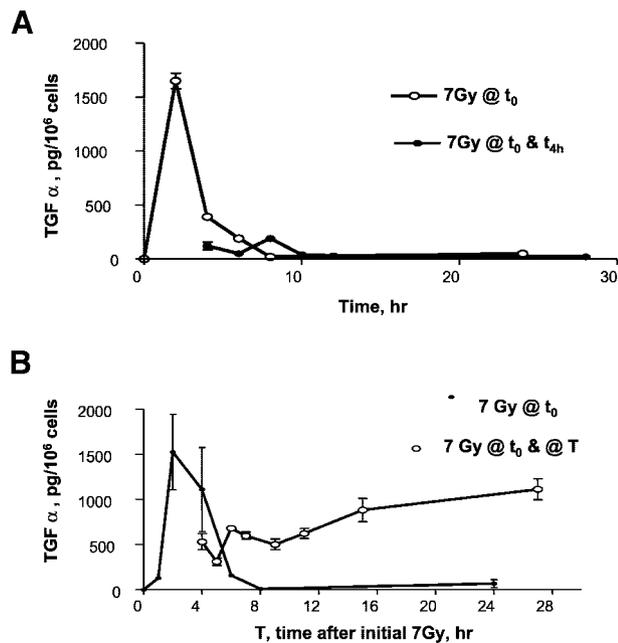
A

B

Fig. 3 Less TGF α is released after reirradiation. Du145 cells were cultured and irradiated as described for Fig. 1*A*. Afterward, the overlying media was sampled for the TGF α ELISA. *Open symbols* depict the TGF α concentration after a single 7-Gy exposure, whereas *closed symbols* show the TGF α released after a second exposure delivered 4 hours after the first. Fresh media were added at the time of the second irradiation. The data represent the mean of three to six determinations, and *error bars* represent +/− *SE*). *B*, *open symbols* depict the TGF α levels determined 3 hours after a second exposure. Here, the interval between the two exposures is varied, and the media unchanged throughout the procedure. *Closed symbols* represent the TGF α released after a single exposure of 7 Gy. Data represent the mean of three determinations and *error bars* (+/− 2SD).

exposure. Ligand activation of EGFR, however, remains intact during period of the radiation exposures. After irradiation, ERK1/2 phosphorylations and XRCC1 expression, which are downstream effects of EGFR activation, are also intact to ligand activation but not radiation-induced transactivation (Fig. 4*C*).

Radiation-Induced TGF α Release and ERK1/2 Activation in Xenografts. Having demonstrated radiation-induced release of TGF α from cells in culture, we next examined DU145 xenografts. In these experiments, we examined ERK1/2 activity and TGF α production after irradiation DU145 cells grown as xenografts in athymic nu/nu mice. Tumors were initiated as described in Materials and Methods. When tumors reached 8 mm in diameter, animals were irradiated or sham-irradiated, sacrificed, and their tumors excised. As illustrated in Fig. 5*A*, substantial levels of human TGF α were detected in the peripheral blood samples from irradiated mice bearing DU145 xenografts but not controls. For these measurements, peripheral blood samples from individual mice, three to five per data point, were separately analyzed and the mean values reported on the figure. Greater than 11,000 (+/− 330 SD)pg TGF α per ml serum/g tumor were found in the peripheral blood of irradiated hosts bearing DU145 xenografts, whereas <250 (+/− 20 SD)pg/ml serum/g tumor was detected in unirradiated xenograft

controls. No human TGF α was detectable (<25 pg/100 μL) in peripheral blood samples from mice without xenografts, irradiated or unirradiated.

In agreement with our *in vitro* findings, reirradiation of DU145 xenografts produced no significant re-release of TGF α. Also shown in Fig. 5*A*, when flank tumors were reirradiated (3 Gy) 3 hours after the initial exposure and followed for TGF α production. Maximal TGF α production, noted 3 hours later,
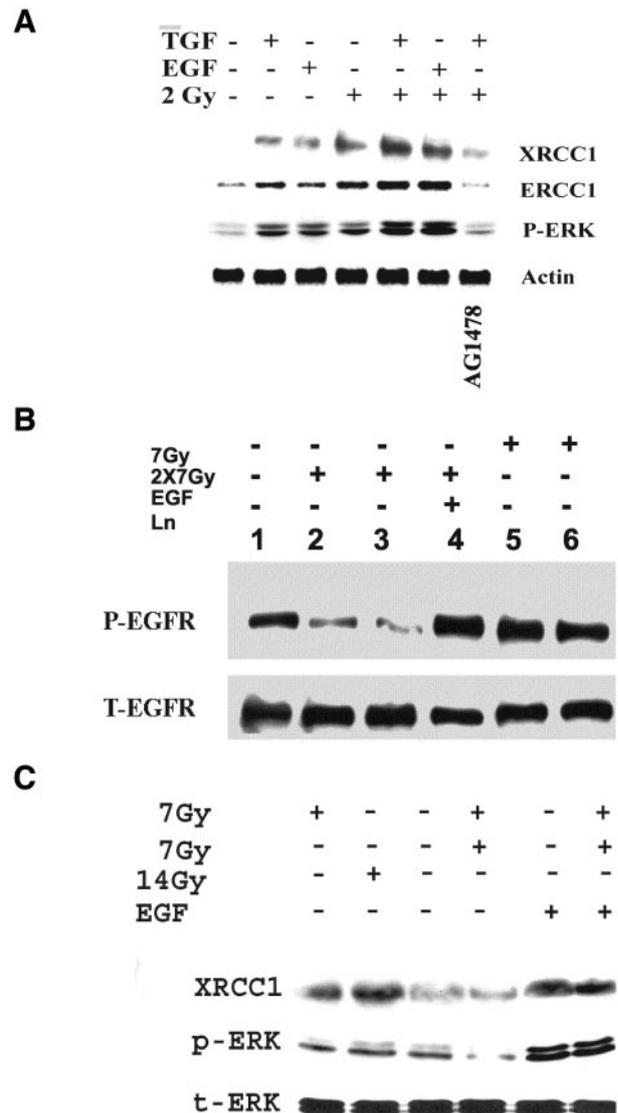

Fig. 4 EGFR and ERK1/2 are phosphorylated after a single radiation exposure but not after reirradiation. *A*. XRCC1 and ERCC1 are up-regulated and ERK1/2 activated after exposure to the EGFR ligands TGF α or EGF (90 minutes before cell harvesting), ionizing radiation (30 minutes prior), or a combination of irradiation and ligand exposure. Inactivation of the EGFR by the tyrphostin AG1478, however, blocks these downstream effects. *B*. Western analysis of EGFR-P (tyr 1173) and total EGFR 30 minutes after a single 7 Gy exposure or two exposures of 7 Gy separated by 3 hours. *C*. Western analysis of XRCC1, pERK1/2, and total ERK 30 minutes after a single 7 or 14 Gy exposure or two exposures of 7 Gy separated by 3 hours. Also shown is the effect of EGF addition 1 hour before the second irradiation.
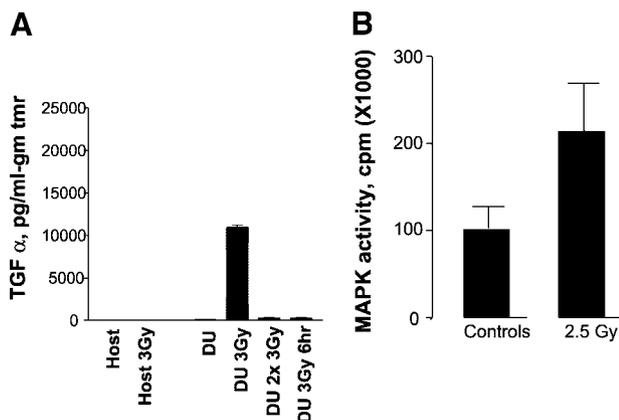
Fig. 5 Irradiation causes the release of TGF α and increased ERK1/2 activity in DU145 xenografts. DU145 flank tumors and peripheral blood samples were taken 3 hours after irradiation from athymic nu/nu mice bearing xenografts as described in Materials and Methods. *A*, serum concentration of TGF α present in peripheral blood samples from three to five mice, irradiated or sham-irradiated. Individual TGF α values are normalized based upon the tumor mass (range, 0.2 to 0.4 g). *B*. These data show the ERK1/2 activity determined in samples of tumor homogenates, three to five tumors per data point (+/− 2SE).

was only 260 (+/−30 SD) pg/ml serum/g tumor. For comparison, the serum level of TGF α 6 hours after a single 3-Gy exposure was 233 (+/− 65) pg/ml serum/g tumor. ERK1/2 activity was also increased in tumor homogenates from irradiated mice, when compared with tumors from sham-irradiated controls (Fig. 5*B*).

Exogenous TGF α Accelerates Xenograft Growth. Serum TGF α levels found in the peripheral blood of mice bearing irradiated DU145 xenografts were substantially above physiologic levels associated with enhanced growth, *i.e.,* 0.02 to 0.1 ng/mL (11). Therefore, we examined the possibility that exogenous TGF α could alter tumor growth. For these experiments, osmotically active minipumps containing TGF α (0.5 ng/μL) were implanted on the dorsum of athymic nu/nu females 5 days after DU145 xenografts were initiated. Releasing 0.5 ng of TGF α per hour, these minipumps were active for 72 hours. Control mice were implanted with minipumps containing saline. Mice were examined every third day until tumor presence was confirmed. Thereafter, tumor growth was measured as indicated in Materials and Methods. The data in Fig. 6 show accelerated tumor growth, albeit with greater variability, for mice receiving exogenous TGF α.

TGF α Is Produced as a Result of Palliative Irradiation in Prostate Cancer Patients with Hormone-Refractory Disease. Because a peripheral source of TGF α accelerated the growth of DU145 xenografts, the shedding of TGF α from irradiated deposits of hormone-refractory prostate cancer may also be important for the palliation of patients with metastatic progression. We therefore have initiated a pilot study to examine serum levels of TGF α in patients receiving palliative radiation therapy as the first treatment for metastatic, hormone-refractory prostate cancer. Patient characteristics, eligibility criteria, and treatment are included in the methods section. For each patient, peripheral blood samples were drawn before and 3 hours after

the initial radiation treatment. As of the time of this writing, six patients have been enrolled as a part of this study. The data depicted in Fig. 7 show that sera from all patients contained markedly elevated TGF α levels after irradiation. One patient, number 320, had elevated levels before irradiation, which were unchanged after irradiation. This patient was treated for painful lymphadenopathy, whereas the other five had experienced painful bony metastases. Three of the five patients with bony metastases were irradiated for extremity metastases, reducing the likelihood that the observed TGF α originated from irradiated nontumor tissues. TGF α levels from the sera obtained from unirradiated volunteers (n = 3) was <25 pg/ml (+/− 5 pg/ml).

DISCUSSION

EGFR-regulated growth is important for prostate development and frequently is deranged in prostate proliferative disor-
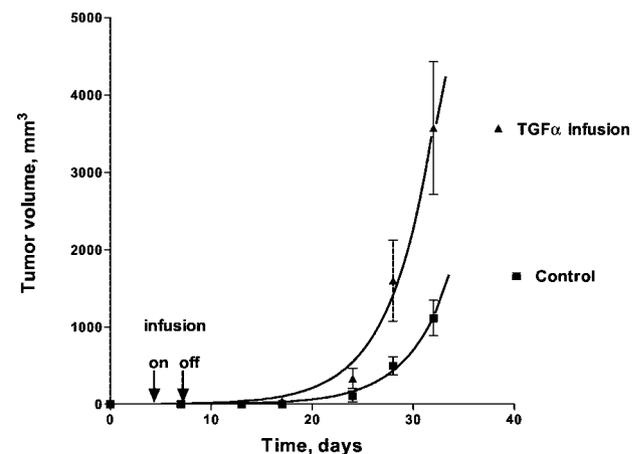

Fig. 6 Exogenous TGF α accelerates tumor development. DU145 xenografts were initiated as described in Materials and Methods. Five days after the initiation of xenografts, 72-hour osmotic minipumps containing TGF α (0.55 mg/ml, ▲) or saline (■) were implanted on the dorsum of the host mice. Tumor volumes shown represent mean values for 8 to 12 tumors (*error bars* reflect +/−2SD).
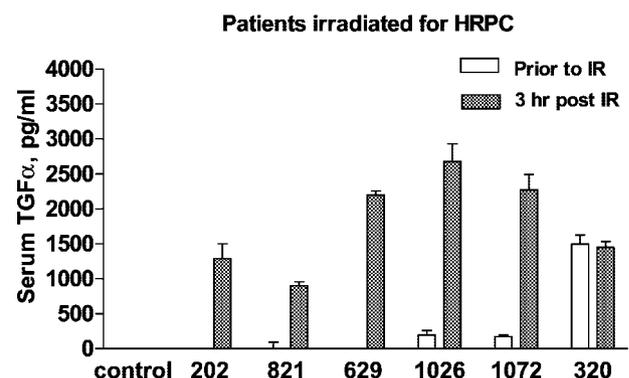

Fig. 7 Serum TGF α levels before and after irradiation (3 Gy) for metastatic hormone refractory prostate cancer (HRPC). Data represent the mean of three determinations made from individual serum samples drawn immediately before or 3 hours after the first treatment of a fractionated course of external beam irradiation.

ders (12–15). First, observable in fetal prostate epithelium after the androgen surge, EGFR expression is normally restricted to the basal layer (12). Although the evidence is limited, EGF and TGF α production is similarly restricted, implying that an autocrine arrangement may be normal (12–15). In benign prostatic hypertrophy, however, the secretory epithelium also appears to secrete both ligands, adding paracrine stimulation (13–15). The same appears to be true for high-grade prostatic interepithelial neoplasia, but in this case, the secretory epithelium also displays EGFR (16).

Adenocarcinomas of the prostate are even less organized, with evidence of EGFR and ligand production throughout tumor foci and in stromal elements as well (13–15). In fact, Scher *et al.* (15) have reported more intense TGF α staining in the stromal elements. Metastases of prostate carcinoma frequently produce both receptor and ligand, with TGF α usually staining more intensely than EGF (15). Thus, with progression, prostate cancer cells appear to become increasingly dependent on TGF α synthesis, which likely contributes to androgen independence, autocrine growth, and unrestrained proliferation (12–18).

Multiple lines of evidence imply TGF α production is involved in cellular recovery after irradiation. For example, TGF α production has been associated with increased likelihood of tumor recurrence after irradiation for cancers of the esophagus (19) and larynx (20), as well as for meningiomas (21). In each series, tumor recurrences were more frequent when the original tumor expressed increased levels of TGF α. Furthermore, there is evidence suggesting that TGF α enhances the proliferative recovery of intestinal crypt cells after irradiation (22, 23). In this regard, however, a recent article from Evans *et al.* (24) showed no difference in the recovery of jejunal crypt cells after irradiation of either wild type of TGF α–null mice. Thus, it is more likely that TGF α enhances recovery after irradiation but is not required.

TGF α is a membrane anchor-dependent growth factor, which is shed as a result of signal transduction initiated proteolysis (25–29). Although the details of TGF α shedding are unknown, proteolysis appears to depend on the "a disintegrin and metalloprotease" family of metalloproteinases, more specifically tumor necrosis factor α converting enzyme (25–27). Recent evidence implicates both ERK1/2 (ERK) and protein kinase C signaling events in the activation of tumor necrosis factor α converting enzyme and resultant TGF α shedding (25–29).

We have shown that ionizing radiation, through ERK1/2 signaling, results in TGF α release (5, 29 and present data). Furthermore, once released, soluble TGF α initiates a second wave of ERK1/2 activation in unirradiated bystander cells, repeating the signaling process (5, 29 and present data). The amount of TGF α released *in vitro* depends on the radiation dose but can be substantially reduced through an interruption in EGFR-ERK1/2 signaling. Once shedding has occurred, however, regardless of the extent of shedding, cells are for several hours refractory to a second round of radiation-induced shedding. Eventually, however, full recovery for TGF α shedding occurs. *In vitro*, this recovery occurs during the period of postirradiation mitotic delay and while cell numbers continue to decline. This suggests that cell proliferation is not required for recovery, implying a process inherent to the irradiated cell.

Although EGFR expression appears to be related to an-

chorage on growth substrate (30), examination of DU145 xenografts showed that they too displayed increased ERK1/2 activity in response to irradiation, releasing substantial TGF α into the serum. Partial body irradiation of mice bearing DU145 xenografts caused a substantial increase in the serum level of TGF α 3 hours after irradiation. After irradiation, the serum concentration of TGF α, ~200 ng/ml per gram of irradiated tumor, was >100 times greater than a normal physiologic serum concentration and also >100-fold greater than that detectable in sham irradiated controls. Moreover, the infusion of exogenous human TGF α at 0.1 ng/hour stimulated tumor development. The refractory period, observed initially in irradiated monolayers, was also a characteristic of irradiated DU145 xenografts. Serum TGF α concentration was not increased in the peripheral blood samples of irradiated mice after a second radiation exposure. These results are consistent with the notion that after irradiation TGF α release *in vivo* is qualitatively similar to that observed *in vitro*. This notion is also supported by the radiation-induced increase in ERK1/2 activity observed *in vivo* and *in vitro*.

The release of a growth factor as a result of irradiation has potential implications regarding patient treatment. As an initial approach, we examined serum levels of TGF α in the peripheral blood samples of patients undergoing palliative irradiation and of normal volunteers. Choosing as subjects patients diagnosed with hormone-refractory prostate cancer was intended to increased the likelihood that some would bear disease-producing TGF α (13, 15, 31). To date, serum samples have been examined for six patients who had received no previous irradiation or chemotherapy. Each produced substantial levels of TGF α, *i.e.,* an average of ~3600 pg/ml serum. For five of the six patients, the TGF α release was radiation dependent. Thus, a substantial proportion of patients diagnosed with hormone-refractory prostate cancer are likely to shed TGF α after irradiation (95% confidence interval, 50–90%). The estimate of the actual incidence will improve as patient accrual continues on this study. This property is likely to be an important consideration when patients presenting with multiple metastases are focally irradiated. Although these data do not prove the TGF α produced by irradiated patients originated from their metastases, those with metastases involving an extremity were unlikely to have released TGF α at these levels from irradiated neighboring tissues. The dose dependence of TGF α release and the kinetics of the refractory period imply that large daily doses may not be the ideal treatment. We are currently investigating altered fractionation schemes, EGFR signal interruption, and MEK1/2 inhibition as methods of reducing TGF α shedding in these patients.

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