

Combination Chemotherapy and Radiation of Human Squamous Cell Carcinoma of the Head and Neck Augments CTL-Mediated Lysis

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Abstract Purpose: The combination of systemic multiagent chemotherapy (5-fluorouracil + cisplatin) and tumor irradiation is standard of care for head and neck squamous cell carcinoma (HNSCC). Furthermore, it has been shown that sublethal doses of radiation or chemotherapeutic drugs in diverse cancer types may alter the phenotype or biology of neoplastic cells, making them more susceptible to CTL-mediated cytotoxicity. However, little is known about the potential synergistic effect of drug plus radiation on CTL killing. Here, we examined whether the combination of two chemotherapeutics and ionizing radiation enhanced CTL-mediated destruction of HNSCC more so than either modality separately, as well as the basis for the enhanced tumor cell lysis.

Experimental Design: Several HNSCC cell lines with distinct biological features were treated with sublethal doses of cisplatin and 5-fluorouracil for 24 hours and with 10-Gy irradiation. Seventy-two hours postirradiation, tumor cells were exposed to an antigen-specific CD8⁺ CTL directed against carcinoembryonic antigen or MUC-1.

Results: In three of three tumor cell lines tested, enhanced CTL activity was observed when the two modalities (chemotherapy and radiation) were combined as compared with target cells exposed to either modality separately. CTL-mediated lysis was MHC restricted and antigen specific and occurred almost entirely via the perforin pathway. Moreover, the combination treatment regimen led to a 50% reduction in Bcl-2 expression whereas single modality treatment had little bearing on the expression of this antiapoptotic gene.

Conclusions: Overall, these results reveal that (a) CTL killing can be enhanced by combining multiagent chemotherapy and radiation and (b) combination treatment enhanced or sensitized HNSCC to the perforin pathway, perhaps by down-regulating Bcl-2 expression. These studies thus form the rational basis for clinical trials of immunotherapy concomitant with the current standard of care of HNSCC.

Each year, >500,000 new malignancies of the head and neck are diagnosed worldwide, with nearly 42,000 of them in the United States alone (1). Approximately 90% of this cancer type are of squamous origin (2) and more than two thirds of these patients will present with stage III or IV disease. Despite significant progress in the modalities of treatment used, and an increased reliance on collaborative multidisciplinary efforts in treatment

planning, patients with advanced disease have an overall 5-year survival rate below 50% (3). This rate has remained relatively unchanged for nearly three decades (4–6). Current therapies permit organ preservation in selected individuals; however, ablative surgical resection, followed by postoperative radiotherapy and concurrent chemotherapy, is required in many patients (7–9). Despite this highly aggressive therapy, local or regional disease still recurs in 30% of patients and distant metastases appear in 25% (3). Indeed, each year, >12,500 people in the United States will die from their disease (10, 11).

The mortality associated with disease and the morbidity associated with its treatment have encouraged the pursuit of alternate therapeutic strategies. Novel radiotherapy regimens offer potential enhancements in local control with less associated morbidity. Additionally, the development of biological and small-molecule inhibitors targeting the epidermal growth factor receptor pathway (12, 13) and vascular endothelial growth factor in angiogenesis (14) is an area of intense development. Another approach to preferentially destroy malignant populations is to harness the intrinsic elements of the immune system. This modality has produced a significant body of work in several subtypes of human malignancy (15). However, the application of active specific immunotherapy for malignancies of the head and neck has received relatively little attention (16).

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Currently, the standard of care in the management of squamous cell carcinoma of the head and neck combines surgery, radiation, and multiagent chemotherapy (17). Investigations are just beginning to assess the efficacy of active vaccination regimens and passive adoptive transfer protocols alone in the treatment of head and neck squamous cell carcinoma (HNSCC). Whereas investigators have analyzed alterations in the absolute levels of immune effector cells following therapy, no previous study has examined if or how the current standard of care therapies modulate HNSCC cells in terms of their ability to be killed by CD8⁺ effector T cells. In this regard, radiation has been shown to alter malignant phenotypes in other human cancer types, rendering them more susceptible to immune-mediated cell killing (18–24). Chemotherapy regimens, particularly platinum-based therapies, have also resulted in phenotypic modification and enhanced T-cell-mediated lysis of other tumor cell types (25–28).

In this study, we show that concomitant use of multiagent chemotherapy and radiation further enhances the susceptibility of HNSCC cell lines to CTL-mediated lysis. Evidence is provided that enhanced susceptibility to CTL-mediated killing is via the perforin pathway and is correlated with reduced transcription of the antiapoptotic *Bcl-2* gene. These studies show for the first time that the combined use of multiagent chemotherapy and radiation enhances antigen-specific CTL-mediated killing in human tumor cells. Most importantly, these findings suggest that current treatment strategies for HNSCC can potentially be complemented by active specific immunotherapy approaches. This work thus serves as a rational basis for clinical trials employing the standard of care in HNSCC concurrent with immunotherapy.

Materials and Methods

Tumor cell lines. The HNSCC cell lines HN-4, HN-12, HN-26, and HN-30 were cultured in DMEM (low glucose) for propagation and maintenance as previously described (29). Cells were incubated at 37°C with 5% CO₂. These cell lines were obtained from the laboratory of Dr. S. Gutkind.

Tumor chemotherapy. Human tumor cells were harvested while in log growth phase. Cells were then replated into 75-cm² tissue culture flasks in 15-mL medium. To this medium were added 0.5 µg/µL *cis*-diammineplatinum(II) dichloride (CDDP; Sigma, St. Louis, MO) and 0.05 µg/µL 5-fluorouracil (5-FU; Sigma). Control cells received buffer (PBS). Cells were incubated with drug for 24 hours, then washed and replated into fresh 75-cm² flasks. After 72 hours, cells were washed and harvested for the CTL lytic assay and surface molecule analysis by flow cytometry.

Tumor irradiation. Human tumor cells were harvested while in log growth phase. Tumor cells in suspension were placed on ice and irradiated (10 Gy) by a ¹³⁷Cs 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 medium and seeded in 75-cm² tissue culture flasks. After 72 hours, cells were harvested for phenotypic and functional analyses.

Combination therapy—chemotherapy/irradiation. Human tumor cells were harvested while in log growth phase. Cells were then replated into 75-cm² tissue culture flasks in 15-mL medium. To this medium were added 0.5 µg/µL CDDP and 0.05 µg/µL 5-FU. Control cells received PBS alone. Cells were incubated in chemotherapy for 24 hours, then washed and harvested. Tumor cells in suspension were placed on ice and irradiated as above. After 72 hours, cells were harvested for phenotypic and functional analyses.

Flow cytometric analysis. Cell surface staining of tumor cells was done using the following primary labeled monoclonal antibodies (mAb): CD95-FITC, CD54-PE, CD66-FITC, COL-1-FITC (30), CD227-FITC, HLA-ABC-PE, and the appropriate isotype-matched controls. 7AAD staining was used as a measure of cell death following the instructions of the manufacturer. 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. For intracellular staining of carcinoembryonic antigen (CEA), cells were permeabilized (BD PharMingen) according to the instructions of the manufacturer. CEA staining was done using CD66-FITC.

Functional Fas assay. Human tumor cells were incubated for 3 hours with varying concentrations of agonistic anti-Fas antibody, clone CH11 (MBL, Watertown, MA). Control cells were incubated with immunoglobulin M isotype control antibody (BD PharMingen). Cells were then fixed and permeabilized before staining for intracellular caspase-3 with a FITC-labeled mAb (BD PharMingen). The level of activated caspase-3 was quantified via flow cytometry as described above and compared with immunoglobulin M isotype control.

CEA-specific CTL. The HLA-A2-restricted, CEA-specific CD8⁺ CTL line, designated V8T, recognizes the CEA peptide epitope YLSGANLNL (CAP-1). It was maintained and propagated as previously described (31, 32).

MUC-1-specific CTL. Modification of the protocol described by Tsang et al. (33) was used to generate MUC-1-specific CTL. This CTL line, designated T-VLG-P93L, was generated from a colon carcinoma patient and recognizes the MUC-1 peptide epitope ALWGQDVTSV.

Cytotoxicity assays. V8T (CEA CTL) and T-VLG-P93L (MUC-1 CTL) cells were used on day 4 of the restimulation cycle following Ficoll purification. Tumor cells received no treatment, chemotherapy, 10-Gy radiation, or the combination of chemotherapy and radiation (as described above). Human tumor cells were then cultured for 72 hours and subsequently used as targets in a standard cytotoxicity assay. Initially they were labeled using ¹¹¹Indium oxine (Amersham Health, Silver Spring, MD) for 30 minutes at 37°C. Radiolabeled tumor cells at 2×10^3 were then incubated with 6×10^4 CTL [effector-to-target ratio (E/T) of 30:1] for 18 hours at 37°C with 5% CO₂. Targets and CTL 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. The percentage of specific lysis was determined by the standard equation % specific lysis = [(experimental – spontaneous) / (maximum – spontaneous)] × 100. For MHC-I blocking studies, HNSCC cells received no treatment or the combination of chemotherapy and radiation as described. Before being used as targets in the CTL assay, the cells were incubated with anti-HLA-A2 mAb (20 µg/mL, Serotec, Raleigh, NC) or isotype control mAb (immunoglobulin G2b, 20 µg/mL, Serotec) for 1 hour at 37°C. Target tumor cells were then incubated with CTL as above.

Concanamycin A treatment. For indicated experiments, the CEA-specific CTLs were preincubated for 2 hours in the presence of 100 nmol/L concanamycin A (to specifically inhibit perforin-dependent lysis) and incubated with target cells; concanamycin A was present during the assay.

RNA isolation and real-time PCR. HN-4 cells received no treatment, chemotherapy, 10-Gy radiation, or the combination of chemotherapy and radiation (as described above). Human tumor cells were then reseeded in 75-cm² tissue culture flasks. After 24, 72, and 168 hours, cells were harvested from flasks and total RNA was extracted and purified from 5×10^7 cells using the RNeasy midi kit (Qiagen, Inc., Valencia, CA) according to the instructions of the manufacturer. Real-time reverse transcription-PCR reactions were done essentially as described previously (34).

Statistical analysis of the data. Where indicated, the results of tests of significance are reported as *P* values and are derived from Student's *t*

test using a two-tailed distribution. *P* values were calculated at 95% using StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA) software package.

Results

The doses of chemotherapy alone, radiation alone, and the combination of chemotherapy and radiation used were sublethal. The combination of systemic multiagent chemotherapy and tumor irradiation is standard of care for HNSCC. To determine the phenotypic and functional consequences of sublethal chemotherapy and radiation to HNSCC tumors, four biologically distinct HNSCC tumor cell lines were chosen (Table 1). These lines varied on their location of primary tumor and previous therapies. To determine doses of chemotherapy and radiation that were sublethal, dose-response curves were carried out using several concentrations of cisplatin (CDDP) and 5-FU (not shown). The dose of chemotherapy chosen for these studies (0.5 $\mu\text{g}/\mu\text{L}$ CDDP, 0.05 $\mu\text{g}/\mu\text{L}$ 5-FU) had a minimal effect on the proliferation and cellular viability. Cell growth was also minimally slowed in some, but not all, cell lines during the 3 days following 10-Gy radiation. No significant increases in cell death were observed using these doses of radiation or chemotherapy alone or in combination, as determined by 7AAD dye uptake and trypan blue staining for all cell lines (not shown). To further confirm that this combination treatment was sublethal on cells that received no therapy or the combination of chemotherapy and radiation, serial cell counts were done on HN-12 cells at 1, 3, 5, and 7 days posttreatment (Fig. 1). There was no statistical difference in the number of viable cells present at either 5 or 7 days ($P = 0.91$).

The combination of chemotherapy and radiation increased tumor cell sensitivity to antigen-specific CTL. To ascertain if treatment of HNSCC cells with radiation and chemotherapy rendered them more susceptible to T-cell-mediated destruction, we examined tumor susceptibility to CTL lysis following no treatment or treatment with multiagent chemotherapy alone, radiation alone, or the combination of chemotherapy and radiation. We have previously described the CEA-specific human CTL line derived from a patient vaccinated with a CEA-based vaccine (31). These CTLs recognize an HLA-A2-restricted epitope on human CEA-expressing carcinoma cells. It has been previously shown that 87% of HNSCC express CEA (35). HNSCC lines positive for HLA-A2 and CEA (HN-4, HN-12, and HN-26) were subjected to no treatment, chemotherapy (0.5 $\mu\text{g}/\mu\text{L}$ CDDP concurrent with 0.05 $\mu\text{g}/\mu\text{L}$ 5-FU), 10-Gy radiation, or the combination of the two modalities. Seventy-two hours after treatment, these target cells were coincubated with the CEA-specific CTL (Fig. 2).

Table 1. Characteristics of HNSCC cell lines used in these studies

| Cell line | Site of origin | Prior therapy | HLA-A2 |
|-----------|-----------------------|----------------------|----------|
| HN-4 | Base of tongue | None | Positive |
| HN-12 | Lymph node metastasis | None | Positive |
| HN-26 | Vocal chord | 6 cycles CDDP + 5-FU | Positive |
| HN-30 | Pharynx | None | Negative |

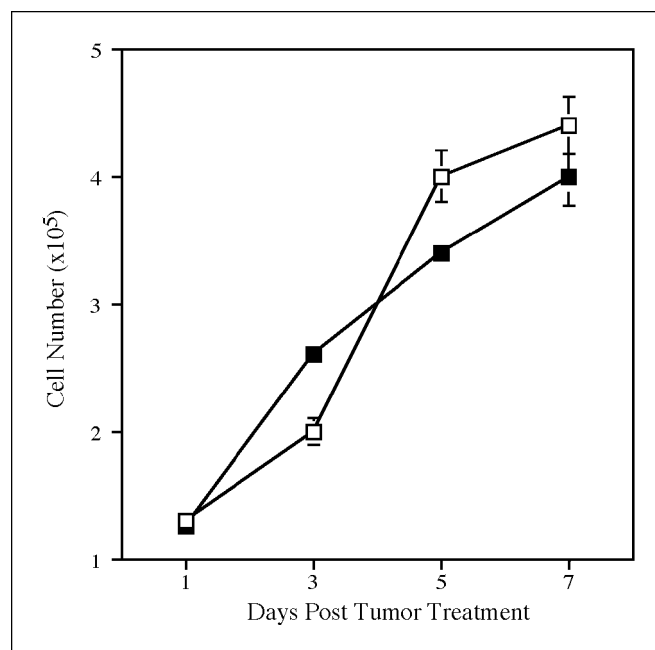


Fig. 1. Combination therapy with multiagent chemotherapy (CDDP and 5-FU) and irradiation (10 Gy) is sublethal. Serial cell counts of a representative cell line (HN-12) were obtained to confirm that the combination of chemotherapy and radiation was sublethal. Tumor cells received no treatment (□) or the combination of chemotherapy and 10-Gy external beam radiation (■). Following treatment, cells were recultured and serial cell counts were obtained at 1, 3, 5, and 7 days posttherapy. Bars, SD.

All (three of three) HLA-A2-positive, CEA-positive tumor cell lines showed substantially enhanced killing by CTL after the combination of multiagent chemotherapy and irradiation when compared with either modality alone or to their untreated counterparts (Fig. 2A-C). HN-4 was not killed at baseline or by chemotherapy or irradiation alone. The combination of the two modalities, however, resulted in a significant enhancement of tumor cell lysis (Fig. 2A; $P = 0.0006$, versus no treatment). HN-12 also showed minimal lysis of control treated cells. However, for this cell line, both chemotherapy alone and radiation alone offered significant enhancement of CTL-mediated killing ($P = 0.002$ and $P = 0.006$, versus no treatment, respectively). This cell line showed the highest level of killing by CTL when both sublethal modalities were combined together ($P < 0.0001$, versus no treatment). This killing was a significant improvement over either chemotherapy ($P = 0.0005$) or radiation alone ($P = 0.0002$; Fig. 2B). HN-26 showed the highest level of killing for untreated control cells (Fig. 2C). As with HN-12, however, both chemotherapy alone ($P = 0.0082$) and radiation alone ($P = 0.0034$) offered improvements in CTL-mediated killing. HN-26 also showed the highest level of killing by CTL when both sublethal modalities were combined together ($P < 0.0001$). This killing was a significant improvement over either chemotherapy ($P = 0.0009$) or radiation alone ($P = 0.0018$). Thus, in three of three CEA⁺ HLA-A2⁺ cell lines tested, the highest level of killing was seen when chemotherapy and irradiation were combined together. HNSCC cells also commonly express the tumor antigen, MUC-1 (36). To determine whether the combination treatment of HNSCC tumor cells improved their lytic sensitivity to another antigen-specific CTL line, we tested them against a MUC-1-specific CTL. This CTL line mediated

moderate lysis of untreated tumor cells. However, for this tumor cell line, either the chemotherapy or radiation alone offered substantial enhancement of CTL-mediated killing. This tumor cell line, however, showed the highest level of killing by CTL when the two sublethal modalities were combined together ($P < 0.0001$, versus no treatment).

To confirm that enhanced CTL sensitivity of HNSCC tumor cells after combination treatment was MHC restricted, cell line HN-4 was incubated with the CEA-specific CTL in the presence of anti-MHC (HLA-A2) blocking antibody (Fig. 2E). As seen before, untreated HN-4 cells were killed at low levels whereas treatment with radiation and chemotherapy greatly enhanced their sensitivity to CTL killing ($P < 0.0001$). This increased sensitivity to CTL killing was abrogated in the presence of anti-HLA-A2 blocking antibody. HN-30 is a CEA-positive, HLA-A2-negative tumor. This cell line, however, was not killed by the HLA-A2-restricted, CEA-specific CTL used in these experiments (Fig. 2F). These data, taken together, indicated that the

enhanced CTL sensitivity of the HNSCC tumor cells after combination treatment was MHC restricted.

Tumor therapy altered surface protein expression. As several cell-surface proteins on tumor target cells have previously been shown to be critical for interactions with CD8⁺ T cells, we next examined the potential role of altered tumor phenotype in CTL sensitivity. Each of the five surface molecules monitored in this study [Fas, intercellular adhesion molecule 1 (ICAM-1) and MHC class I, and tumor-associated antigens CEA and MUC-1] 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 CTL to directly kill specific targets (37). ICAM-1 has both cell adhesion and costimulatory molecule properties. It has been shown that increased expression of adhesion molecules on tumor cells correlates with increased T-lymphocyte binding and killing of tumor cells (38, 39). 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 antigens CEA and MUC-1 have previously been shown to be differentially expressed in tumors versus normal tissues (22). In addition, MHC class I molecule/peptide complexes are important for presentation of antigenic epitopes to T cells (40–42). We sought to quantify the expression of these proteins in each of the four biologically distinct HNSCC tumor cell lines (Table 1) pre- and post-treatment. Tumor cell lines were subjected to no treatment, chemotherapy, radiation, or the combination of the two modalities. Cell-surface expression of Fas, MUC-1, CEA, ICAM-1, and MHC class I molecules was monitored by flow cytometry (Fig. 3). For these analyses, the population of cells positive for isotype control antibody staining never exceeded 5%. Whereas the combination of chemotherapy and radiation was nonlytic (Fig. 1), both modalities induced some alterations in the expression of the surface proteins analyzed. Treatment of HN-4 cells with the combination of chemotherapy and radiation, for example, increased the expression of ICAM-1 2-fold in terms of mean fluorescence intensity over that of control treated cells. In addition, mean fluorescence intensity of the tumor-associated antigen CEA on these cells increased 3-fold after the combination treatment (Fig. 3). In total, each of the four tumor cell lines responded to low-dose irradiation or low-dose chemotherapy by up-regulating one or more surface molecules. Whereas the combination did not induce up-regulation, which was already noted from either modality alone, the combination of chemotherapy and radiation generally further potentiated these surface changes.

It should be pointed out that CEA-specific T-cell killing was seen in cells with low surface expression of CEA protein. T-cell killing, however, is mediated by peptide-MHC expression. In a previous study, Kass et al. (35) showed that a majority of HNSCC lines or tumor samples express CEA intracellularly. To examine the effects of chemotherapy and irradiation on intracellular CEA levels, HNSCC tumor cells that received no therapy (control) and HNSCC tumor cells that received the combination of chemotherapy and irradiation were immunostained for intracellular CEA. HN-4 cells were 60% positive for intracellular CEA (Fig. 3B). After treatment with sublethal levels of chemotherapy and irradiation, however, 99% of the cells became positive. HN-26 cells, although weakly positive for surface CEA (35%), were strongly positive for intracellular CEA

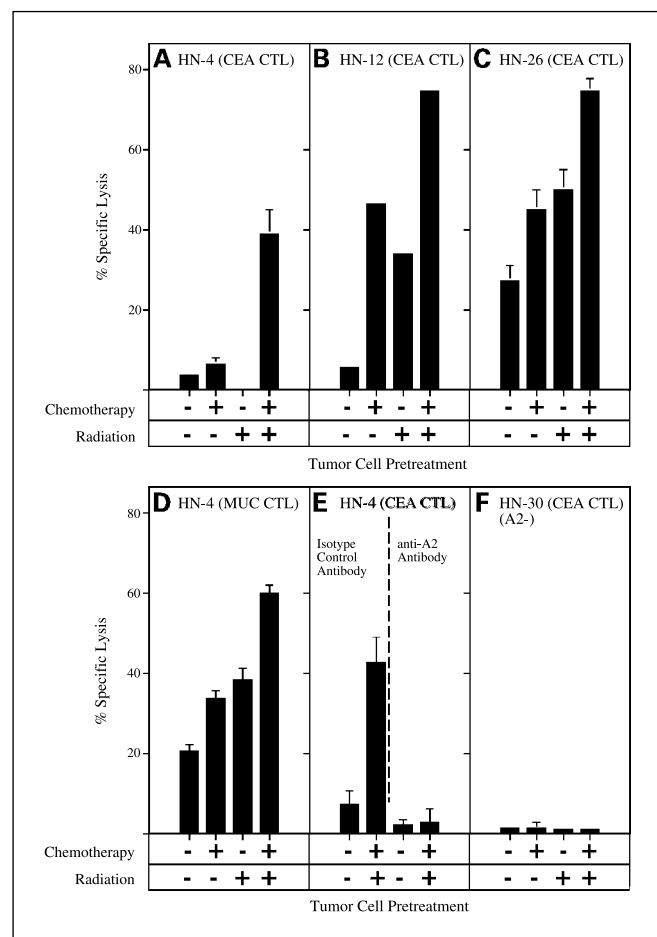
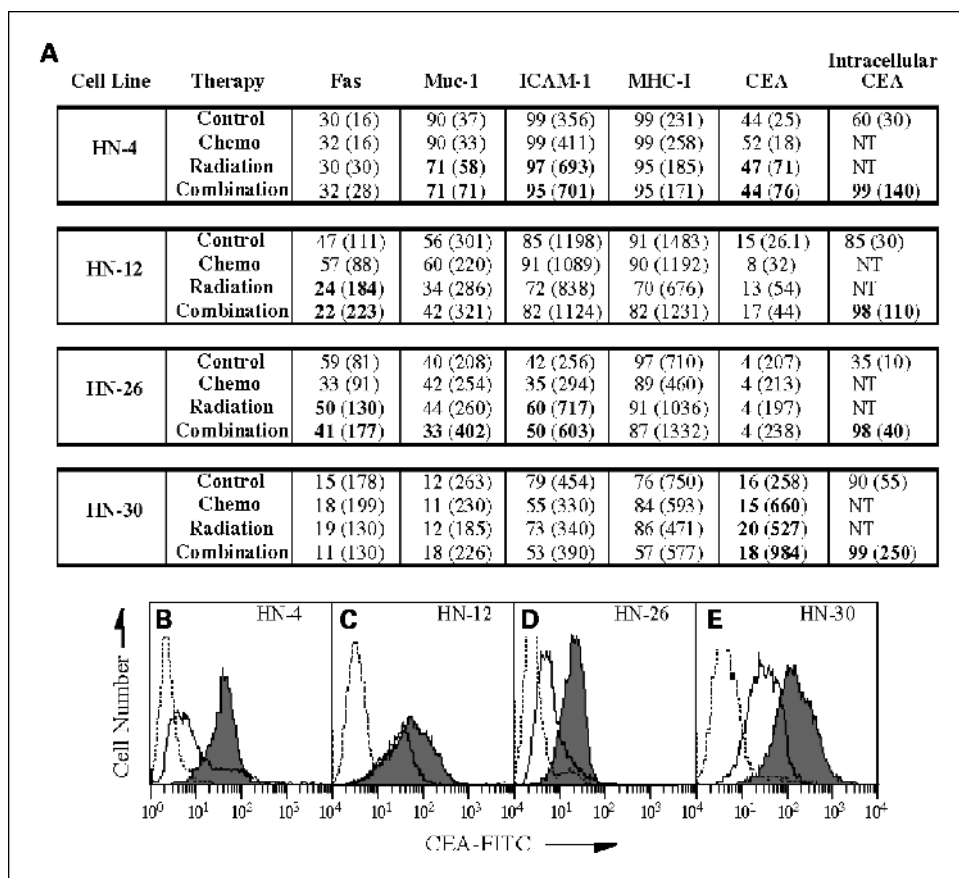


Fig. 2. HNSCC cells treated with chemotherapy and radiation show significantly increased sensitivity to antigen-specific cytotoxic T-cell killing. HNSCC tumor cells received no treatment (control), chemotherapy (CDDP and 5-FU), irradiation (10 Gy), or the combination of chemotherapy and irradiation. Following therapy, cells were recultured for 72 hours. Cells were then labeled with ¹¹¹In and coincubated with HLA-A2-restricted, CEA-specific CTL for 18 hours at an E/T ratio of 30:1. A to C, cell lines shown were both CEA and HLA-A2 positive. D, treated cells were coincubated with HLA-A2-restricted, MUC-1-specific CTL for 18 hours at an E/T ratio of 30:1. E, blocking of CEA-specific cytotoxicity of HN-4 cells with anti-HLA-A2 mAb. F, HN-30 is a CEA-positive, HLA-A2-negative cell line and is shown as a negative control. Bars, SD.

Fig. 3. Treatment of HNSCC tumor cells with chemotherapy and irradiation modulates tumor phenotype. HNSCC tumor cells received no therapy (control), chemotherapy (CDDP and 5-FU), irradiation (10 Gy), or the combination of chemotherapy and irradiation. *A*, cells were recultured for 72 hours and then analyzed by flow cytometry. Cells were analyzed for Fas, MUC-1, ICAM-1, and MHC class I surface expression after each treatment. Intracellular levels of CEA expression were also measured. Numbers indicate the percentage of positive cells. Numbers in parentheses denote the mean fluorescent intensity. Isotype control antibody staining was 5% in all samples. Boldface, marked up-regulation (increased cell-surface levels of $\geq 10\%$ or a >1.5 -fold increase of mean fluorescent intensity not observed in isotype control). *B* to *E*, cells were analyzed for intracellular CEA levels. Dashed lines, staining with isotype control antibody; solid lines, CEA expression of HNSCC tumor cells that received no therapy (control); solid histograms, CEA expression of HNSCC tumor cells that received the combination of chemotherapy and irradiation.



(98%; Fig. 3D, *solid lines*). These cells further increased their intracellular CEA levels following treatment with chemotherapy and irradiation (Fig. 3D, *solid histograms*). Similar observations were made with the other HNSCC cell lines HN-12 (Fig. 3C) and HN-30 (Fig. 3E).

Fas cross-linking assay revealed a nonfunctional death receptor pathway. CTLs kill their targets through two principal mechanisms (43). One pathway employed by CTLs involves ligation of tumor necrosis factor receptor-like molecules, such as Fas/CD95, by their cognate ligands, leading to activation of proapoptotic caspases (44). The second pathway involves the toxic contents of secretory vesicles of the CTL released toward the target cell, with perforin and/or granzymes penetrating into the target cell cytoplasm and nucleus (45, 46).

Each HNSCC tumor cell line was initially assayed for functional Fas using a Fas cross-linking assay. Tumor cells were incubated in the presence of anti-Fas mAb clone CH11 or an isotype-matched control immunoglobulin M antibody. Following incubation, tumor cells were stained for intracellular caspase-3 protein using a fluorescently labeled mAb. Active caspase-3 levels were then quantified via flow cytometry. The flow cytometry results for HN-4 (Fig. 4A) show no activation of caspase-3—a key downstream effector of the Fas death pathway—following surface cross-linking of the Fas receptor. These results are similar to that obtained with cell lines HN-12, HN-24, and HN-30 (not shown).

Blockade of perforin secretion abrogated CTL killing of treated HNSCC cells. The absence of functional Fas activity in tumor cell targets led us to examine other killing modalities such as the perforin/granzyme pathway. Perforin insertion into the

target cell membrane is a stimulus that amplifies the endocytic uptake of other granule constituents and their delivery into the target cell cytosol and nucleus (43). Concanamycin A inhibits a vacuolar type H^+ -ATPase, thereby abrogating perforin-based cytotoxic activity, mostly due to accelerated degradation of perforin by an increase in the pH of lytic granules (47). HNSCC cell line HN-4 received no therapy or the combination of chemotherapy and radiation. Tumor cells incubated with concanamycin A-treated CTL showed significantly ($P = 0.001$) reduced killing (Fig. 4B and C), suggesting that this killing was solely dependent on perforin.

The combination of chemotherapy and irradiation led to a persistent reduction in the expression level of antiapoptotic protein Bcl-2. After observing that treated HNSCC cell lines were killed by CTL via the perforin pathway, we sought to gain insight into the molecular basis for this enhanced lytic response by examining the expression of proapoptotic and antiapoptotic genes. Five genes were analyzed: three proapoptotic genes, *Bcl-X_s*, *Bax*, and *Bak*, and two antiapoptotic genes, *Bcl-X_L* and *Bcl-2*. Tumor cells received no treatment, chemotherapy (0.5 $\mu\text{g}/\mu\text{L}$ CDDP concurrent with 0.05 $\mu\text{g}/\mu\text{L}$ 5-FU), 10-Gy radiation, or the combination of the two modalities. Twenty-four hours after treatment, RNA was harvested, cDNA was transcribed, and real-time PCR reactions were conducted. Gene expression levels were normalized to the housekeeping gene *GAPDH*. Posttreatment *Bcl-X_s*, *Bax*, and *Bak* and *Bcl-X_L* levels were relatively unchanged in cells receiving the combination of both modalities compared with untreated cells (Fig. 5A-D). Posttreatment *Bcl-2* levels were unchanged in cells receiving chemotherapy or radiation alone compared with untreated

cells (Fig. 5E). However, cells receiving the combination of both modalities together showed a 50% reduction in the relative expression level of Bcl-2 compared with control treated cells (Fig. 5E; $P < 0.001$).

Studies indicate that resistance to perforin-mediated killing may be conferred by up-regulation of the expression of the antiapoptotic protein Bcl-2 (48). We next sought to determine the persistence of Bcl-2 down-regulation following treatment with chemotherapy and radiation. We repeated the treatment and harvested cells for analysis at 3 and 7 days postexposure. Levels of Bcl-2 remain depressed for at least 7 days following treatment, decreasing to 0.0026% over this time course (Fig. 5F).

Discussion

Ionizing radiation and multiagent chemotherapy protocols are primary modalities in cancer treatment, particularly in cancers of the head and neck. The "curative" doses of radiation and chemotherapy used clinically are typically the maximum tolerated human doses and may or may not directly induce tumor regression. Multiple investigations suggest that many patients undergoing primary treatment for their malignancy have populations of malignant cells that ultimately receive sublethal doses of radiation and chemotherapy. Sublethal doses of radiation, however, have been associated with the

up-regulation of several classes of molecules on tumor cells including HLA molecules, costimulatory molecules, adhesion molecules, tumor-associated antigens, heat shock proteins, inflammatory mediators, cytokines, and death receptors (49). These alterations have been shown *in vitro* to enhance immune-mediated killing in non-HNSCC human cell lines (24). Similarly, non-HNSCC tumor cells subjected to sublethal chemotherapy have shown multiple alterations leading to enhanced immune-mediated killing (28).

Cisplatin (CDDP) and 5-FU are among the most important combinations of chemotherapeutic drugs for HNSCC. Clinically, the dose of combination chemotherapy used for HNSCC is 120 mg/m² CDDP and 2 g/m² 5-FU. The chemotherapy is administered according to several schedules, with the most common regimen being an intermittent standard-dose bolus schedule every 3 to 4 weeks. The peak plasma concentrations after such a regimen have been reported to be 4.9 μmol/L CDDP and 4.1 μmol/L 5-FU (50). The dose of CDDP used *in vitro* for the studies reported here was 1.66 mmol/L, 338-fold greater than the level expected to be present in a patient's plasma. The dose of 5-FU used *in vitro* here was twice greater than the level expected to be present in a patient's plasma. Although it is difficult to correlate the therapeutic plasma levels with the concentrations of the chemotherapeutic drug combination used for the *in vitro* treatment of the HNSCC cell lines due to variability in liver metabolism (for the 5-FU) and the repeated dosing, it is important to note that the doses chosen for our studies were sublethal (Fig. 1).

Under these experimental conditions, we show for the first time that sublethal doses of chemotherapy combined with radiation (Fig. 1) enhance CTL-mediated killing (Fig. 2) and modulate phenotype (Fig. 3). It was observed that all (three of three) HLA-A2-positive HNSCC cell lines were killed to significantly higher levels by CEA-specific CTL following treatment with chemotherapy and radiation as compared with treatment with either modality separately. It is also interesting to note that one cell line (HN-26), which was derived from a patient who received six cycles of CDDP and 5-FU, was still killed to a significantly greater degree after CDDP/5-FU and irradiation. This cell line, although low for the expression of surface CEA (4%), was highly positive for intracellular CEA (98%; Fig. 3D). This confirms and extends the observations of Kass et al. (35), who noted that a majority of 69 cases of HNSCC tissues expressed CEA protein as determined by immunohistochemical analysis of tumor tissue. HNSCC cells commonly express other tumor antigens such as MUC-1 (36), which was confirmed by flow cytometry (Fig. 3). It was observed that HN-4 tumor cells were killed to significantly higher levels by MUC-1-specific CTL following treatment with chemotherapy and radiation, as compared with treatment with either modality separately, thus extending these findings to a second CTL line. For all tumor cell lines tested, the only discernable pattern of up-regulation or down-regulation of any surface molecule examined that correlated with enhanced sensitivity to CTL (Fig. 2) was CEA, and to a lesser extent, MUC-1 expression (Fig. 3). Whereas it is possible that the other three molecules examined could contribute to enhanced CTL killing of tumor targets, it is also likely that many other proteins could contribute to these observations.

It is currently established that the ratio of proapoptotic and antiapoptotic proteins, in particular proteins of the Bcl-2 family (51), plays critical roles in preventing apoptosis. Proteins of the

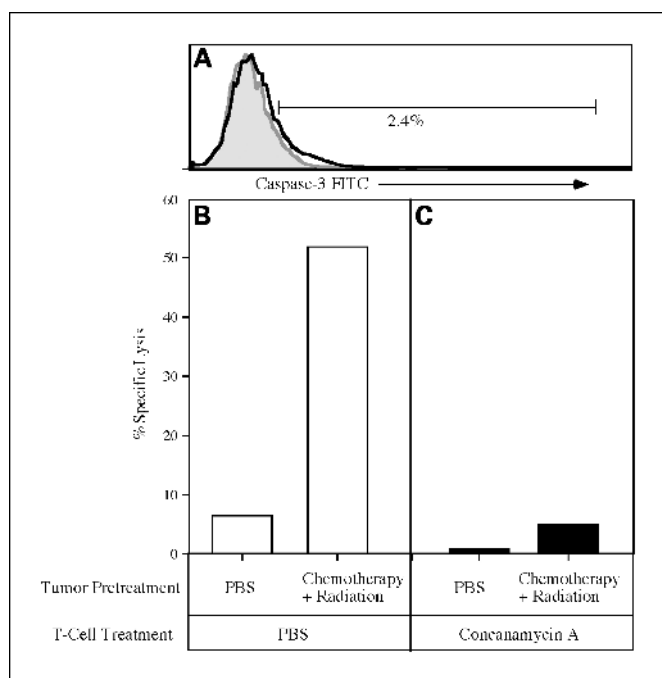
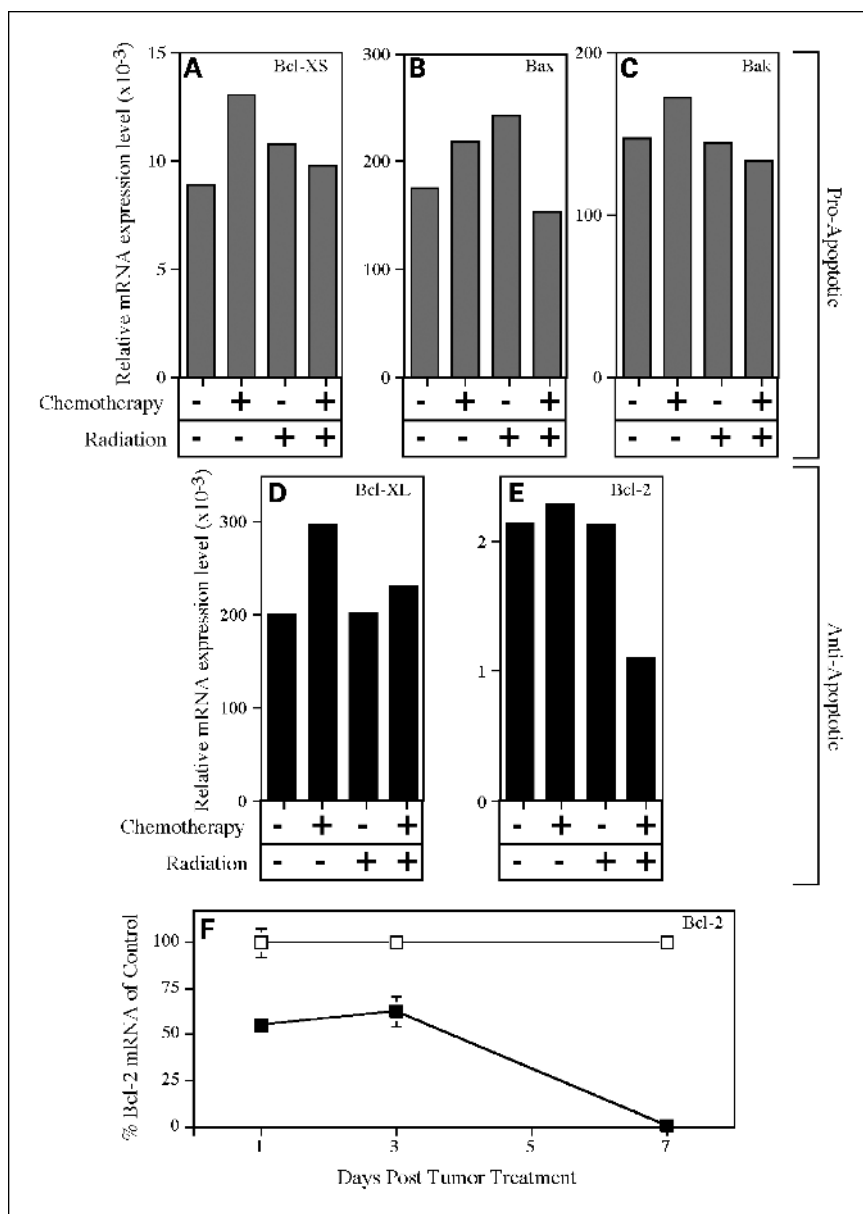


Fig. 4. The enhanced CTL killing of HNSCC after combination therapy is perforin dependent. *A*, the Fas signaling pathway of tumor cell line HN-4 was determined to be defective. HN-4 cells were incubated with Fas cross-linking antibody CH11 or immunoglobulin M isotype control antibody (shaded area) for 3 hours. Cells were then fixed and intracellular staining of caspase 3 (solid thick line) was done with FITC-labeled fluorescent antibody. Caspase expression was quantified via flow cytometry. *B* and *C*, functional abrogation of perforin pathway resulted in the loss of enhanced sensitivity of treated tumor cells to CTL killing. Tumor cells received no treatment (control) or the combination of chemotherapy and 10-Gy external beam radiation. Following treatment, cells were recultured for 72 hours. CTLs were then incubated with either PBS (*B*) or concanamycin A (100 nmol/L; *C*) for 3 hours at 37°C. Following incubation, tumor cells were labeled with ¹¹¹In and coincubated with the CTL for 18 hours at an E/T ratio of 30:1.

Fig. 5. The combination of chemotherapy and radiation reduces tumor cell expression of antiapoptotic gene Bcl-2. HN-4 tumor cells received no treatment (control), chemotherapy, 10-Gy external beam irradiation, or the combination of chemotherapy and radiation. Following treatment, cells were recultured for 24 hours. Cellular mRNA was then harvested and real-time PCR was done. Proapoptotic and antiapoptotic gene expression levels were normalized to housekeeping gene *GAPDH* mRNA levels for each sample. *F*, persistence of Bcl-2 down-regulation. HN-4 cells received no treatment (□) or the combination of chemotherapy and radiation (■). At the indicated time points, mRNA was harvested and real-time PCR was done for Bcl-2.



Bcl-2 family have been shown to be elevated in HNSCC and are significantly associated with more aggressive disease and the loss of differentiation in tumors (52). Additionally, such tumor cells overexpressing Bcl-2 showed resistance to immune-mediated destruction *in vivo* (53). Recent studies have confirmed that central molecular mediators of apoptosis can impair the tumor suppressive activity of antigen-specific CTL (54). Taken independently, this would seemingly limit the ability of immunotherapy to destroy malignant populations preselected to express high levels of antiapoptotic proteins. However, based on the implications of our study, conventional therapies in HNSCC may be able to lower the expression levels of antiapoptotic elements and thereby confer enhanced sensitivity to CTL-mediated lysis.

Although this study was conducted using human HNSCC tumor cell lines *in vitro*, similar results showing enhanced immune-mediated destruction after radiation have been observed in murine models *in vivo*. Chakraborty et al. examined

the effect of localized irradiation of s.c. growing tumors on the efficiency of CTL adoptive immunotherapy in a murine tumor model system. There, irradiation alone significantly potentiated tumor rejection by antigen-specific CTL (55) and by vaccine-induced CTL (56). In another study, the dynamic changes in tumor antigen CEA expression in human colonic xenografts in response to radiation were investigated using radiolabeled antibodies (22). Immunohistology showed that radioantibody-delivered sublethal radiation (35 Gy) 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. These studies provide support for the idea that tumor cells respond to radiation and chemotherapy *in situ* in a manner similar to cells treated *in vitro*.

This study shows for the first time the synergistic effect of chemotherapy and radiation on tumor cell susceptibility to antigen-specific CTL killing (Fig. 2). In our *in vitro* system, these effects were mediated by the cytotoxic granule perforin/

granzyme pathway (Fig. 4) and inversely correlated with Bcl-2 expression (Fig. 5). Overexpression of Bcl-2 in other tumor models has been shown to confer resistance to this perforin/granzyme pathway of apoptosis (48). Thus, it seems that alterations in tumor cell expression of antiapoptotic proteins could be one potential mechanism responsible for the enhanced susceptibility of HNSCC to the perforin/granzyme pathway of lymphocyte-mediated cytotoxicity seen following treatment with chemotherapy and radiation (Fig. 5).

Despite highly aggressive standard of care combination chemotherapy with CDDP and 5-FU with external-beam radiation, local or regional disease still recurs in 30% of patients and distant metastases appear in 25%. We could envision a translational path to clinically test these findings by immunizing patients with a CEA- and MUC-1-based tumor vaccine to generate tumor-specific T cells. These vaccines are presently in phase II and phase III clinical trials. They consist of recombinant poxviruses expressing the transgenes *CEA* and *MUC-1* and three T-cell costimulatory molecules (57). The patient could then undergo definitive combination chemotherapy with CDDP and 5-FU concurrent with external-beam

radiation (bolus or fractionated doses). The control group would receive standard of care combination chemotherapy with CDDP and 5-FU with external-beam radiation. The patient groups could then be monitored for toxicity, clinical responses, and immune responses to vaccine. Agents that more recently have shown activity in recurrent head and neck cancer are paclitaxel (58) and docetaxel (59). Future studies should focus on the role of additional chemotherapeutic therapies, with or without radiation, on tumor phenotype modulation and immune activation. Multimodal therapy thus offers the potential to make human tumors more amenable to immune system recognition. Taken together, these results form the rational basis for clinical trials using the current standard of care in HNSCC in combination with T-cell-mediated immunotherapy.

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