[CANCER RESEARCH 64, 7985–7994, November 1, 2004]

Sublethal Irradiation of Human Tumor Cells Modulates Phenotype Resulting in Enhanced Killing by Cytotoxic T Lymphocytes

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ABSTRACT

Local radiation of tumor masses is an established modality for the therapy of a range of human tumors. It has recently been recognized that doses of radiation, lower than or equal to those that cause direct cytolysis, may alter the phenotype of target tissue by up-regulating gene products that may make tumor cells more susceptible to T-cell–mediated immune attack. Previously, we demonstrated that radiation increased Fas (CD95) gene expression in carcinoembryonic antigen (CEA)-expressing murine tumor cells, which consequently enhanced their susceptibility to CEA-specific CTL-mediated killing. The present study was designed to determine whether these phenomena also occur with human tumor cells. Here, 23 human carcinoma cell lines (12 colon, 7 lung, and 4 prostate) were examined for their response to nonlytic doses of radiation (10 or 20 Gy). Seventy-two hours postirradiation, changes in surface expression of Fas (CD95), as well as expression of other surface molecules involved in T-cell–mediated immune attack such as intercellular adhesion molecule 1, mucin-1, CEA, and MHC class I, were examined. Twenty-one of the 23 (91%) cell lines up-regulated one or more of these surface molecules postirradiation. Furthermore, five of five irradiated $CEA^+/A2^+$ colon tumor cells lines demonstrated significantly enhanced killing by CEA-specific HLA-A2–restricted $CD8^+$ CTLs compared with nonirradiated counterparts. We then used microarray analysis to broaden the scope of observed changes in gene expression after radiation and found that many additional genes had been modulated. These up-regulated gene products may additionally enhance the tumor cells' susceptibility to T-cell–mediated immune attack or serve as additional targets for immunotherapy. Overall, the results of this study suggest that nonlethal doses of radiation can be used to make human tumors more amenable to immune system recognition and attack and form the rational basis for the combinatorial use of cancer vaccines and local tumor irradiation.

INTRODUCTION

Recent studies have demonstrated that an enhancement in the immune system's response to tumor antigens can be achieved using various cancer vaccines targeting tumor-associated antigens (TAAs; ref. 1). It is becoming clear, however, that neoplastic cells may evade the adaptive immune system by altering expression of specific molecules on the target cell. Fas expression on tumors is often used by activated Fas ligand-expressing CTLs to directly kill tumor targets (2). MHC class I is responsible for direct presentation of tumor antigen peptides to CTLs via peptide-MHC complexes, and intercellular adhesion molecule 1 (ICAM-1) has been implicated in enhancing T-cell ability to kill targets because of better cell-to-cell adhesion (3, 4). ICAM-1 can also function to directly costimulate activated T cells (5). Additionally, carcinoembryonic antigen (CEA) and mucin-1 (MUC-1) are overexpressed on a wide variety of tumor cells *in vivo*, including prostate, colorectal, and lung carcinomas (6); subsequently, they are being used as tumor-associated antigenic targets for vaccine-mediated immunotherapies (7–12). Therapies aimed at modulating each of these molecules in tumors, used in combination with immunotherapies, are therefore potentially an important approach to cancer treatment. Cytokines, chemotherapy agents and radiation have all been examined for this purpose (6, 13–15).

Radiation is the standard of care for many cancers and has conventionally been exploited for its direct cytotoxic effect on tumors or palliative effects in patients. Interestingly, numerous classes of genes have been reported to be modulated after irradiation of both murine and human tissues (16) and has led to our interest in the use of radiation therapy of tumors in combination with active immunotherapy approaches. Recent preclinical work from our laboratory has shown that irradiation of murine tumors enhanced their ability to be killed by specific CTLs in a Fas-dependent mechanism (6).

Although human colon, lung, and prostate are among the most prevalent cancers, very few studies, typically limited to one or two cell lines, have focused on the phenotypic changes in response to irradiation (17–22). In addition, most of these studies examined changes in only one or two surface molecules. Less than one third of the cell lines used in this study have been examined by others in similar phenotypic assays (16). In fact, to our knowledge, none have reported the functional significance, as it relates to CTLs, of phenotypic changes observed after irradiation of human colon, lung, or prostate carcinoma.

Here, we examine the effects of sublethal radiation on molecules that have each been implicated in enhancing T-cell–mediated tumor cell killing through diverse mechanisms: functional Fas, the cell adhesion/costimulatory molecule ICAM-1, MHC class I, and the human carcinoma-associated antigens CEA and MUC-1. Results of this study also examine whether changes induced by irradiation of tumor cells could improve CTL-mediated tumor killing. Lastly, microarray analysis of colorectal tumor cells also revealed changes in numerous additional genes that could serve as additional potential targets for immunotherapy.

Overall, these studies show, for the first time (1), a comparison of the effects of nonlethal radiation on surface molecule expression in a large panel of human colon, lung, and prostate carcinoma cell lines (2), the use of radiation to functionally enhance Ag-specific CTL-mediated killing in several human cell lines, and (3) the use of microarray to identify additional potential immune targets enhanced by irradiation. Thus, these findings have potentially important implications on the combined use of local tumor irradiation and cancer immunotherapies in the clinic.

MATERIALS AND METHODS

Tumor Cell Lines. Human colorectal carcinoma (Caco-2, HCT116, WiDr, HT-29, LS 174T, SW1463, SW403, SW480, SW620, T84, LoVo, and COLO 205), lung carcinoma (A549, SK-LU-1, SW900, HLF-a, NCI-H23, NCI-H647, and Calu-1), and prostate carcinoma (22Rv1, DU 145, PC-3, and LNCaP) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in media designated by American Type Culture Collection for propagation and maintenance. Cells were incubated at 37°C incubator with 5% CO_2.

Tumor Irradiation. Human tumor cells were harvested while in log-growth phase. Tumor cells, in suspension, were placed on ice and irradiated (0

Received 4/30/04; revised 7/7/04; accepted 8/24/04.

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to 20 Gy) by a cesium-137 source (Gammacell-1000; AECL/Nordion, Kanata, Ontario, Canada) at a dose rate of 0.74 Gy/min. Control samples were also placed on ice but not irradiated. Both irradiated and nonirradiated cells were then washed in fresh media and seeded in 75-cm^2 tissue culture flasks. After 72 hours, cells were harvested for surface molecule analysis by flow cytometry.

Flow Cytometric Analysis. Cell surface staining of tumor cells was done with the following primary-labeled monoclonal antibodies: CD95-FITC, CD54-PE, CD66-FITC, COL-1-FITC (23), CD227-FITC, HLA-ABC-PE, and the appropriate isotype matched controls. 7-amino actinomycin D staining was used as a measure of cell death following the manufacturer's instructions. All antibodies, with the exception of COL-1, were purchased from BD PharMingen (San Diego, CA). Stained cells were acquired on a FACScan flow cytometer using CellQuest software (BD PharMingen). Isotype control staining was <5% for all samples analyzed. Dead cells were excluded from the analysis based on scatter profile.

Functional Fas Assay. Human tumor cells were nonirradiated (0 Gy) or irradiated (10 and 20 Gy) and recultured. After 72 hours, cells were harvested and counted. A total of 2×10^6 tumor cells was labeled with 75 μCi ^{111}Indium Oxine (Amersham Health, Silver Spring, MD) for 30 minutes at 37°C. Cells were washed and subsequently incubated for 18 hours with varying concentrations of anti-Fas antibody, clone CH11 (MBL, Watertown, MA). Control cells were incubated with IgM isotype control antibody (BD PharMingen), and Jurkat cells were used as a positive control for Fas-mediated cytotoxicity. Functional Fas was determined as described previously (6).

CTL Cell Line. The A2-restricted, CEA-specific, $CD8^+$ CTL line, designated V8T, recognizes the CEA peptide epitope YLSGANLNL (CAP-1; refs. 24, 25).

Cytotoxicity Assays. V8T cells were used on day 4 of the restimulation cycle after Ficoll purification. Irradiated (10 Gy) and nonirradiated (0 Gy) human tumor cells were cultured for 72 hours and subsequently used as targets in a standard cytotoxicity assay using indium-111. A total of 2×10^3 radiolabeled tumor cells were incubated with 6×10^4 V8T CTLs (E:T of 30:1) for 18 hours at 37°C with 5% CO_2. Targets and CTLs were suspended in complete medium supplemented with 10% human AB serum in 96-well U-bottomed plates (Costar, Cambridge, MA). After incubation, supernatants were collected and assayed as above.

RNA Isolation. WiDr (human colon carcinoma) cells in suspension were irradiated (20 Gy) or nonirradiated (0 Gy) on ice and reseeded in 150-cm^2 tissue culture flasks. After 8 and 24 hours, cells were harvested from flasks, and total RNA was extracted and purified from 5×10^7 cells with the RNeasy midi kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

cDNA Microarray. Dual-color microarray hybridizations were performed as follows: RNA isolated from irradiated (20 Gy) WiDr cells was compared with RNA isolated from nonirradiated WiDr cells at two different time points after irradiation (8 and 24 hours). Analysis was carried out as previously described (26). Genes were considered to be up-regulated or down-regulated, after irradiation, if their normalized intensity ratio was ≥2 or ≤0.5 (2-fold cutoff) and ≥3 or ≤0.33 (3-fold cutoff). Hierarchical clustering (27) was applied to those genes that had a normalized intensity ratio ≥3 or ≤0.33. Pearson correlation was used as distance matrix.

RESULTS

Tumor Irradiation Alters Surface Protein Expression. We examined whether sublethal doses of radiation altered surface molecule expression in a panel of 23 human carcinoma cell lines. Tumor cells were subjected to 0, 10, and 20 Gy of radiation, and cell surface expression of Fas, ICAM-1, CEA, MUC-1, and MHC class I molecules was monitored by flow cytometry after 72 hours. For these analyses, the population of cells positive for isotype control staining never exceeded 5%. Cell growth was minimally slowed in some but not all cell lines during the 3 days after irradiation. This cytostatic effect of radiation typically resulted in a 25% decreased yield in the number of cells harvested from the irradiated flasks compared with the nonirradiated flasks. No significant increases in dead cells were observed with these doses of radiation as determined by 7-amino actinomycin D dye uptake and trypan blue staining (data not shown). Radiation survival curves were carried out for 14 days on HCT116 and WiDr cells. Although growth of cells with or without radiation treatment was the same for 72 hours, at day 7, the growth of the irradiated cells decreased. However, the cells remained viable for a total of 14 days as analyzed by vital staining, confirming that these radiation doses were sublethal. These doses of irradiation, however, did induce enhanced expression of surface proteins in a dose-dependent manner. HCT116 human colon carcinoma cells, for example, increased surface Fas from 39% when nonirradiated, up to 89 and 97% after 10 and 20 Gy of irradiation, respectively. Likewise, surface ICAM-1 increased, in terms of percentage of cells expressing this molecule, from 51% without irradiation up to 72% (10 Gy) and 78% (20 Gy) after irradiation. In addition, MHC class I increased in surface density after radiation with a change in mean fluorescence intensity from 247 without radiation up to 624 after 20 Gy of radiation. No changes in surface MUC-1 were observed after irradiation of HCT 116 cells, whereas a slight increase in CEA expression was observed. For this and subsequent analysis, proteins were reported as up-regulated if detection levels were increased by ≥10% or if the mean fluorescence intensity doubled after irradiation. To confirm that increased cell surface levels of proteins reflected an increase in overall levels rather than increased movement to the cell surface, WiDr and HCT116 cells were subjected to 0 and 20 Gy of radiation, and protein extracts were made after 72 hours. CEA protein levels were quantified by enzyme linked immunosorbant assay. The detection limit for CEA protein was 1 ng/mL. For both cell lines, the CEA level was undetectable before radiation. After radiation, for WiDr cells the CEA level was 90 ng/10^6 cells, and for HCT116, the CEA level was 85 ng/10^6 cells.

The results obtained using 11 additional human colorectal tumor cell lines, 7 human lung cell lines, and 4 human prostate cell lines are summarized in Fig. 1. In total, 21 of 23 (91%) human tumor cell lines responded to low-dose irradiation by up-regulating one or more surface molecules (Fig. 1*A–C*). Of the colorectal cells lines tested, 12 of 12 up-regulated at least one of the monitored surface proteins (Fig. 1*A*). CEA was up-regulated most often, and an increase in this molecule was observed in 83% of these lines. In the lung tumor lines, CEA and ICAM-1 were the molecules increased most often after radiation (Fig. 1*B*); two lung cancer cell lines, HLF-a and Calu-1, showed no up-regulation after radiation. Taken as a whole, prostate cell lines were the least altered after irradiation. Although each prostate line did respond by up-regulating at least one protein, no single line responded by increasing more than three proteins, as was observed in both colon and lung carcinomas. In addition, none of the prostate carcinomas showed an increase in surface MHC class I expression. Only one cell line, colon carcinoma WiDr, responded to irradiation by up-regulating all five of the monitored surface molecules. Overall, ICAM-1 and CEA, up-regulated 61 and 69%, respectively, were the most commonly up-regulated molecules. Twenty-six percent up-regulated functional Fas, 74% (17 of 23) up-regulated a TAA (either CEA or MUC-1), and 35% up-regulated MHC class I (Fig. 1). In addition, no clear distinctions, in response to radiation, could be made between lines derived from primary tumor sites and those derived from metastatic sites.

Fas Up-Regulation in Response to Irradiation Is Often Functional. Tumors have numerous escape mechanisms including the down-regulation of MHC class I, Fas receptor down-regulation, or loss of Fas function (28, 29). We thus sought to examine if up-regulated surface Fas in human carcinoma cell lines, as a consequence of radiation, was biologically active. The cell lines that up-regulated Fas after irradiation were HCT116, WiDr, LS 174T, SW480, SW620, LoVo, A549, NCI-H647, 22Rv1, and LNCaP. Each of these 10 lines

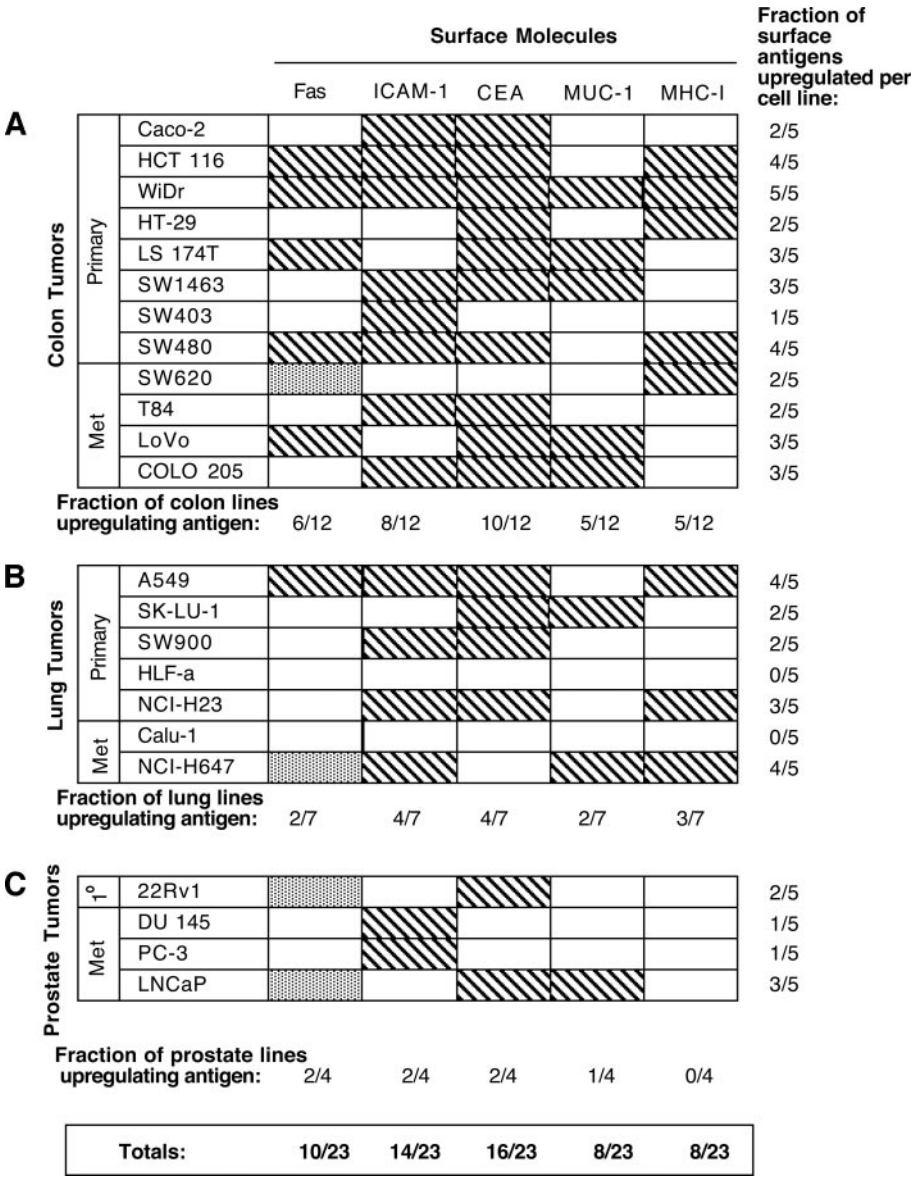


Fig. 1. Multiple changes in surface protein expression in human colon, lung, and prostate cell lines after irradiation. Twenty-three human colon (*A*), lung (*B*), and prostate (*C*) tumor cell lines received 0, 10, and 20 Gy of radiation. Cells were recultured for 72 hours and then analyzed by flow cytometry for Fas, ICAM-1, CEA, MUC-1, and MHC class I surface expression. Cell surface changes after irradiation are represented. Increased cell surface levels, of ≥10% (or a doubling of mean fluorescent intensity not observed in isotype control) after irradiation, are indicated by ▧. Increased surface Fas after irradiation, which was not functional as defined by antibody cross-linking assay, is shown as *shaded boxes*. Protein expression that remained unchanged after irradiation is indicated by □. MHC-I, MHC class I; Met, metastasis; 1°, primary.

was assayed for functional Fas with an agonistic antibody cross-linking assay. Tumor cells were subjected to 0, 10, and 20 Gy of radiation and then incubated, 72 hours later, in the presence of anti-Fas monoclonal antibody clone CH11 as described in the Materials and Methods. A549 and LNCaP cells are shown in Fig. 2 as Fas functional and nonfunctional examples, respectively. Fas-sensitive Jurkat cells were used as a positive control; the antibody efficiently mediated lysis of 45% of these cells in an 18-hour assay. Overall, 6 of 10 cell lines that up-regulated surface Fas after irradiation underwent enhanced lysis (ranged from 35 to 65%) in response to anti-Fas

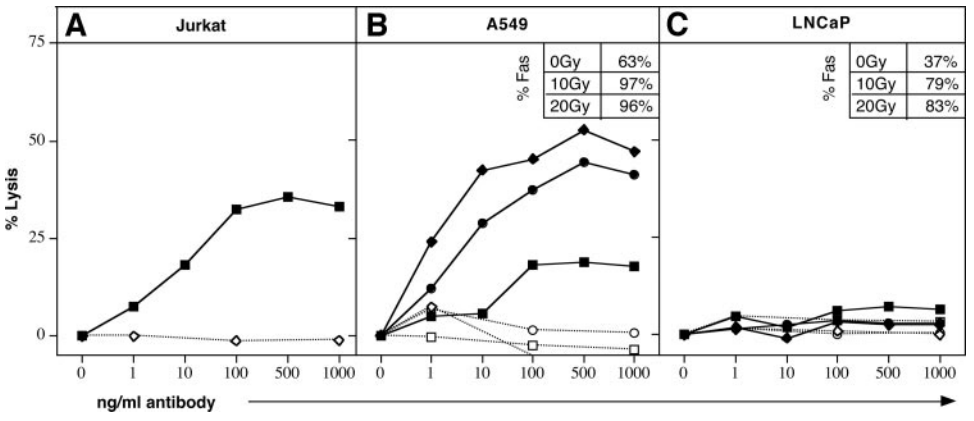
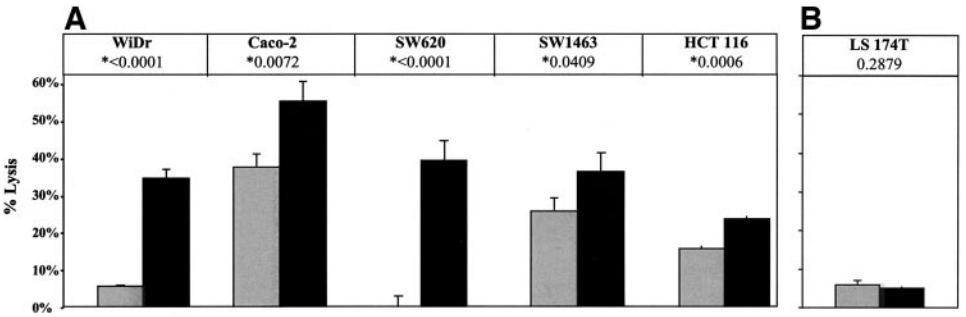


Fig. 2. Functional analysis of up-regulated surface Fas after irradiation. Human tumor cells were mock-irradiated (*squares*) or irradiated with 10 Gy (*diamonds*) or 20 Gy (*circles*) and recultured for 72 hours. Cells were harvested, labeled with ^{111}In, and incubated with the indicated concentrations of Fas cross-linking antibody CH11 (*closed symbols*), or IgM isotype control antibody (*open symbols*) for 18 hours. *Inset*, surface Fas levels, as determined by flow cytometry of unlabeled cells.



C

Cell line		Fas	Funct'l	ICAM-1	CEA	MUC-1	MHC-1	HLA-A2
		0Gy/10Gy		0Gy/10Gy	0Gy/10Gy	0Gy/10Gy	0Gy/10Gy	
WiDr	%+:	**39%/86%**	Yes	**62%/74%**	**17%/46%**	11%/12%	99%/99%	+
	(MFI):	(19/138)		(38/232)	(82/127)	**(21/140)**	**(189/612)**	
Caco-2	%+:	5%/8%	N/A	95%/94%	80%/84%	11%/18%	97%/93%	+
	(MFI):	(34/42)		**(192/356)**	**(347/638)**	(75/108)	(247/400)	
SW620	%+:	**10%/24%**	No	91%/78%	94%/83%	69%/60%	99%/98%	+
	(MFI):	(11/19)		(50/57)	(19/37)	(23/36)	**(92/177)**	
SW1463	%+:	5%/13%	N/A	**27%/61%**	**18%/28%**	**17%/27%**	98%/99%	+
	(MFI):	(46/43)		(90/102)	(90/90)	(75/96)	(541/628)	
HCT116	%+:	**38%/89%**	Yes	**51%/72%**	25%/28%	4%/7%	99%/99%	+
	(MFI):	(34/179)		(40/160)	**(61/182)**	(59/100)	**(247/616)**	
LS174T	%+:	**36%/85%**	Yes	12%/8%	56%/61%	8%/13%	95%/91%	-
	(MFI):	(34/74)		(27/50)	**(78/273)**	**(48/116)**	(69/128)	

Fig. 3. Irradiation increases tumor cell sensitivity to Ag-specific cytotoxic T-cell killing. CEA-positive tumor cells were mock-irradiated (▒) or irradiated with 10 Gy (■) and recultured for 72 hours (*A* and *B*). Cells were then labeled with ^{111}In and were co-incubated with HLA-A2–restricted CEA-specific CTLs for 18 hours at an E:T ratio of 30:1. LS 174T (*B*) is a CEA-positive, HLA-A2–negative cell line and is shown as a negative control. All other cell lines shown were both CEA and HLA-A2 positive. * denotes statistical significance. *C*, numbers indicate % positive and those in parentheses indicate mean fluorescent intensity of cells expressing molecule on cell surface after receiving 0 Gy/10 Gy of radiation, respectively. Numbers in bold represent molecules classified as up-regulated after irradiation.

stimulation when compared with their nonirradiated counterparts (Fig. 1, hatched boxes). Interestingly, Fas up-regulation following radiation did not always result in increased Fas-mediated lysis. Specifically, A549 and LNCaP cells both up-regulated Fas to similar levels (Fig. 2); however, only A549 cells were killed upon surface Fas cross-linking. No lysis was observed when irradiated cells were incubated in the presence of an isotype-matched control IgM antibody (Fig. 2, open symbols). Functional Fas was subsequently confirmed, in selected cell lines, by active caspase-3 detection after Fas receptor cross-linking (data not shown).

Irradiation Increases Tumor Cell Sensitivity to Ag-specific Cytotoxic T-Cell Killing. To determine the functional significance of phenotypic alterations after irradiation of tumor cells, we examined tumor susceptibility to CTL lysis with and without irradiation of human target cells. We previously generated a human CTL line from a patient vaccinated with a CEA-based vaccine (24). These CTLs recognize a HLA-A2–restricted epitope on human CEA-expressing carcinoma cells. Colon cell lines positive for HLA-A2 and CEA were thus irradiated and incubated with the CEA-specific CTLs as described in Materials and Methods. Five of five HLA-A2–positive, CEA-positive tumor cell lines demonstrated significantly enhanced killing by CTLs after 10 Gy of irradiation when compared with their nonirradiated counterparts (Fig. 3). LS 174T is a CEA-positive, HLA-A2–negative tumor that greatly up-regulated surface Fas in response to radiation. This cell line, however, was not killed by the HLA-A2–restricted, CEA-specific CTLs used in these experiments and was included as a negative control (Fig. 3*B*). Enhanced CTL killing was not always linked to Fas up-regulation in this study. For example, two of the five cell lines (Caco-2 and SW1463) showed significantly enhanced killing after irradiation, although they did not up-regulate surface Fas after irradiation (Fig. 3). Furthermore, SW620 up-regulated surface Fas that was not functional, yet these cells demonstrated the most significant enhancement of killing by CTLs. In fact, of the five molecules observed, MHC class I was the only other molecule observed to increase after irradiation of this cell line. Although it is possible that the five molecules examined could contribute to enhanced CTL killing of tumor targets, it is also likely that many other proteins could contribute to these observations.

Microarray Reveals Numerous Additional Genes Differentially Expressed after Irradiation of Tumor Cells. Studies were then undertaken to broaden the scope of examined genes beyond Fas, ICAM-1, CEA, MUC-1, and MHC class I. We used cDNA microarrays to compare changes in gene expression patterns of the colon carcinoma cell line (WiDr) with and without 20 Gy of radiation, as described in Materials and Methods. In an attempt to recognize early-response *versus* long-term radiation-response genes, RNA from irradiated WiDr cells was prepared at 8 and 24 hours after irradiation and then compared with RNA from nonirradiated WiDr cells. Using a Hs-UniGEM2 chip, quantitative analysis of the difference in gene expression of 9000 genes, between irradiated and nonirradiated WiDr cells, resulted in a total of 104 genes up-regulated by at least 2-fold 8 hours after irradiation. In contrast, 465 genes have at least 2-fold altered mRNA levels in response to radiation at a later time point of 24 hours. Of these genes, 348 genes (75%) were up-regulated at least 2-fold in response to radiation, and 117 (25%) of these genes were repressed at least 2-fold after irradiation of WiDr cells.

Additional analysis focused on those genes that exhibited a change in expression of at least 3-fold, which corresponded to 22 genes up-regulated at 8 hours after irradiation and 90 genes differentially expressed at 24 hours after irradiation. A hierarchical clustering has placed these 90 genes into two groups (Fig. 4, *A* and *B*). Cluster A contains 84 genes up-regulated after irradiation, and cluster B contains the only six genes whose expression was repressed at 24 hours after irradiation. The set of 90 genes that was differentially expressed at 24 hours included very few genes whose expression was also modified at a shorter time after irradiation, suggesting that many of those 90 genes belong to a category of late-responsive genes because their RNA levels were only modified at the later time point. Exceptions were *CDKN1A*, *CTBS*, and *FSLT3* genes that were also induced at least 3-fold at 8 hours after irradiation.

All genes that had at least a 3-fold difference in expression at either 8 or 24 hours after irradiation were then grouped in accordance with their presumed function, as shown in Table 1. Also shown in the table is the normalized relative intensity ratio for each individual gene. Of a total of 109 genes differentially expressed at 8 or 24 hours, 10 genes encoded proteins that participate in the control of cell cycle and cell

Fig. 4. Hierarchical cluster of differentially expressed genes in irradiated *versus* nonirradiated colon carcinoma (WiDr) cells. WiDr cells were irradiated (20 Gy) or nonirradiated (0 Gy). After 8 and 24 hours, a cDNA microarray was performed. Genes were considered differentially expressed if their levels of expression differed at least 3-fold. Each row represents a particular gene, each column indicates an individual experiment. The corresponding levels of expression at 8 hours after irradiation are also shown for each gene.

proliferation. Among them was *CDKN1A* (*p21*), which is p53 dependent and was previously described as one of the acute-response genes in irradiation responses (30). *p21* was detected early and was 12-fold up-regulated at 8 hours after irradiation and maintained its high level of expression in irradiated cells at 24 hours after irradiation. A group of seven genes encoding for apoptosis-related proteins was also differentially expressed after irradiation, among them the apoptosis inhibitors *Bcl-2* and *MCL-1* (a *Bcl-2*–related gene) were both 3.3-fold

Table 1 *Genes differentially expressed after irradiation of WiDr cells, organized by function*

Gene name	Description	8 hours	24 hours
Cell cycle/cell proliferation control			
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	11.9	10.6
FSTL3	Follistatin-like 3 (secreted glycoprotein)	3.1	3.2
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate	4.0	
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250 kDa	3.7	
CNK	Cytokine-inducible kinase		7.2
FRAP1	FK506 binding protein 12-rapamycin associated protein 1		4.2
CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2		4.2
NDN	Necdin homologue (mouse)		3.9
PPP6C	Serine/threonine phosphatase catalytic subunit		3.5
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs		0.3
Apoptosis			
PTPN6	Protein tyrosine phosphatase, nonreceptor type 6		4.0
TRAF2	TNF receptor-associated factor 2		3.8
PLAB	Prostate differentiation factor		3.7
SPTAN1	Spectrin, α, nonerythrocytic 1 (α-fodrin)		3.3
SERPINB6	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6		3.6
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)		0.3
BCL2	B-cell CLL/lymphoma 2		0.3
Signaling pathways			
STC2	Stanniocalcin 2		4.4
RGS19IP1	Regulator of G-protein signalling 19 interacting protein 1		3.3
MAP3K11	Mitogen-activated protein kinase kinase kinase 11		3.2
AKT2	v-akt murine thymoma viral oncogene homologue 2		3.2
PIK4CB	Phosphatidylinositol 4-kinase, catalytic, beta polypeptide		3.2
MAPKAPK5	Mitogen-activated protein kinase-activated protein kinase 5		3.2
RPS6KB2	Ribosomal protein S6 kinase, 70 kDa, polypeptide 2		3.1
PTK9L	PTK9L protein tyrosine kinase 9-like (A6-related protein)		3.1
DNA repair			
MGMT	*O*-6-methylguanine-DNA methyltransferase		6.1
MPG	*N*-methylpurine-DNA glycosylase		4.9
C11orf13	Chromosome 11 open reading frame 13		4.0
DDB2	Damage-specific DNA binding protein 2, 48 kDa		3.8
Transcription/chromatin remodeling			
TGIF	TGFB-induced factor (TALE family homeobox)	4.3	
POLRMT	Polymerase (RNA) mitochondrial (DNA directed)	3.8	
SUPT6H	Suppressor of Ty 6 homologue (*S. cerevisiae*)	3.5	
REL	v-rel reticuloendotheliosis viral oncogene homologue (avian)	3.5	
TNKS1BP1	Tankyrase 1 binding protein 1, 182 kDa		4.6
CGI-18	CGI-18 protein		4.2
ZNF385	Zinc finger protein 385		3.9
SREBF2	Sterol regulatory element binding transcription factor 2		3.4
CTDP1	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase 1		3.3
E4F1	E4F transcription factor 1		3.0
IRX5	Iroquois homeobox protein 5		0.3
HESX1	Homeobox (expressed in ES cells) 1		0.3
RNA processing/protein modification and turnover			
RNU3IP2	RNA, U3 small nucleolar interacting protein 2	3.8	
RABGGTA	Rab geranylgeranyltransferase, α subunit		3.8
Receptor/signal transduction			
BZRP	Benzodiazapine receptor (peripheral)	4.0	
GPC1	Glypican 1	3.3	
RAMP1	Receptor (calcitonin) activity modifying protein 1		4.9
MTVR1	Mouse mammary turmor virus receptor homologue 1		4.5
CD40	Tumor necrosis factor receptor superfamily, member 5 (TNFRSF5)		4.5
PL6	Placental protein 6		3.3
ADORA2B	Adenosine A2b receptor		3.2
Transporters			
TETRAN	Tetracycline transporter-like protein	4.2	
STX8	Syntaxin 8		4.3
SLC12A3	Solute carrier family 12 (sodium/chloride transporters), member 3		3.9
EVER1	Epidermodysplasia verruciformis 1		3.6
PEX10	Peroxisome biogenesis factor 10		3.5
SLC9A3R2	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 2		3.4
AP4M1	Adaptor-related protein complex 4, μ 1 subunit		3.4
SCNN1A	Sodium channel, nonvoltage-gated 1 α		3.0
SLC35D2	Solute carrier family 35, member D2		3.3
Oxidoreductase activity			
COX8	Cytochrome *c* oxidase subunit VIII	3.9	
FDXR	Ferredoxin reductase		4.7
LEPREL2	Leprecan-like 2 protein		4.2
GPX2	Glutathione peroxidase 2 (gastrointestinal)		3.3
H105E3	NAD(P) dependent steroid dehydrogenase-like		3.2
RDH10	Retinol dehydrogenase 10 (all-trans)		0.3
Metabolism			
CTBS	Chitobiase, di-N-acetyl-	6.4	8.7
CTSS	Cathepsin S		4.1
TST	Thiosulfate sulfurtransferase (rhodanese)		3.8
MAN1B1	Mannosidase, α, class 1B, member 1		3.7
FDFT1	Farnesyl-diphosphate farnesyltransferase 1		3.7
DPYSL3	Dihydropyrimidinase-like 3		3.6
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1		3.6

Table 1 *Continued*

Gene name	Description	8 hours	24 hours
Metabolism			
SGSH	*N*-sulfoglucosamine sulfohydrolase (sulfamidase)		3.3
PYGB	Phosphorylase, glycogen; brain		3.3
PEPD	Peptidase D		3.2
GYG	Glycogenin		3.1
ALDH6A1	Aldehyde dehydrogenase 6 family, member A1		3.0
Cell adhesion			
LMAN2	Lectin, mannose-binding 2	3.9	
NINJ1	Ninjurin 1		4.1
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor		3.5
CLC	Charot-Leyden crystal protein		3.3
Cytoskeleton/structural molecule			
PCM1	Pericentriolar material 1	3.8	
ABLIM1	Actin binding LIM protein 1	3.8	
BICD1	Bicaudal D homologue 1 (*Drosophila*)	3.1	
EVPL	Envoplakin		3.4
INA	Internexin neuronal intermediate filament protein, α		3.3
Tumorigenesis			
HSRTSBETA	rTS beta protein		4.2
PLAU	Plasminogen activator, urokinase		3.6
KLK10	Kallikrein 10		3.2
EPHA2	EphA2		3.3
Miscellaneous			
PTD008	PTD008 protein	4.1	
SYNGR2	Synaptogyrin 2	3.6	
HSU15552	Acidic 82 kDa protein mRNA	3.1	
	Homo sapiens, clone IMAGE:4754514, mRNA	3.0	
KIAA0685	KIAA0685		7.8
	Incyte EST		5.1
	Incyte EST		4.9
	Homo sapiens similar to hypothetical protein MGC16385 (LOC376553), mRNA		4.7
	EST, moderately similar to VATM_human vascular ATP synthase subunit H		4.5
20D7-FC4	Hypothetical protein 20D7-FC4		3.9
	Homo sapiens, clone 24694, mRNA		3.6
PP2447	Hypothetical protein PP2447		3.6
FLJ22301	Hypothetical protein FLJ22301		3.6
KIAA0842	KIAA0842 protein		3.6
FLJ13868	Hypothetical protein FLJ13868		3.5
TOM1L2	Target of myb1-like 2 (chicken)		3.4
HSPC242	Hypothetical protein HSPC242		3.2
FLJ10377	Hypothetical protein FLJ10377		3.1
	Incyte EST		3.1

NOTE. Genes whose expression levels differed at least 3-fold between irradiated (at either 8 or 24 hours after irradiation) and nonirradiated WiDr cells were organized by their presumed function. The normalized relative intensity for each gene is indicated only when its value is either ≥ 3 or ≤ 0.33.

repressed at 24 hours postirradiation. Also in this group were *PTPN6* (*Sph-1*), a negative regulator of signaling that is downstream of a wide range of receptors, including cytokine receptors, growth factor receptors, and receptor complexes of the immune system (31), and *PLAB/MIC-1*, a member of the transforming growth factor β superfamily that can block cell proliferation and induce apoptosis (32). As would be expected, a set of genes that participate in the DNA repair process was also up-regulated at 24 hours postirradiation, such as *DDB2* that encodes a p53-inducible damage-specific DNA binding protein. Similarly, oxidative stress genes involved in p53-mediated apoptosis, such as *ferredoxin reductase*, which generates oxidative stress in mitochondria (33), were also differentially expressed 24 hours after irradiation. Additionally, many genes whose expression was modified in response to radiation belonged to the category of receptors, membrane transporters, and adhesion molecules, such as CD40, a member of the tumor necrosis factor receptor superfamily that is known for mediating costimulatory signals to the T cells, as well as for its ability to modulate the viability of various tumor cells (34). Several adhesion molecule-encoding genes were also up-regulated in irradiated WiDr cells, such us *LMAN2*, *ICAM-1*, and *Ninjurin-1*. Included in this cell adhesion category is *Mesothelin*, whose gene expression was increased by 2.5-fold and therefore missed the 3-fold cutoff used in Table 1. This gene is of particular interest because it has recently been identified as a potential target for immune-mediated attack of carcinoma cells (35, 36). The up-regulation of *ICAM-1* gene expression was in accordance with the observations at the protein level observed previously (Fig. 1). Two genes observed as up-regulated by flow cytometry, CEA, and HLA-B were also both up-regulated by microarray analysis (CEA, 1.4-fold; HLA-B, 1.7 fold), however, not to 3-fold levels reported in Table 1. No change in MUC-1 mRNA expression was detectable by microarray. The gene chips used in this assay did not include Fas cDNA. This discrepancy, however, could be due to factors such as RNA turnover and/or stability or the fact that the RNA was analyzed only at 8 and 24 hours after irradiation, whereas the flow cytometry was carried out at 72 hours after irradiation.

DISCUSSION

Each of the five surface molecules monitored in this study (Fas, CEA, MUC-1, ICAM-1, and MHC class I) has been implicated in enhancing antitumor T-cell responses through diverse mechanisms. The Fas receptor is a mediator of apoptosis and is one of the mechanisms used by CTLs to directly kill specific targets (2). ICAM-1 has both cell adhesion and costimulatory molecule properties. It has been demonstrated that increased expression of adhesion molecules on tumor cells correlates with increased T lymphocyte binding and killing of tumor cells (3, 4). In addition, increased ICAM-1 expression could enhance immune destruction via direct costimulation of T cells, thus making tumor cells better immunogens. The tumor-associated molecules (TAA) CEA and MUC-1 are differentially expressed in tumors *versus* normal tissues (17), and MHC class I molecules are important for antigen presentation of tumor molecule epitopes to

circulating T cells (37–39). Several reports have demonstrated that the down-regulation of these genes is a common mechanism used by tumors to escape immune recognition and elimination (28, 29). It therefore seems likely that the up-regulation of these genes by radiation could make tumor cells more susceptible or amenable to attack by the immune system.

Previous studies with human tumor lines have shown that doses of radiation, lower than those that cause direct damage, may up-regulate MHC class I (40–42), tumor-associated molecules (21, 43), adhesion molecules (3, 44), and death receptors (45, 46). Few, however, have focused on the phenotypic changes in colorectal (17–20), lung (21), or prostate (22) human tumor cells' response to irradiation. These studies have typically been limited to one or two cell lines. Specifically, previous phenotypic studies on human colon carcinoma cells' response to radiation *in vitro* have been limited to three cell lines. In addition, each of these studies typically reported on a single surface molecule per cell line (20, 47). In a more recent study, LOVO colon carcinoma cells were observed by protein microarray analysis after receiving 4 to 8 Gy of radiation (19). CEA protein levels were decreased 4 hours after irradiation but were subsequently shown to increase 24 hours after irradiation. We extended our observations to include 12 colon carcinomas in a single study 72 hours after irradiation. Findings reported here confirm and extend those of Sheard *et al.* (18), who demonstrated HCT116 up-regulation of surface Fas 24 hours after irradiation, and the lack thereof in HT-29. In contrast, however, increased MUC-1 expression was not observed by us in HT-29 cells as observed by Kang *et al.* (20). These investigators reported increased MUC-1 expression by flow cytometry with an antibody to the glycosylated form of the protein. This difference in results could be attributed to the monoclonal antibodies used for detection.

In vitro studies of human lung carcinoma cells' response to irradiation are more limited. Matsumoto *et al.* (21) reported CEA expression on GLL-1 cells increased after irradiation at a single dose of 10 Gy, with the peak of CEA expression observed on day 4 after irradiation. One additional study reported enhancement in the expression of MHC class I in cultured human lung A549 cells after low-dose radiation. We examined phenotypic changes in response to radiation in a total of seven lung carcinomas and found that ICAM-1 and CEA were up-regulated most often in response to irradiation.

Phenotypic studies of this kind are currently the most limited for prostate carcinomas. A single study examined the effects of radiation in human prostate carcinoma cells.

In a very recent study, Shimada *et al.* (22) reported that surface Fas expression increased 24 hours after 5 Gy of irradiation. We observed the same modulation of Fas for this cell line and extended observations to three additional prostate carcinomas, as well as examining other surface markers. Although this carcinoma type was the least responsive to tumor irradiation, all four lines did up-regulate at least one surface molecule.

Overall, the data reported here are the first to show a comparison of the effects of nonlethal radiation on surface Fas, ICAM-1, CEA, MUC-1, and MHC class I expression in a large panel of human colon, lung, and prostate tumor cell lines. In contrast to previous observations, here, we extended our phenotypic observations to include, in a single study, five surface molecules and 23 cell lines, representing three categories of cancer. As it has been previously observed that surface molecule up-regulation after irradiation is durable and can be maintained for long periods of time (6), we examined phenotypic changes 72 hours after irradiation. Although the number and magnitude of molecules up-regulated in response to sublethal radiation varied between cell lines, an overwhelming majority (91%) of human tumor lines demonstrated changes in surface molecule expression in response to irradiation. Perhaps not surprisingly, of the 21 that responded by surface molecule up-regulation, no consistent pattern of up-regulation was observed across the group.

Also reported here for the first time is the use of radiation to functionally enhance Ag-specific CTL-mediated killing in numerous human colon cell lines. We observed enhanced Ag-specific killing of human tumor cells by CTL after tumor irradiation. Five of five irradiated $CEA^+/A2^+$ colon tumor cells lines were killed better by CEA-specific HLA-A2–restricted $CD8^+$ CTL than their nonirradiated counterparts. In addition, we additionally demonstrated that we could observe a significant enhancement in killing by CTLs, regardless of the surface molecule expression pattern observed in the five molecules monitored. However, in contrast to *in vivo* and *in vitro* observations in a single murine tumor cell line (6), we show that enhanced sensitivity to CTL attack was not solely dependent on Fas because it did not always correlate with functional Fas up-regulation or high expression levels. In fact, one of the tumor cell lines exhibiting significantly greater lysis by CTL (SW620) expressed surface Fas that was not functional after irradiation. In addition, two cell lines that did not up-regulate Fas after irradiation were subsequently killed better by CTLs. Whether the enhanced killing by CTLs, in these instances, reflects increased presentation of TAAs by MHC class I molecules or increased ICAM expression, resulting in enhanced cell adhesion, is unknown. The enhanced killing could even be a result of other accessory molecules not observed in this study. Observations in this study were extended beyond surface staining and enhanced killing by CTLs to include microarray analysis of a colon carcinoma cell line. To our knowledge, this is the first time a cell line has been examined so extensively after radiation. RNA from the colon carcinoma cell line WiDr was used in these analyses at 8 and 24 hours after irradiation. Using this broad-based approach, we were able to identify additional molecules modulated by radiation that could potentially be targets for immunotherapy or cancer vaccine strategies (Table 2). For instance, numerous up-regulated molecules, such as Ninjurin-1, PLAU, mesothelin, glypican, EphA2, CYP1A1, or HSRTS-β, could serve as potential tumor-associated molecules and targets for tumor-specific CTLs. CD40 up-regulation could serve to enhance costimulatory signals to tumor-specific T cells and as a result stimulate better T-cell killing. The antiapoptotic protein bcl-2 was down-regulated upon tumor irradiation. This alteration could increase cell susceptibility to apoptotic signals given to the tumor, whereas the increase in kallikrein 10 could complement this process by slowing tumor growth. Of particular interest is mesothelin, which has recently been identified as a potential target for immune mediated attack of carcinoma (35, 36). Future studies will explore the functional significance that some of these genes may play in enhanced tumor immunogenicity and sensitization to T-cell attack.

Although this study was conducted with human tumor cell lines *in vitro*, similar results involving up-regulation after radiation have been observed in murine models *in vivo*. Others have looked into the efficacy of radiation therapy in solid tumors. Chakraborty *et al.* (6) examined the effect of localized irradiation of s.c. growing tumors on the efficiency of CTL adoptive immunotherapy in a murine tumor model system. Irradiation caused up-regulation of Fas by these tumors *in situ* and significantly potentiated tumor rejection by Ag-specific CTLs (6). In another study, the dynamic changes in CEA and MUC-1 expression in human colonic xenografts in response to radiation were investigated using radiolabeled antibodies (17). Immunohistology showed that sublethal radiation, delivered by targeted radioantibody therapy, increased CEA expression in HT-29 and LS174T tumor xenografts. This up-regulation was in fact maintained over a 4-week period in HT-29 tumors. An increase in MUC-1 expression was also seen in LS174T tumors by day 5 after radiation therapy. Lastly, it has

Table 2 *Genes with potential therapeutic use after tumor irradiation*

Gene	Function	Potential in enhancement of immunotherapy	Fold
Fas	Apoptosis signalling receptor	Enhanced Fas-mediated killing	2.2†
Bcl-2	Prevents Fas mediated killing	Enhanced Fas-mediated killing	0.3*
PLAB/MIC-1	Induction of apoptosis	Modulate tumor cell death	3.7*
	Tumor growth inhibition	Slowed tumor growth/balance tumor growth and T-cell killing	
	Macrophage inhibitor		
ICAM-1	Costimulation and cell adhesion	Enhanced T-cell binding to tumor targets	1.2†
		Enhanced costimulatory signals to T cells	3.5*
CD40	Costimulation	Direct tumor growth inhibition	4.5*
		Could enhance costimulatory signals to T cells	
CEA	Homophilic adhesion molecule	TAA/CTL target	2.7†
Ninjurin 1	Homophilic adhesion molecule	Potential TAA/CTL target	4.1*
PLAU	Plasminogen activator	Potential TAA/CTL target	3.6*
Mesothelin	Glycoprotein	Potential TAA/CTL target	2.5*
EphA2	Endothelial cell angiogenesis	Potential TAA	3.3*
CYP1A1	Conversion of precarcinogens into carcinogens	Potential TAA	3.6*
Glypican 1	Proteoglycan	Potential TAA	3.3*
HSRTS Beta	Antisense thymidilate synthase	Potential TAA	4.2*
HLA Class I	Presentation of intracellular Ags to class I-restricted T cells	Enhance presentation of TAA epitopes to CTLs	3.2†
Kallikrein 10/NES1	Potential tumor suppressor	Slowed tumor growth/balance tumor growth and T-cell killing	3.2†

NOTE. Summary of genes that demonstrated altered expression by microarray * and/or fluorescence-activated cell sorting† after irradiation (20Gy) of WiDr cells.

been reported that Fas molecule expression was induced by radiotherapy for malignant lymphoma (48). Immunohistochemistry staining was performed on clinical biopsies taken before and after treatment at doses of 4, 10, and 20 Gy. These studies provide support for the idea that tumor cells respond to radiation *in situ* in a manner similar to cells treated *in vitro*.

It is estimated that during their illnesses, 50 to 60% of patients with cancer will undergo radiation therapy, either for cure or palliation (49). Standard of care doses of radiation vary depending upon the cancer being treated, ranging from 45 to 50 Gy for residual prostate tumors to 56 to 60 Gy for lung cancer, both delivered fractionated over a period of weeks (49). However, it is unclear what dose of radiation is given to each tumor cell, and the result may be that some tumor cells are killed while others are phenotypically modified. Although a single, sublethal, low dose of radiation ($\leq$20Gy) was used in this study, previous studies suggest that similar protein level increases are observed at higher doses of radiation (6, 40). In addition, fractionation, as radiation is often given in the clinic, achieved similar response rates both *in vitro* and *in vivo* similar to that of a bolus dose in a preclinical model (6, 47). These results suggest that radiation doses need not necessarily be lowered below those typically used for patient care to benefit from radiation immune enhancing strategies. Results also suggest that radiation could be beneficial for treating tumors along with vaccines for which radiation is currently not the standard of care. Overall, results of this study suggest that nonlethal doses of radiation can be used to make human tumors more amenable to immune system recognition and attack and form the rational basis for the combinatorial use of cancer and vaccines and local tumor irradiation.

ACKNOWLEDGMENTS

We thank Marion Taylor for excellent technical assistance. We also thank Debra Weingarten for her editorial assistance in the preparation of this manuscript.

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Sublethal Irradiation of Human Tumor Cells Modulates Phenotype Resulting in Enhanced Killing by Cytotoxic T Lymphocytes

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Cancer Res 2004;64:7985-7994.