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Increased tumor necrosis factor α mRNA after cellular exposure to ionizing radiation

(gene expression)

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ABSTRACT **We report that tumor necrosis factor α (TNF-α) mRNA is increased after treatment with x-rays in certain human sarcoma cells. An increase in TNF-α mRNA is accompanied by the increased production of TNF-α protein. TNF-α enhances radiation lethality in both TNF-α-producing and -nonproducing tumor cells. These data suggest that, in addition to the direct cytotoxic effects of x-rays, production of TNF-α may add to radiation lethality through autocrine and paracrine mechanisms. Combinations of TNF-α and therapeutic radiation may be useful in clinical cancer therapy.**

Tumor necrosis factor α (TNF-α) is a polypeptide mediator of the cellular immune response with pleiotropic activity. TNF-α acts directly on vascular endothelium to increase the adhesion of leukocytes during the inflammatory process (1). This *in vivo* response to TNF-α may be responsible for hemorrhagic necrosis and regression of transplantable mouse and human tumors (2). TNF-α also has a direct effect on human cancer cell lines *in vitro*, resulting in cell death and growth inhibition (3, 4). The cytotoxic effect of TNF-α correlates with free-radical formation, DNA fragmentation, and microtubule destruction (5–10). Cell lines that are resistant to oxidative damage by TNF-α also have elevated free-radical buffering capacity (11, 12).

Much investigation of the cytotoxic effects of ionizing radiation has focused on the repair of DNA damage or the modification of radiation lethality by hypoxia (13, 14). DNA-damaging agents other than x-rays induce expression of a variety of genes in higher eukaryotes (15–17). In prokaryotes and lower eukaryotes, ionizing radiation has been shown to induce expression of several DNA repair genes (18); however, induction of gene expression by ionizing radiation has not been described in mammalian cells. The induction of a cytotoxic protein by x-rays was suspected when medium decanted from irradiated cultures of some human sarcoma cell lines was cytotoxic to these as well as other tumor cell lines. The level of TNF-α in the irradiated tumor cultures was elevated over that of nonirradiated cells when analyzed by the ELISA technique (19). Therefore, we investigated whether elevated TNF-α protein after irradiation potentiates x-ray killing of cells by an unusual undescribed mechanism.

MATERIALS AND METHODS

Cell Lines. Methods of establishment of human sarcoma and epithelial cell lines have been described (20, 21). Culture medium for epithelial tumor cells was 72.5% Dulbecco's modified Eagle's medium/22.5% Ham's nutrient mixture F-12 [DMEM/F-12 (3:1)]/5% fetal bovine serum (FBS), transferrin at 5 μg/ml/10^{-10} M cholera toxin/1.8 × 10^{-4} M adenine, hydrocortisone at 0.4 μg/ml/2 × 10^{-11} M triiodo-L-thyronine/penicillin at 100 units/ml/streptomycin at 100 μg/ml. Culture medium for sarcoma cells was DMEM/F-12 (3:1)/20% FBS, penicillin at 100 units/ml/streptomycin at 100 μg/ml.

TNF-α Protein Assay. Human sarcoma cells were cultured as described above and grown to confluence. The medium was analyzed for TNF-α 3 days after feeding and again 1–3 days after irradiation. Thirteen established human sarcoma cell lines were irradiated with 500-centigray (cGy) x-rays with a 250-kV Maxitron generator (20). TNF-α was measured by ELISA with two monoclonal antibodies that had distinct epitopes for TNF-α protein (19); the assay detects TNF-α from 0.1 to 2.0 units/ml.

RNA Isolation and Northern (RNA) Blot Analysis. Total cellular RNA was isolated from cells by using the guanidine thiocyanate–lithium chloride method (22). RNA was size-fractionated by formaldehyde-1% agarose gel electrophoresis, transferred to nylon membranes (GeneScreen*Plus*, New England Nuclear), hybridized as previously described to the 1.7-kilobase (kb) *Bam*HI fragment of the PE4 plasmid containing TNF-α cDNA (19, 23), and autoradiographed for 16 days at −85°C with intensifying screens. Northern blots were also hybridized to 7S rRNA and β-polymerase plasmids as described (15). Ethidium bromide staining revealed equal amounts of RNA applied to each lane. RNA blot hybridization of TNF-α was analyzed after cellular irradiation with 500 cGy. Cells were washed with cold phosphate-buffered saline and placed in ice at each time interval. RNA was isolated at 3, 6, and 12 hr after irradiation.

Treatment of Cells with X-Irradiation and TNF-α. Exponentially growing cells were irradiated by using a 250-kV x-ray generator. The colony-forming assay was used to determine cell survival (20). The multitarget model survival curves were fit to a single-hit multitarget model [$S = 1 - (-e^{-D/D_0})^n$] (24). Concentrations of recombinant human TNF-α (10 units/ml) (2.3 × 10^6 units/mg) and (1000 units/ml) (Asahi Chemical, New York) were added 24 hr before irradiation.

RESULTS

TNF-α Protein Production. To investigate TNF-α protein production after x-irradiation, the levels of TNF-α in the medium of human tumor cell lines and fibroblasts were quantified by the ELISA technique (19) before and after exposure to 500-cGy x-rays (Table 1). Five of 13 human bone and soft tissue sarcoma cell lines (STSAR-5, -13, -33, -43, and

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Abbreviations: TNF-α, tumor necrosis factor α; cGy, centigray; PE, plating efficiency.

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Table 1. Production of TNF-α in human sarcoma cell lines

Cell line	Origin	TNF-α level, units/ml	
		Control	X-ray
STSAR-5	MFH	0.4	>2.0
STSAR-13	Liposarcoma	0.0	0.34
STSAR-33	Ewing sarcoma	0.17	>2.0
STSAR-43	Osteosarcoma	0.41	1.3
STSAR-48	Neurofibrosarcoma	0.28	0.43

TNF-α levels were measured in medium from confluent cell cultures (control) and in irradiated confluent cells (x-ray). TNF-α levels increased as measured by the ELISA technique. MFH, malignant fibrous histiocytoma.

-48) release TNF-α into the medium after irradiation, whereas TNF-α levels are not elevated in supernatant from normal human fibroblast cell lines (GM-1522 and NHF-235) and four human epithelial tumor cell lines (HN-SCC-68, SCC-61, SCC-25, and SQ-20B) after exposure to radiation. The assay accurately measures TNF-α levels between 0.1 and 2.0 units per ml (2.3×10^6 units/mg) (19). Tumor cell line STSAR-13 produces undetectable amounts of TNF-α before x-irradiation and 0.35 units/ml after x-ray exposure. Cell lines STSAR-5 and -33 respond to x-irradiation with increases in TNF-α concentrations of >5- to 10-fold; however quantities above 2 units/ml exceed the range of the assay (19). Cell lines STSAR-43 and -48 demonstrate increases in TNF-α of 1.5- to 3-fold (Table 1). TNF-α protein in the medium is first elevated at 20 hr after x-ray treatment, reaches maximal levels at 3 days, and remains elevated beyond 5 days. Furthermore, supernatant from irradiated, but not control STSAR-33, is cytotoxic to TNF-α-sensitive cell line SQ-20B (data not shown).

RNA Analysis. Increased levels of TNF-α mRNA are detected in the TNF-α-producing sarcoma cell lines after irradiation relative to unirradiated controls (Fig. 1). For example, TNF-α transcripts are present in unirradiated STSAR-13 and -48 cell lines. TNF-α mRNA levels in cell line STSAR-13 increase by >2.5-fold as measured by densitometry 3 hr after exposure to 500 cGy and then decline to baseline levels by 6 hr (Fig. 1). These transcripts increase at 6 hr after irradiation in cell line STSAR-48, thus indicating

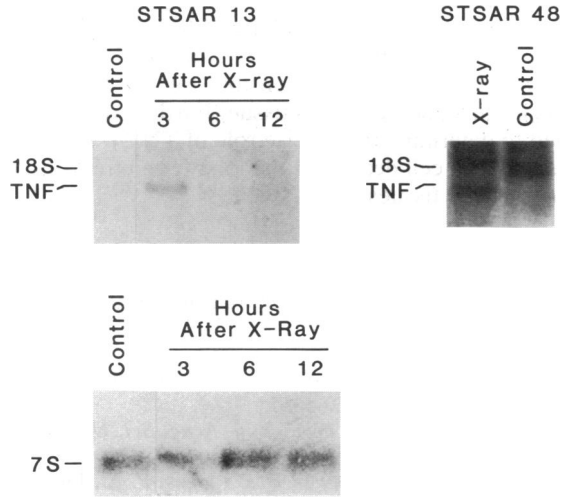

FIG. 1. Effects of irradiation on TNF-α gene expression. RNA from untreated cells (control) and irradiated cells was size-fractionated and hybridized to ^{32}P-labeled TNF-α cDNA (STSAR-13) and PE4 plasmid containing TNF-α cDNA (STSAR-48). Autoradiograms show increased expression of TNF-α mRNA 3 hr after irradiation in cell line STSAR-13 and at 6 hr in cell line STSAR-48. 7S RNA is hybridized to show equally loaded lanes.

some heterogeneity between cell lines in terms of the kinetics of TNF-α gene expression (Fig. 1). In contrast, irradiation had no detectable effect on 7S RNA levels (Fig. 1) or expression of the polymerase β gene (data not shown).

Interaction Between TNF-α and X-Irradiation. To investigate the influence of TNF-α on radiation-induced cytotoxicity in TNF-α-producing cell lines, recombinant human TNF-α was added to cultures before irradiation (Fig. 2). Recombinant human TNF-α (1000 units/ml) (2.3×10^6 units/mg) is cytotoxic to four of five TNF-α-producing sarcomas (STSAR-5, -13, -33, and -43). The plating efficiency (PE) is reduced by 60–90% at 1000 units/ml in these lines. Radiation-survival analysis of cell line STSAR-33 was performed with TNF-α (10 units/ml). The radiosensitivity (D_0), defined as the reciprocal of the terminal slope of the survival curves is 80.4 cGy for cell line STSAR-33. When TNF-α is added 20 hr before irradiation, the D_0 is 60.4 cGy. Surviving fractions are corrected for the reduced PE with TNF-α. Thus, the interaction between TNF-α and radiation in STSAR-33 cells is synergistic (25) (Fig. 2*A*). Sublethal concentrations of TNF-α (10 units/ml) enhance killing by radiation in cell line STSAR-33, suggesting a radiosensitizing effect of TNF-α. The surviving fraction of cell line STSAR-5 at 100–700 cGy is lower than expected by the independent killing of TNF-α and x-rays, although the D_0 values are similar (data not shown). Thus, the interaction between TNF-α and radiation is additive (25) in STSAR-5 cells. Cell lines STSAR-13 and STSAR-43 are independently killed with x-rays and TNF-α, and no interaction was observed (data not shown).

To determine the possible interactions between TNF-α and x-rays in non-TNF-α producing cells, human epithelial tumor cells (SQ-20B and HNSCC-68) were irradiated 20 hr after TNF-α was added. These cell lines do not produce TNF-α in response to ionizing radiation. TNF-α (1000 units/ml) is cytotoxic to SQ-20B and SCC-61 cells, reducing the PE by 60–80%. The radiation survival of SQ-20B cells with and without TNF-α is shown in Fig. 2*B*. The D_0 for cell line SQ-20B is 239 cGy. With TNF-α (1000 units/ml) added 24 hr before x-rays, the D_0 is 130.4 cGy. Therefore, a synergistic interaction (25) between TNF-α and x-rays is demonstrated in this cell line. TNF-α added after irradiation did not enhance cell killing by radiation in cell line SQ-20B. Nonlethal concentrations of TNF-α (10 units/ml) result in enhanced radiation killing in cell line HNSCC-68 (Fig. 2*C*), suggesting that TNF-α may sensitize some epithelial as well as mesenchymal tumor cell lines to radiation.

DISCUSSION

DNA-damaging agents other than ionizing radiation have been observed to induce expression of a variety of prokaryotic and mammalian genes (26, 27). However, the TNF-α gene is the only mammalian gene found to have increased expression after exposure to ionizing radiation. Studies comparing transcription in DNA repair-deficient cells to that of normal cells suggest that damaged DNA may be the event that triggers transcription (26). This situation is analogous to the proposed mechanism for x-ray induction of some genes after the appearance of DNA-strand breaks in prokaryotes (18). Activation of the TNF-α gene by ionizing radiation may be a model for the study of x-ray-induced gene expression in higher eukaryotes. Induction of TNF-α gene expression in some human sarcoma cell lines by x-irradiation is similar to that seen in human monocytes after exposure to tetradecanoylphorbol acetate and lipopolysaccharide (19).

TNF-α causes hydroxyl radical production in cells sensitive to killing by TNF-α (9). Cell lines sensitive to the oxidative damage produced by TNF-α have diminished radical-buffering capacity after TNF-α is added (8). Lower levels of hydroxyl radicals have been measured in cells resistant to

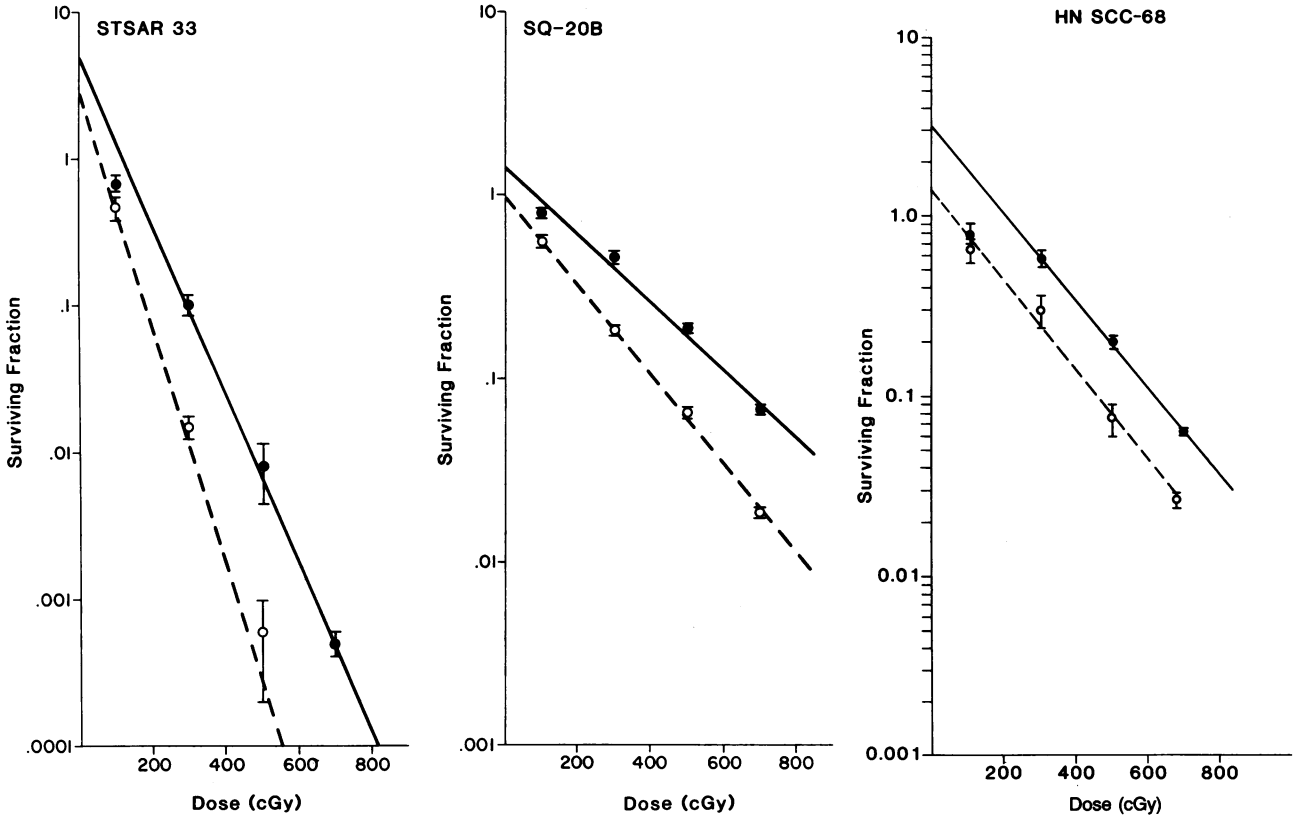

FIG. 2. Influence of TNF-α on radiation lethality of TNF-α-producing human sarcomas and TNF-α-nonproducing human tumor cells. Solid lines indicate radiation alone, and dashed lines indicate TNF-α and irradiation. (*A*) Representative survival data for cell line STSAR-33. Lower dashed line represents survivals with TNF-α at 1000 units/ml, corrected for a PE of 30%. (*B*) Human epithelial tumor cells (SQ-20B) irradiated with TNF-α (10 units/ml and 1000 units/ml). Survival data for SQ-20B show an additive effect of TNF-α (1000 units/ml). Survivals with TNF-α are corrected for 85% killing with TNF-α alone. (*C*) Radiation survival data for HNSCC-68. A nonlethal dose of TNF-α (10 units/ml) was added 24 hr before irradiation.

TNF-α cytotoxicity when compared with cells sensitive to TNF-α killing (9). Thus, the interaction between TNF-α and ionizing radiation seen herein may be from saturation of the radical-scavanging systems within the cell. Cells that do not exhibit interactive killing between TNF-α and x-rays may be inherently more resistant to oxidative damage. This concept is further supported by the fact that TNF-α-producing sarcoma cells (D_0 = 110.5 ± 10.3 SEM) are more radiosensitive than a group of squamous cell carcinoma cell lines (D_0 = 178.6 ± 13) and normal human fibroblasts (D_0 = 146 ± 5) previously measured in our laboratory (20, 21, 28). Non-TNF-α-producing sarcoma cell lines have a D_0 of 139 ± 15.

Increased TNF-α production by human sarcomas after x-irradiation is interesting because of the direct cytotoxic effects of this polypeptide on human tumor cells (3, 4). We propose that the intracellular production of TNF-α within irradiated tumor cells may result in lethality after x-ray exposure that is greater than killing produced by the direct effects of ionizing radiation alone. Cells that produce TNF-α after radiation exposure may also potentiate radiation damage in adjacent cells. Further evidence to support paracrine killing by TNF-α production is the observation that the antibody to TNF-α reversed the reduction of the PE of TNF-α-sensitive tumor cells when conditioned medium from irradiated cell cultures was added. The production of a protein (TNF-α) that affects cell survival after exposure to ionizing radiation by possible autocrine and paracrine mechanisms (in addition to the direct cytotoxic effects of x-rays) is an emerging concept in this study of x-ray-induced lethality.

The additive and synergistic effects of TNF-α on tumor killing by radiation suggest potential applications for the use of TNF-α in clinical radiotherapy. TNF-α potentiates the cellular immune response (1, 29). *In vivo* studies have shown that TNF-α enhances tumor control by x-rays in mice with implanted syngeneic tumors by the augmentation of the host's immune system (29). Therefore, TNF-α may reverse immune suppression, which may accompany radiotherapy. TNF-α causes proliferation of fibroblasts and endothelial destruction, suggesting that TNF-α production by tumors may be one component responsible for the late radiation effects in surrounding normal tissue. Further investigation is needed to determine the precise role of TNF-α in radiation-induced tumor cell death and the long-term effects of radiation on normal tissues.

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