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Effect of EGFR Antagonists Gefitinib (Iressa) and C225 (Cetuximab) on MnSOD-Plasmid Liposome Transgene Radiosensitization of a Murine Squamous Cell Carcinoma Cell Line

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Abstract. *Radiation therapy of tumors of the head and neck region is compromised by dose limiting toxicity of normal tissues including the oral cavity and oropharyngeal mucosa. MnSOD-Plasmid Liposome (MnSOD-PL) intraoral gene therapy has been demonstrated to decrease normal tissue toxicity and also improve survival in mice with orthotopic SCC-VII squamous cell tumors on the floor of the mouth. Furthermore, intravenous administration of MnSOD-PL in mice with orthotopic tumors, or addition of MnSOD-PL to tumor cell lines in vitro produces a radiosensitizing effect attributable to differences in antioxidant pool responses of tumor cells compared to normal tissues following irradiation. To determine whether EGF receptor (EGFR) antagonists Iressa, or Cetuximab provided further improvement of radiation killing of squamous cell tumors, MnSOD-PL transfected or control SCCVII tumor cells were irradiated in vitro, and then the effect of EGFR receptor antagonists was tested. Cells transfected with MnSOD-PL were relatively radiosensitive D_0=1.244±0.126 Gy compared to control D_0=3.246±0.087 ($p<0.0001$). Clonogenic radiation survival curves of SCCVII cells demonstrated radiosensitization by Iressa D_0=2.770±0.134 Gy ($p=0.0264$), but no significant radiosensitizing effect of Cetuximab D_0=3.193±0.309 ($p=0.7338$). The combination of MnSOD-PL plus Iressa further increased radiosensitivity of SCC-VII cells in vitro D_0=0.785±0.01064 ($p<0.0001$). The results suggest some synergy of the effectiveness of the EGFR antagonist Iressa on increasing the radiation killing of SCC-VII cells that supplements MnSOD-PL tumor radiosensitization.*

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The epidermal growth factor receptor (EGFR) signal transduction cascade has been shown to be important in the etiology of squamous cell carcinomas of the head and neck region (1). Expression of EGFR has been found to be of particular importance in the diagnosis of squamous cell carcinomas of the head and neck (2, 3). Targeted therapies against squamous cell tumors have been developed in recent years with a focus on inhibiting the EGFR signal transduction pathway (4, 5). In particular two categories of cytotoxic therapies have been developed. Gefitinib (Iressa) is a small molecule targeting the EGFR (5-7). Another targeted therapy approach has utilized a monoclonal antibody to the EGFR C225, Cetuximab (8), and this agent has been shown to target EGFR positive cells.

Gefitinib (Iressa) has significant effects at inhibiting both squamous cell and hematopoietic cell growth *in vitro* (9-10), and, in early clinical trials, this agent has successfully elicited a therapeutic response in head and neck (11, 12) and lung cancers (13). Clinical trials with Cetuximab for squamous cell carcinoma of the head and neck region have also been very promising (14-17). While there is controversy as to the potential mechanism of action and general utilization of Iressa or Cetuximab (18-22), the relevance of the EGFR signal transduction pathway in human squamous cell cancers has been established (22-24).

Radiotherapy of squamous cell tumors of the head and neck regions is known to produce significant tumor responses, but normal tissue side-effects reflect as mucositis and salivary gland dysfunction (24-28). Tumor cell responses to irradiation include stimulation of cellular repopulation (24, 25) and induction of angiogenic pathways which serve to restimulate tumor recovery from radiation. Angiogenic responses in squamous cell tumors of the head and neck, as well as lung cancer, are known to involve the induction of endothelial cell recruitment by both irradiation and the hypoxic tumor microenvironment (29-32).

Modern radiotherapy techniques including conformal radiotherapy fields (33) and intensity modulation (IMRT)

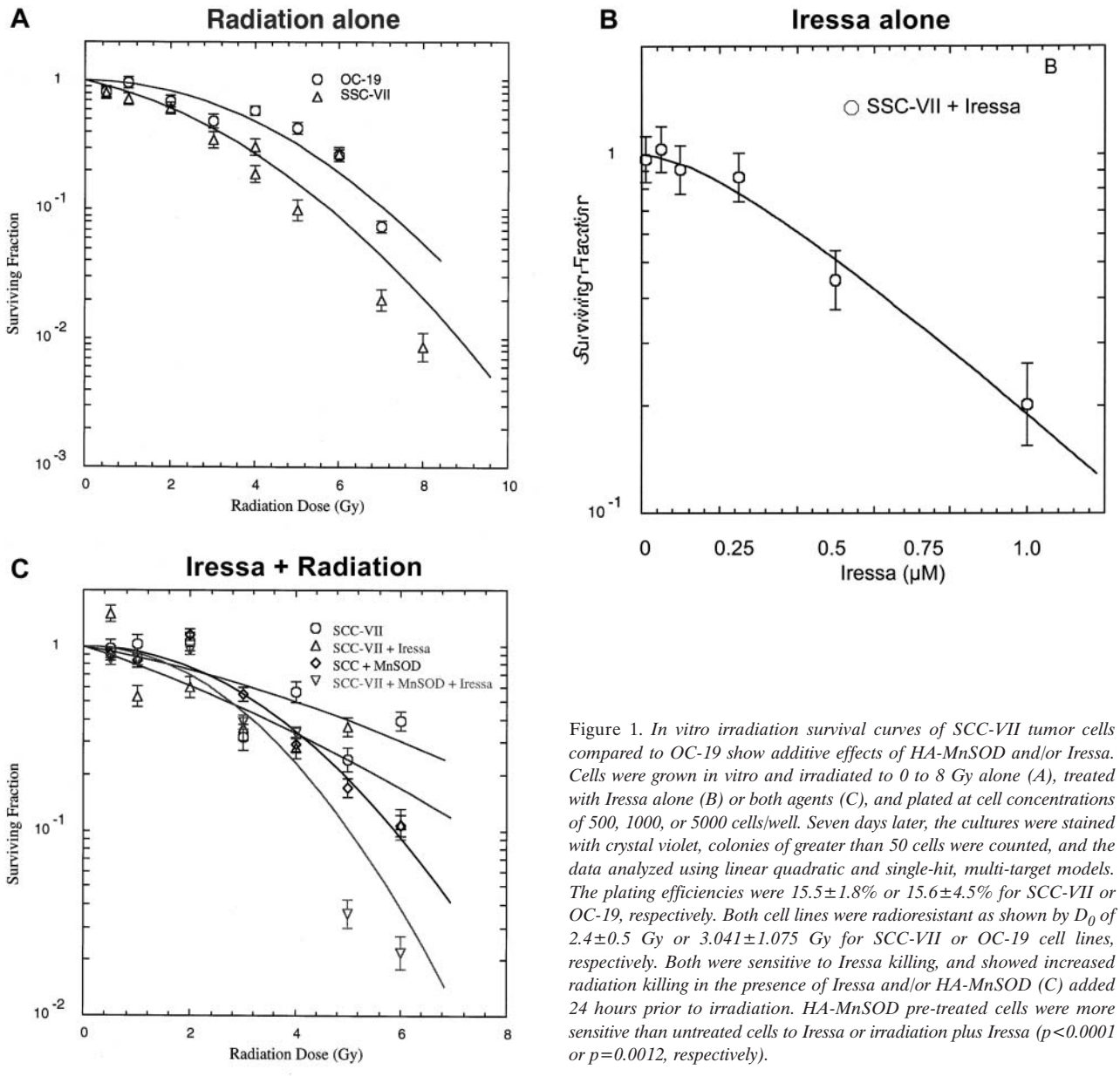

Figure 1. *In vitro irradiation survival curves of SCC-VII tumor cells compared to OC-19 show additive effects of HA-MnSOD and/or Iressa. Cells were grown in vitro and irradiated to 0 to 8 Gy alone (A), treated with Iressa alone (B) or both agents (C), and plated at cell concentrations of 500, 1000, or 5000 cells/well. Seven days later, the cultures were stained with crystal violet, colonies of greater than 50 cells were counted, and the data analyzed using linear quadratic and single-hit, multi-target models. The plating efficiencies were 15.5±1.8% or 15.6±4.5% for SCC-VII or OC-19, respectively. Both cell lines were radioresistant as shown by D_0 of 2.4±0.5 Gy or 3.041±1.075 Gy for SCC-VII or OC-19 cell lines, respectively. Both were sensitive to Iressa killing, and showed increased radiation killing in the presence of Iressa and/or HA-MnSOD (C) added 24 hours prior to irradiation. HA-MnSOD pre-treated cells were more sensitive than untreated cells to Iressa or irradiation plus Iressa ($p<0.0001$ or $p=0.0012$, respectively).*

(34-40), have facilitated irradiation dose escalation to treatment volumes in the head and neck region. However, the toxicity of concomitant or sequential chemotherapy necessitates the development of better targeted therapies that can both radiosensitize the tumor and prevent the growth of distant metastasis (41-45). Tumor cells adjacent to normal tissue areas respond to ionizing irradiation with the production of free radicals including: hydroxyl, superoxide, nitric oxide, peroxynitrite and hydrogen peroxide (ROS) causing initial DNA strand breaks, and apoptosis resulting from mitochondrial damage (46, 47). The secondary elaboration of cytokines from irradiation damaged tissues causes secondary normal tissue damage effects (48, 49). The ROS response to ionizing irradiation may secondarily stimulate the EGF signal transduction pathway as part of the repopulation response to irradiation (50-52).

Normal tissue radioprotective antioxidant gene therapy using MnSOD-Plasmid Liposomes (MnSOD-PL) has been demonstrated to produce paradoxical tumor radiosensitization in orthotopic tumors, perhaps by indirect effects on the tumor repopulation response (46), but also by other mechanisms which may involve different responses of tumor cells compared to normal cells to the oxidative stress induced by irradiation (47).

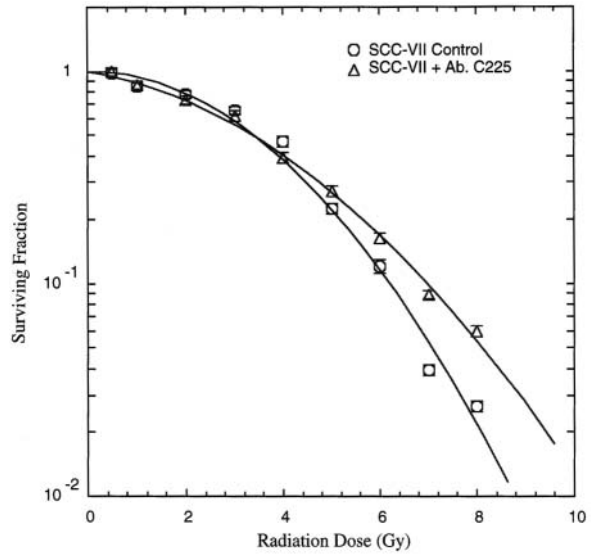

Figure 2. *Effect of Cetuximab in irradiation killing of SCC-VII cells in vitro. Cells were irradiated after 24 hours incubation in 40 μg/ml C225 or control antibody. Cells were plated at 5x10³, 1x10⁴, or 5x10⁴ cells per dish in 4 well tissue culture plates and counted colonies of ≥50 cells scored at 7 days. Results are presented as described in the methods.*

In the present study the SCC-VII squamous cell floor of the mouth murine cancer cell line (63) was utilized to evaluate the potential tumor radiosensitizing effects of Iressa, compared to Cetuximab, and also to determine whether radiosensitization of tumor cells by either agent might further enhance MnSOD-PL mediated tumor radiosensitization (64).

Materials and Methods

Cell lines. The SCC-VII murine squamous cell carcinoma cell line and OC-19, a human squamous cell carcinoma cell line, were passaged *in vitro* according to previously published methods (63). SCC-VII and OC-19 cells were generously provided by Dr. Jennifer Grandis, University of Pittsburgh Cancer Institute, USA.

Fractionated irradiation. Cells were irradiated *in vitro* using a 4 MeV linear accelerator (Varian) over doses from 0 to 800 cGy as previously reported (65). Radiation survival curves were plotted according to previously published methods (65, 66) using single-hit multi-target and linear quadratic models.

Iressa and Cetuximab. Clinical grade EGFR antagonists Iressa and Cetuximab were generously provided by Dr. Jennifer Grandis, University of Pittsburgh Cancer Institute, and prepared for *in vitro* culture according to the manufacturer's guidelines.

MnSOD-Plasmid Liposomes (MnSOD-PL). MnSOD-PL were prepared according to previously published methods (62, 63). SCC-VII cells were transfected *in vitro* 24 hours prior to irradiation. An epitope-tagged HA-MnSOD was utilized to confirm that over 80% of cells were transfected and were producing transgene protein at the time of the radiation survival curve assays (67).

Statistics. Radiation survival curves were evaluated by statistical analysis according to previously published methods (65, 66). Student *t*-test was used to compare the different experimental groups (65, 66).

Results

SCC-VII murine squamous cell carcinoma cells are sensitive to Iressa-mediated cytotoxicity. The radiation survival curve of SCC-VII squamous cell carcinoma cells, and a human squamous cell carcinoma cell line OC-19 were first carried out. Results, shown in Figure 1A, demonstrate significant radioresistance of both cell lines *in vitro*. D_0 of SCC-VII cells were 3.246±0.087 Gy, and that for OC-19 cells was 3.460±0.419 Gy.

Treatment of SCC-VII cells with Iressa (Figure 1B) showed significant cytotoxicity over the dose range of 0.25 to 1.0 micromolar Iressa.

Iressa supplements MnSOD-PL transfection-mediated radiosensitization of SCC-VII cells in vitro. SCC-VII cells were transfected with MnSOD-PL as reported in "Materials and Methods". Radiation survival curves demonstrated significant radiosensitivity of transfected compared to untreated cells (Figure 1C). HA-MnSOD transfection was utilized to confirm transgene expression in over 80% of the transfected cells and significant radiosensitization production by MnSOD-PL transfection (D_0 1.249±0.126 Gy ($p<0.0001$).

The addition of Iressa to MnSOD-PL treatment (24 hours prior to irradiation) further increased radiosensitization of SCC-VII cells (Figure 1C) D_0 0.785±0.010 ($p<0.0001$). These results were significant compared to MnSOD-PL alone or Iressa alone ($p=0.0216$ and 0.0001, respectively).

Cetuximab is not detectably radiosensitizing for SCC-VII cells in vitro. SCC-VII cells were treated *in vitro* with Cetuximab at a concentration of 10 to 40 μg/ml. Over the dose range tested there was no significant spontaneous cell killing by Cetuximab. Irradiation of SCC-VII cells in the presence of 40 μg/ml Cetuximab produced no significant radiosensitization (Figure 2).

Discussion

Targeted therapies have shown great potential in the treatment of tumors of the head and neck region (19-22). A concern for the addition of targeted therapies to chemoradiotherapy has been over the possible toxicity of these agents, or their exacerbation of radiotherapy/chemotherapy toxicity (53-55). Other targeted therapies including hypoxic

cell sensitizers (Tirapazamine) have shown encouraging results when added to chemotherapy or radiotherapy (56).

Attempts to decrease the toxicity of chemoradiotherapy-induced mucositis have included addition of free radical scavenger compounds, such as Amifostine (Ethyol) (57), Pilocarpine (58), or combinations of agents (58-61).

A recently reported radioprotector of potential therapeutic value is the antioxidant transgene MnSOD, delivered by plasmid liposomes (62-64). MnSOD-PL gene therapy, by targeting transgene product to the mitochondria of oral mucosa cells (65, 66), protects normal tissues, in part through preventing the irradiation-induced cell cycling (67). In the present study MnSOD-PL transfection of a squamous cell tumor cell line of SCC-VII, murine squamous cell carcinoma, induced radiosensitization *in vitro*. These results confirm and extend previously conducted studies (63). Targeted EGFR antagonist Iressa was synergistic with MnSOD-PL in providing further radiosensitization of this tumor cell line *in vitro*. In contrast the monoclonal antibody anti-EGFR Cetuximab was not radiosensitizing for SCC-VII cells *in vitro*. This could be due to the fact that the small molecule Iressa entered cells more rapidly *in vitro* and was able to exert a specific therapeutic toxic effect on the murine squamous cell tumor line resulting in further radiosensitization. This effect was not seen with the antibody treatment. Alternatively, lack of significant expression of an epitope of EGFR, responsive to the Cetuximab monoclonal antibody, on the surface of murine SCC-VII cells may have been responsible for the lack of further radiosensitization. Further studies are required to determine how the successful radiosensitization by Iressa can be translated to potential therapeutic benefit in orthotopic tumor models and normal tissue protection and simultaneous tumor radiosensitization by MnSOD-PL.

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