Targeted Molecular Therapy for Oral Cancer With Epidermal Growth Factor Receptor Blockade

A Preliminary Report

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Background: Overexpression of epidermal growth factor receptor (EGF-R) is associated with increased malignant potential and correlates with poor clinical outcome in head and neck cancer. Therefore, inhibition of the EGF-R pathway provides an ideal target for molecular therapy. We examined in vitro and in vivo effects of PKI166, an orally administered EGF-R inhibitor, on 2 human squamous cell carcinoma of the oral cavity cell lines, Tu159 and MDA1986.

Study Design: Basic science, laboratory investigation.

Results: For Western blotting, Tu159 and MDA1986 cells were pretreated for 1 hour and then stimulated with EGF. The EGF-R–specific tyrosine kinase autophosphorylation was inhibited completely by PKI166 at all doses tested (1-10 µg/mL). By means of a tetrazolium-based viable cell assay, PKI166 was shown to arrest the growth of Tu159 cells, and MDA1986 cells. The inhibitory concentration (50%), calculated from regression lines on the linear portion of the growth inhibition graphs, was 0.18µM (R=0.98) for Tu159 cells and 0.23µM (R=0.97) for MDA1986 cells. Nude mice were inoculated subcutaneously with 1 × 10⁶ Tu159 tumor cells and observed for 7 days. Next, daily doses of PKI166 (0, 10, or 50 mg/kg) were delivered by orogastric lavage for 28 days and the animals were observed for tumor growth. PKI166 significantly reduced tumor growth in mice treated for 1 month with oral PKI166 in a dose-dependent fashion.

Conclusions: Targeted molecular therapy with EGF-R blockade arrests the growth of oral cancer in vitro and reduces its proliferation in an experimental xenograft animal model.

MATERIALS AND METHODS

ANIMALS

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 US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center, Houston. They were 8 to 12 weeks old when they were used for this study.

CELL LINES AND CULTURE CONDITIONS

Tu159 and MDA1986 are human SCCOC cell lines derived from individual patients undergoing surgery, primarily at M. D. Anderson Cancer Center.\(^\text{16,17}\) The cells were grown in vitro in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and l-glutamine. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of Mycoplasma species and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler encephalitis virus, Sendai virus, minute virus, ectromelia virus, and lactate dehydrogenase virus (assayed by MA Bioproducts, Walkersville, Md). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

INHIBITION OF EGF-R AUTOPHOSPHORYLATION IN HUMAN SCCOC

In our first experiment, we determined whether treatment of Tu159 and MDA1986 cells with PKI166 could inhibit EGF-stimulated tyrosine phosphorylation of the EGF-R. Tu159 and MDA1986 cells, incubated 15 minutes with serum-free medium but containing EGF, exhibited high levels of autophosphorylated EGF-R as detected by antiphosphotyrosine antiserum on Western blots of anti–EGF-R–immunoprecipitated cell lysates. Next, pretreatment of cells with PKI166 for 60 minutes, followed by a 15-minute treatment with EGF, inhibited the autophosphorylation in a dose-dependent manner (0-10 µg/mL). Expression of the 170-kd EGF-R protein was found to be down-modulated by the addition of EGF in the absence of PKI166. Receptor autophosphorylation was found to be maximal under these conditions, and the EGF-R–specific tyrosine autophosphorylation was inhibited completely by PKI166 at all doses tested (1-10 µg/mL) in both cell lines (Figure 1 and Figure 2).

INHIBITION OF EGF-R AUTO-PHOSPHORYLATION IN HUMAN SCCOC

We present a preclinical evaluation of an EGF-R tyrosine kinase inhibitor and its antitumoral effects on in vitro and in vivo proliferation of human SCCOC.

RESULTS

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MEDIATION OF IN VITRO CYTOTOXICITY OF SCCOC

Tu159 and MDA1986 cells were incubated for 5 days in medium, either with or without PKI166. As seen in
similar patterns of growth inhibition by PKI166. The IC50 was seen with the addition of PKI166. Both cell lines had MDA1986 cells. 

Figure 3 and Figure 4, a dose-dependent cytotoxicity was seen with the addition of PKI166. Both cell lines had similar patterns of growth inhibition by PKI166. The IC50 was calculated from regression lines on the linear portion of the log-transformed data.

**MTT CELL PROLIFERATION ASSAY**

PKI166 was tested against the Tu159 and MDA1986 cell lines by means of an MTT-based assay. The MTT assay measures cell proliferation, based on the ability of living cells to use MTT and convert it into dark-blue formazan.30 One thousand cells were plated into 38-mm² wells of 96-well tissue culture plates. The cells were grown in Dulbecco modified Eagle medium supplemented with sodium pyruvate, essential amino acids, and 10% fetal bovine serum. After a 24-hour attachment period, the cells were refed with medium (negative control with dimethyl sulfoxide alone) or medium containing PKI166. After a 5-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 96-well microtiter plate reader at an optical density at 570 nm (MR-5000; Dynatech Laboratories Inc, Chantilly, Va). Growth inhibition was calculated from the following formula: cytostasis (%) = ([1 – (A/B)] × 100), where A is the absorbance of treated cells and B is the absorbance of control cells.

**REDUCTION OF IN VIVO GROWTH OF Tu159 SCCOC XENOGRAFTS**

On the basis of these in vitro data, a pilot study was performed to determine the effect of PKI166 on the growth of SCCOC in nude mice. Groups of 5 mice were inoculated subcutaneously with 1 × 10⁶ Tu159 cells each and observed for 7 days. Then, the animals were treated daily with oral PKI166 (0, 10, or 50 mg/kg) for 28 days. As shown in Figure 5, the growth of Tu159 xenografts was reduced in a dose-dependent manner. With the repeated-measures analysis (log-transformed data), differences in the growth in tumor size over time (as measured by the time × dose interaction) were statistically significant (P = .008). Differences between the control animals and the 50-mg dose group (P = .004) and between the 10-mg and 50-mg groups (P = .02) were statistically significant. Differences between control animals and the 10-mg dose group were not statistically significant. Inferences drawn from the untransformed tumor volume data and the square root–transformed data were similar to those observed for the log-transformed data.

**IN VIVO TUMOR XENOGRAFTS**

Tu159 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% edetic acid. Trypsinization was reversed with the addition of medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Hanks balanced saline solution. Tumor cells were then implanted subcutaneously in the flanks of nude mice at a concentration of 1 × 10⁶ cells per mouse. One week elapsed, at which time subcutaneous tumors could be palpated. The mice were then treated for 28 days with daily oral doses of 0, 10, or 50 mg of PKI166 per kilogram. Tumor sizes were measured by calipers and recorded weekly. Measurements were recorded as the products of the length and width of tumors.

**STATISTICS**

Sigma Plot software (SPSS Science, Chicago, Ill) was used to calculate the inhibitory concentration (50%) (IC50) by means of equations based on an exponential rise algorithm. SPSS software (SPSS Science) was used for statistical analysis. A repeated-measures regression analysis was used to assess the effects of time, dose, and time × dose interaction on growth of in vivo tumor Tu159 xenografts in mice. The primary analysis was a repeated-measures analysis. Repeated-measures analysis was performed on the log-transformed data to mitigate skewness observed in the tumor volume data. Repeated-measures analysis was also performed on the original data and on the square root–transformed data to ensure consistent inferences. The effects in the model were assessed at an significance level of .05. All computations were carried out on a DELL personal computer (DELL Computer Corp, Austin, Tex) with Windows NT operating system (Microsoft Corp, Redmond, Wash) and using the SAS Proc Mixed procedure (SAS Institute Inc, Cary, NC).

Blockade of the EGF-R signaling pathway by the novel tyrosine kinase inhibitor PKI166 suppressed the growth of human SCCOC. PKI166 specifically inhibited the autophosphorylation mediated by the EGF-R tyrosine kinase pathway, as measured by Western blotting. Furthermore, the in vitro cytotoxicity of PKI166 on 2 SCCOC cell lines was confirmed by MTT assay. Finally, we present the first report, to our knowledge, of in vivo suppression of SCCOC tumor growth in an experimental animal model using oral tyrosine kinase inhibitor–EGF-R blockade.

The proliferation of HNSCC has been correlated with increased expression of EGF-R and its ligands, EGF and
transforming growth factor α (TGF-α). The HNSCCs express higher levels of EGF-R and TGF-α than corresponding normal tissues.21 Furthermore, the concomitant expression of both EGF-R and its ligand TGF-α suggests that an autocrine control mechanism may be important in the development of these tumors.22,23 In head and neck cancer, overexpression of EGF-R and TGF-α has been shown to correlate with aggressive malignant progression and poor clinical outcome.5,24

Targeted molecular therapy for HNSCC has focused on the use of anti-EGF-R antibody preparations. Tumor proliferation in cell culture and tumor xenografts in athymic mice have been inhibited by these antibodies, which block EGF binding to EGF-R.25,26 When injected into mice bearing tumor xenografts, mouse anti-EGF-R antibody preparations can cause partial tumor regression. The addition of concomitant chemotherapeutic agents (cisplatin or doxorubicin) is necessary for a more complete tumor response.27 A chimeric version of the 225 monoclonal antibody (C225) in which the mouse antibody variable regions are linked to human constant regions exhibited an improved in vivo therapeutic effect at high doses. These promising results with C225 led to phase 1 and 2 clinical trials that are now under way.13

The use of an orally administered compound that inhibits the proliferation of HNSCC has several advantages over treatment with receptor-specific antibodies. These advantages include drug availability, no immunogenic reactivity, and direct intracellular effects on EGF-R. Phase 1 trials are currently under way to assess the pharmacokinetic bioavailability and toxicity of PKI166.28 Early reports29,30 suggest that this class of compounds has low levels of systemic toxic effects, limited to fatigue, nausea, and rash. The most common complications from monoclonal antibody therapy targeted at EGF-R signaling were fever, asthenia, elevation of aminotransferase levels, nausea, and rash.30

The crucial biological activity of EGF-R pathway inhibition may be derived from its ability to suspend cell proliferation. Blockade of the EGF-R 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. While halted by EGF-R inhibition, cancer cells may be more susceptible to concomitant cytotoxic agents (paclitaxel or cisplatin) and adjuvant radiotherapy. Studies are under way in our laboratory to evaluate the efficacy of PKI166 when given in combination with paclitaxel, with the use of an orthotopic nude mouse model of oral cancer.

In summary, the blockade of the EGF-R signaling pathway with a tyrosine kinase inhibitor arrests the growth of oral cancer in vitro and reduces its proliferation in an experimental xenograft animal model. These preliminary results require further confirmation with an orthotopic model of oral cancer as well as preclinical studies to determine the safety of tyrosine kinase inhibitors in humans. These studies are now under way in our laboratory.

Accepted for publication January 18, 2002.

This study was presented at the Fifth International Conference on Head and Neck Cancer, San Francisco, Calif, July 31, 2000.

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REFERENCES