Epidermal Growth Factor Receptor Blockade Potentiates Apoptosis Mediated by Paclitaxel and Leads to Prolonged Survival in a Murine Model of Oral Cancer

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ABSTRACT

Purpose: Because survival for patients with oral cancer has not improved over the past 25 years, new approaches for treatment are needed. Targeted molecular therapy against epidermal growth factor receptor (EGFR) has shown promise as an adjuvant therapy in preliminary studies in several solid tumors, including head and neck cancer. The objective of this study was to determine the efficacy of paclitaxel and PKI166, a novel inhibitor of EGFR, against oral cavity cancer.

Experimental Design and Results: JMAR human oral cancer cells were pretreated for 1 h with PKI166 and then stimulated with epidermal growth factor. EGFR-specific tyrosine kinase autophosphorylation measured by Western immunoblotting was inhibited by PKI166 in a dose-dependent fashion at all doses tested (0.01–1 μM). Next, the induction of apoptosis in JMAR cells treated with paclitaxel (0.001 to 0.1 μM) with or without PKI166 (0, 1, or 2 μM) was determined using a propidium iodide assay. The addition of 2.0 μM PKI166 significantly increased tumor cell death, shifting the amount of paclitaxel needed to induce apoptosis in 50% of cells from 0.1 to 0.001 μM. JMAR oral cancer cells were implanted into the tongues of nude mice. After lingual tumors developed, mice were randomized into four groups (n = 10): (a) oral PKI166 (100 mg/kg); (b) i.p. paclitaxel (200 μg/wk); (c) PKI166 and paclitaxel; or (d) placebo. Mice treated with PKI166/paclitaxel demonstrated a significant increase in survival (P = 0.028). After necropsy, all tongue tumors were evaluated for apoptosis by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay. A greater apoptotic fraction of tumor cells was found in tumors of mice treated with paclitaxel and PKI166 as compared with the other treatment groups (136.4 versus 37.8; P = 0.016).

Conclusions: Combination therapy with paclitaxel and PKI166 prolongs survival in an orthotopic preclinical model of tongue cancer by increasing programmed cell death of oral cancer.

INTRODUCTION

Targeted molecular therapy offers an exciting, new approach to treat human malignancy (1) and represents a fundamental change in cancer therapeutics (2). Current treatment relies on cytotoxic drugs that, for the most part, lack specificity for tumor cells. A better understanding of the molecular pathogenesis of cancer has identified targets for therapeutic intervention that are specific against tumor cells and hence may reduce toxicity commonly associated with adjuvant treatment. The success of the TKI STI-571 in early clinical trials for the treatment of chronic myeloid leukemia (3) and gastrointestinal stromal tumors (4) highlights the unique potential for novel therapy based on specific molecular abnormalities present in a human cancer.

The EGFR pathway provides an attractive target for molecular therapy of HNSCC. Most work has focused on the use of anti-EGFR antibody preparations. Tumor proliferation in cell culture and tumor xenografts in athymic mice have been inhibited by these antibodies (5, 6). When injected into mice bearing tumor xenografts, anti-EGFR antibody preparations caused partial tumor regression. A chimeric version of the 225 MAb (C225) in which the mouse antibody variable regions are linked to human constant regions exhibited an improved in vivo therapeutic effect at high doses (6). These promising results with

The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HNSCC, head and neck squamous cell cancer; TKI, tyrosine kinase inhibitor; MAb, monoclonal antibody; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; PKB, phosphatidylinositol 3-kinase.
C225 led to Phase I and II clinical trials that are now under way (7).

In addition to parenteral targeted EGFR therapy, a class of orally administered, low molecular weight compounds that block the EGFR tyrosine kinase has been described (8). The use of a p.o. administered compound that inhibits the proliferation of HNSCC has several advantages over treatment with receptor-specific antibodies. These advantages include drug availability, no immunological reactivity, and direct intracellular effects on EGFR. Early reports (9–11) suggest that this class of compounds has low levels of systemic toxic effects, limited to fatigue, nausea, and aceniform rash. A recent report showed that ZD1839 (Iressa) inhibits EGFR activation and affects downstream receptor-dependent processes at doses well below thresholds producing unacceptable toxicity (12). We have shown that PKI166, an EGFR-specific TKI, could arrest the growth of oral cancer cells in vitro (13) and reduce the growth of oral cancer xenografts in an experimental animal model.

However, regardless of the type of blockade, using a MAb or EGFR-specific TKI, several lines of evidence suggest that EGFR blockade will be most useful when combined with appropriately selected second agents (14–17). In A431 SCC xenografts, anti-EGFR MAb therapy of established tumors was unable to retard growth, but the addition of concomitant chemotherapeutic agents resulted in a more complete tumor response (14). EGFR blockade may act as a radiosensitizer by augmenting radiation-induced apoptosis (15). C225 and radiation resulted in a significant decline in the proliferation of a panel of HNSCC cell lines, when compared with each treatment alone. Furthermore, this reduction in proliferation correlated with reduced EGFR tyrosine phosphorylation and a reduction in STAT-3 protein, a proapoptotic transcription factor and phosphorylated signal transducer (15).

We hypothesize that there will be increased therapeutic efficacy of EGFR blockade with PKI166 when combined with paclitaxel against human oral cancer, in vitro and growing orthotopically in nude mice.

MATERIALS AND METHODS

JMAR, an Aggressive Human Oral Cancer Cell Line.

The invasive oral squamous carcinoma cell line JMAR (18) was cultured in DMEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, t-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY). JMAR expresses wild-type PTEN/MMAC1 and HER-2/neu oncogene but does not exhibit autocrine EGFR/transforming growth factor α stimulation. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3; pneumonia virus; K virus; Thielere’s encephalitis virus; Sendai virus; min virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M.A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Western Blotting.

JMAR cells were plated into 6-well plates at a concentration of 1 × 10⁵ cells/well and then incubated for 24 h. Triplicate experiments were performed using JMAR cells in both serum-free and serum-enriched media. Treated cells were preincubated with inhibitor (0.01–1 μM; controls were preincubated with DMSO alone) for 1 h, and then EGF (40 ng/ml) was added for 15 min; the cells were then washed with PBS containing 5 mM EDTA and 1 mM sodium orthovanadate. Cells were scraped into lysis buffer [1% Triton X-100, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol (v/v), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM aprotonin-leupeptin-trypsin inhibitor, and 2 mM sodium orthovanadate] and centrifuged to remove insoluble protein. Samples were diluted in sample buffer [0.5 mM Tris-HCl (pH 6.8), 10% SDS, 1 μDTT, 10% (v/v) glycerol, and 1% bromphenol blue] and boiled. The proteins (50 μg/ml) were resolved on 10% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The 10% gels were used to probe with anti-EGFR and anti-phosphotyrosine antibodies. The membranes were blocked with 5% (w/v) nonfat milk in TBST [0.1% Tween 20 (v/v) in TBS], probed with mouse monoclonal anti-phosphotyrosine (IgG2bk; 1:5000; Upstate, Inc., Lake Placid, NY) in 5% nonfat milk, and incubated with horseradish peroxidase-conjugated sheep antimouse immunoglobulin (1:2000; Amersham, Inc., Arlington Heights, IL) in 5% nonfat milk. The blots were also probe with sheep anti-phosphorylated EGFR-Tyr845 and anti-phosphorylated Akt-Ser473 (1:1000; Cell Signaling Technology, Beverly, MA) in 5% nonfat milk in TBST and incubated with peroxidase-conjugated donkey antiserum IgG (1:3000; Sigma, St. Louis, MO) in 5% nonfat milk. Finally, all blots were probed with anti-β-actin (1:1000) in 5% nonfat milk (Sigma), followed with horseradish peroxidase-conjugated donkey antirabbit immunoglobulin (1:2000; Amersham) in 5% nonfat milk. Protein bands were visualized by the enhanced chemiluminescence detection system (Amersham).

Measurement of Cell Death.

Cells were plated at a density of 5 × 10⁵ cells in 38-mm² six-well plates (Costar, Cambridge, MA) and maintained for 24 h before treatment with PKI166 and paclitaxel. Seventy-two h later, the extent of cell death was determined by PI staining of hypodiploid DNA (19). For the PI staining, 3 × 10⁵ cells were resuspended in Nicoletti buffer [50 μg/ml PI (Sigma), 0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/ml RNase A (Roche, Basel, Switzerland) in PBS] and analyzed by FACSscan (Becton Dickinson, Mountainview, CA). The fraction of cells with sub-G₁ DNA content was assessed using the Lysis program (Becton Dickinson). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis.

Measurement of Cytotoxicity.

One thousand JMAR cells were plated into 38-mm² wells of 96-well tissue culture plates. The cells were grown in DMEM supplemented with sodium pyruvate, essential amino acids, and 10% FBS. After a 24-h attachment period, the cells were refed with medium (negative control was refed with DMEM alone) or medium containing paclitaxel. After a 3-day incubation, the number of metabolically active cells was determined by MTT assay. The conversion of MTT to formazan by metabolically active cells was measured by a MR-5000 96-well microtiter plate

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reader (Dynatech, Inc., Chantilly, VA) at an absorbance at 570 nm. Growth inhibition was calculated from the following formula: cytostasis (%) = \([1 - (A/B)] \times 100\), where \(A\) is the absorbance of treated cells, and \(B\) is the absorbance of control cells.

Mice. Male athymic nude mice (NCR-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center under a protocol approved by the Institutional Animal Care Use Committee. They were 8 weeks old when used for this study.

Orthotopic Implantation of Tumor Cells. To produce tumors, JMAR cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. One million JMAR cells were suspended in 50 \(\mu\)l of HBSS and injected into the tongue, as described previously (20), using a 30-gauge hypodermic needle and tuberculin syringe. Based on previous experience with this model, a tumor take rate of at least 67% was estimated. Therefore, 60 mice initially received injection. After 7 days, all mice were examined. Forty mice with homogeneous tumors were identified and then randomized into four groups. The mice were fed moistened food after the development of tumors. All treatments were administered by an animal technician.

The mice were killed before succumbing to tumor progression when moribund or after >20% loss of their recorded initial body weight. Clinical judgement regarding morbidity and the need for the sacrifice of the animals was made by the primary investigator. Therefore, the assessment of the animals was partially blinded.

Reagents. PKI166 [4-(R)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrrolo(2,3-d)-pyrimidine] was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). For in vivo administration, PKI166 was dissolved in DMSO (0.5%) and then diluted 1:20 in HBSS (21). The dosing and Monday-Wednesday-Friday administration schedule of PKI166 was chosen based on the work of Solorzano et al. and Bruns et al. (10, 22), which suggested that PKI166 therapy significantly inhibited phosphorylation of the EGFR without affecting EGFR expression. EGFR phosphorylation was restored 72 h after cessation of therapy. Paclitaxel was obtained from Sigma and administered at 200 \(\mu\)g/wk based on previous studies at our institution (22, 23). PI and MTT were both purchased from Sigma. Stock solutions were prepared by dissolving 5 mg of each compound in 1 ml of PBS and filtering the solution to remove particles. The solution was protected from light, stored at 4°C, and used within 1 month.

Therapy of Established Human Oral Tongue Squamous Cell Carcinoma Tumors Growing in the Tongues of Athymic Nude Mice. Mice were randomized into the following treatment groups (\(n = 10\)): (a) thrice weekly oral administration of PKI166 (100 mg/kg); (b) once per week i.p. injections of paclitaxel (2 mg/kg); and (c) thrice weekly oral PKI166 and once per week paclitaxel. Control mice received oral HBSS + DMSO and i.p. HBSS. All mice were killed on day 38, and their body weight was determined. The tongues were excised and weighed. For immunohistochemical and routine H&E staining, one part of the tissue was fixed in formalin and embedded in paraffin. Another part was embedded in OCT compound (Miles Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. H&E staining confirmed the presence of tumor in each of the tongues.

Immunofluorescence Staining for Apoptotic Cells. TUNEL assay was performed using a commercial Apoptosis Detection Kit (Promega, Madison, WI) with modifications. Frozen tissues were randomized and then sectioned (8–10 \(\mu\)m), mounted on positively charged slides, air dried for 30 min, and fixed in cold acetone for 5 min, acetone plus chloroform (1:1) for 5 min, and acetone for 5 min. The slides were then labeled numerically, without reference to the corresponding treatment group. Immunofluorescence staining was therefore performed in a blinded fashion. Samples were fixed with 4% paraformaldehyde (methanol free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After two 5-min washes with PBS, the samples were incubated with equilibration buffer for 10 min at room temperature. The equilibration buffer was drained, and the reaction buffer containing 44 \(\mu\)l of equilibration buffer, 5 \(\mu\)l of nucleotide mix, and 1 \(\mu\)l of terminal deoxynucleotidyl transferase (Promega Kit) was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h, avoiding exposure to light. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were then washed three times for 5 min to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 \(\mu\)g/ml Hoechst stain for 10 min at room temperature. Fluorescent bleaching was minimized by treating slides with glycerol/PBS mounting media containing 0.1 m propyl gallate (Sigma). Immunofluorescence microscopy was performed on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with 100 W HBO mercury lamp and narrow bandpass excitation filters (Chrom Technology Corp., Brattleboro, VT) to individually select for green, red, and blue fluorescence. Images were captured with a cooled charge-coupled device Hamamatsu C5810 camera (Hamamatsu Photonics K.K., Bridgewater, NJ) and Optimas software (Media Cybernetics, Silver Spring, MD) on a Dell computer (Round Rock, TX). Composite photographs were made using Adobe Photoshop (version 5.5; Adobe Systems, Mountainview, CA). Images were further processed using Scion Image software (Scion Corp., Frederick, MD). DNA fragmentation was detected by localized green fluorescence within the nucleus of apoptotic cells.

Statistical Methods. Survival was analyzed using the method of Kaplan and Meier. Differences between treatment and control groups were compared using the log-rank test. For
EGFR Blockade Potentiates Paclitaxel-mediated Apoptosis

**IN VITRO RESULTS**

**Inhibition of Ligand-stimulated EGFR Tyrosine Phosphorylation in JMAR.** We evaluated whether PKI166 inhibits EGFR-stimulated tyrosine phosphorylation of the EGFR in human JMAR oral cancer cells. Under basal conditions in both serum-containing and serum-free media, JMAR cells demonstrated a low level of autophosphorylation, which was enhanced by a dose of 1 μM β-Actin is shown in the bottom panel to confirm equal protein loading. PKI166 potentiates the ability of paclitaxel to induce apoptosis in 50% of cells from 0.01 to less than 0.00125 μM (see Fig. 3).

**DISCUSSION**

We report that the inhibition of EGFR signaling with PKI166 potentiates the ability of paclitaxel to induce apoptosis in human oral cancer cells growing in culture and in the tongues of nude mice. In *in vitro*, PKI166 specifically inhibited the autophosphorylation mediated by the EGFR tyrosine kinase pathway and diminished Akt activation. The addition of 2.0 μM PKI166 significantly increased tumor cell death, shifting the amount of paclitaxel needed to induce apoptosis in 50% of cells from 0.01 to 0.001 μM, using a PI assay. Finally, *in vivo*, combination therapy with paclitaxel and a selective EGFR-specific TKI prolongs survival in an orthotopic preclinical model of tongue cancer by increasing programmed cell death of oral cancer.

The crucial biological activity of EGFR pathway inhibition may be derived from its ability to suspend cell proliferation. Blockade of the EGFR signaling pathway results in cellular arrest at the G1 restriction point, which has been shown to increase sensitivity to cytotoxicity mediated by radiation or chemotherapeutic agents (24). Although halted by EGFR inhibition, cancer cells may be more susceptible to concomitant cytotoxic agents (such as paclitaxel, in this study) and adjuvant radiotherapy. Both *in vitro* and *in vivo* data suggest that there is a therapeutic potential for combining the EGFR inhibitor and paclitaxel for treatment of oral cancer.

The mechanism by which EGFR blockade potentiates paclitaxel remains unknown. Whereas it is well known that paclitaxel exerts its cytotoxic effects by binding β-tubulin and
stabilizing microtubular structures (25, 26), it less clear how paclitaxel induces apoptosis (27). Recent experimental evidence has pointed toward several pathways already implicated in programmed cell death: the PI3K/Akt pathway (28); serine/threonine kinase-dependent phosphorylation of Bcl-2 (29); Raf-1 kinase (30); and c-Jun NH2-terminal kinase/stress-activated protein kinase pathways (31). Regardless, activation of these apoptotic pathways may be the critical event in taxane-mediated cytotoxicity, ultimately resulting in mitotic arrest.

We demonstrate that PI-3k/Akt signaling is downregulated with PKI166 using Western blotting. Whereas PI3K/Akt may exert certain antiapoptotic effects (32), the effect of paclitaxel on this pathway remains unclear. Early reports suggested that overexpression of Akt conferred resistance to taxanes (33), but other work has shown that paclitaxel-induced apoptosis in human ovarian cells is independent of PI3K/Akt signaling (34). Paclitaxel may induce apoptosis via an unknown pathway.

Fig. 2  Dose-dependent growth-inhibitory effects of (A) paclitaxel, (B) EGFR blockade (PKI1166), and (C) varying combinations of paclitaxel and EGFR blockade (PKI1166) on the cultured JMAR oral cancer cells. Data represent the average of triplicate experiments.

Fig. 3  Apoptosis in JMAR cells treated with paclitaxel (0.0003 to 0.01 μM) with or without PKI1166 (0, 1, or 2 μM) was determined using a PI assay. The addition of 2.0 μM PKI1166 significantly increased tumor cell death, shifting the amount of paclitaxel needed to induce apoptosis in 50% of cells from 0.01 to 0.001 μM.

Fig. 4  Survival in mice treated with EGFR blockade (PKI1166) and paclitaxel in an orthotopic nude mouse model. JMAR oral cancer cells were implanted into the tongues of nude mice. After lingual tumors developed, mice were randomized into four groups (n = 10): (a) oral PKI1166 (100 mg/kg); (b) i.p. paclitaxel (200 μg/kg); (c) PKI166 and paclitaxel; or (d) placebo. Mice treated with PKI166/paclitaxel demonstrated a significant increase in survival (P = 0.028). Ps represent the results for a comparison of the four groups using the log-rank test associated with the survival curves.
downstream mediator in the mitogen-activated protein kinase family (35). Identification of this target may also shed light on the mechanism of action of combination therapy with EGFR blockade and paclitaxel.

The therapeutic efficacy of these two drugs in combination is superior to that of each agent alone, but no synergistic effect can be demonstrated. Rather, EGFR blockade potentiated the cytotoxicity of paclitaxel. The combination index isobologram method of Chou and Talalay (36) requires that each drug exert an independent dose-related effect. Previous work has demonstrated that PKI166 can suspend proliferation of oral cancer but does not cause tumor regression (8). Therefore, standard isobologram calculations cannot be used in this setting. Instead, we show that EGFR blockade potentiates the cytotoxic potential of paclitaxel for oral cancer.

In summary, combination therapy with paclitaxel and EGFR-specific TKI arrests the proliferation of oral cancer cells in vitro and prolongs survival in an orthotopic model of oral cancer. We believe these preclinical data argue for Phase I-II studies to assess the safety and toxicity of EGFR-specific TKIs in combination with taxanes. If safe for use, EGFR blockade in combination with paclitaxel may provide an effective, novel approach for adjuvant treatment in patients with oral cancer.

ACKNOWLEDGMENTS

We thank Jerald J. Killion, Director of Animal Studies, for his contribution in administration of EGFR inhibitor and animal care techniques.

REFERENCES


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**Fig. 5** Quantitative image analysis of TUNEL staining of murine tongues treated with EGFR blockade and paclitaxel. In a separate and final experiment, three groups of five mice were treated with PKI166, paclitaxel, and a combination of PKI166 and paclitaxel. After 1 week of treatment, murine tongues were frozen at necropsy and stored at –20°C. TUNEL assay using immunofluorescence was performed, and all slides, pathologically masked, were then viewed under microscopy. The number of cells undergoing apoptosis was compared in four different groups. Image analysis demonstrated a significant induction of apoptosis in mice treated with EGFR blockade and paclitaxel, when compared with control. Between-group comparisons showed a statistically significant difference between PKI-Taxol and control (P = 0.0159). There were no statistically significant differences between PKI166 and control (P = 0.7302) and Taxol and control (P = 0.8571).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>No. of TUNEL + cells (mean ± SD)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>37.792 ± 18.842</td>
<td></td>
</tr>
<tr>
<td>PKI166</td>
<td>5</td>
<td>41.597 ± 17.660</td>
<td>0.7302</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>3</td>
<td>57.111 ± 10.763</td>
<td>0.8571</td>
</tr>
<tr>
<td>PKI166 + paclitaxel</td>
<td>5</td>
<td>136.425 ± 58.528</td>
<td>0.0159</td>
</tr>
</tbody>
</table>

* Ps were calculated relative to control.


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