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Effect of PTK/ZK on the Angiogenic Switch in Head and Neck Tumors

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Abstract

Transformation of small avascular masses of tumor cells into rapidly progressive cancers is triggered by the angiogenic switch, a process that involves vascular endothelial growth factor (VEGF) signaling. We have shown that VEGF enhances the survival and angiogenic potential of endothelial cells by activating the Bcl-2-CXCL8 signaling axis. The purpose of this study was to evaluate the effect of a small molecule inhibitor of VEGF receptors (PTK/ZK) on the initial stages of head and neck tumor angiogenesis. *In vitro*, PTK/ZK blocked head and neck tumor cell (OSCC3 or UM-SCC-17B)-induced Bcl-2 and CXCL8 expression in endothelial cells. Oral administration of PTK/ZK decreased xenograft head and neck tumor microvessel density, and inhibited Bcl-2 and CXCL8 expression in tumor-associated endothelial cells. These data demonstrate that PTK/ZK blocks downstream targets of VEGF signaling in endothelial cells, and suggest that PTK/ZK may inhibit the angiogenic switch in head and neck tumors.

Keywords

Neovascularization; angiogenesis; developmental therapeutics; cancer

INTRODUCTION

Solid tumors do not progress without the oxygen influx and nutrient supply provided by tumor-associated microvascular networks (Folkman, 1971; Carmeliet, 2005; Naumov *et al.*, 2006). This fundamental observation led to the development of anti-angiogenic drugs, some of which have been approved for treatment of patients with cancer (*e.g.* Avastin, Genentech). An emerging concept is the potential use of anti-angiogenic strategies to block the progression of dormant lesions into progressive tumors as a means to convert cancer into a chronic manageable disease (Folkman and Kalluri, 2004). This might be achievable through the control of the angiogenic switch, a process through which an avascular tumor begins to secrete angiogenic factors and becomes vascularized (Hanahan and Folkman, 1996).

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The mechanisms underlying the angiogenic switch are still not well understood. However, enhanced expression of vascular endothelial growth factor (VEGF) appears to be associated with the process that “turns on” the angiogenic phenotype of tumor cells (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003). VEGF is the prototypic angiogenic factor with a well-established role in new vessel formation in both physiological and pathological conditions (Ferrara, 2005; Carmeliet, 2005). VEGF has also a pro-endothelial cell survival function that is regulated by the activity of the anti-apoptotic Bcl-2 (Gerber *et al.*, 1998; Nör *et al.*, 1999). The positive impact of Bcl-2 upregulation in endothelial cells on the overall progression of tumors has been demonstrated in a carcinoma and sarcoma tumor models (Nör *et al.*, 2001a). In contrast, inhibition of Bcl-2 expression in neovascular endothelial cells with RNA interference resulted in significant inhibition of tumor progression (Kaneko *et al.*, 2007).

Bcl-2's classical role is of an anti-apoptotic protein (Reed, 2006). However, we have recently demonstrated that Bcl-2 also functions as a pro-angiogenic signaling molecule through signaling events involving NF- κ B activation and upregulation of expression of the pro-angiogenic CXCL1 (GRO- α) and CXCL8 (Interleukin-8) (Karl *et al.*, 2005). VEGF and CXCL8 were detected at higher concentrations in the serum of patients with head and neck cancer than in controls (Chen *et al.*, 1999). Notably, the expression of Bcl-2 in tumor-associated endothelial cells in patients with head and neck cancer is significantly higher than in endothelial cells of normal oral mucosa (Kaneko *et al.*, 2007). Taken together, these studies provide a conceptual framework for the inhibition of the VEGF-Bcl-2-CXCL8 signaling axis as a therapeutic strategy to prevent the angiogenic switch in head and neck tumors.

PTK/ZK (PTK787/ZK222584; Vatalanib; Novartis/Shering AG) is a small molecule inhibitor of all known tyrosine kinase VEGF receptors (Wood *et al.*, 2000; Drevs *et al.*, 2000, Hess-Stumpp *et al.*, 2005). Its anti-angiogenic and anti-tumor effects have been examined in preclinical studies, and several clinical trials for cancers such as colorectal and thyroid cancer (Wood *et al.*, 2000; Drevs *et al.*, 2005; Yazici *et al.*, 2005; Los *et al.*, 2007; Steeghs *et al.*, 2007). PTK/ZK inhibited the phosphorylation of VEGFR2 in endothelial cells and decreased the microvessel density of xenografted head and neck carcinomas (Kim *et al.*, 2006). Here, we evaluated the effect of therapeutic inhibition of VEGF receptors with PTK/ZK on the early stages of head and neck tumor angiogenesis.

MATERIALS & METHODS

Non-contact co-culture *in vitro* assays

Human Dermal Microvascular Endothelial Cells (HDMEC) (Cambrex, Walkersville, MD) were cultured in endothelial cell growth medium (EGM2-MV, Cambrex). Human head and neck cancer cells (OSCC-3, gift from M. Lingen; and UM-SCC-17B, gift from T. Carey) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% FBS, 1% L-glutamine, and penicillin and streptomycin. Non-contact co-culture *in vitro* assays were performed as previously described (Kaneko *et al.*, 2007). Cells were treated with 0–100 nM PTK/ZK (Novartis/Schering AG; Basel, Switzerland) or equivalent concentrations of vehicle (DMSO; Sigma, St. Louis, MO). For details, see the APPENDIX.

Western Blots

Whole cell lysates were resolved by polyacrylamide gel electrophoresis and membranes were probed overnight at 4°C with the following antibodies: polyclonal anti-human Bcl-2, 1:1000 dilution (51–1513 GR; BD Pharmingen, San Jose, CA); polyclonal anti-human CXCL8, 1:2000 dilution (AF-208-NA; R&D Systems); and monoclonal anti-human GAPDH, 1:100,000

dilution (MAB374; Chemicon International, Temecula, CA). Proteins were visualized with the enhanced chemiluminescence's detection kit (Supersignal West Pico; Pierce, Rockford, IL).

SCID Mouse Model of Human Tumor Angiogenesis

Human xenograft tumors vascularized with human blood vessels were generated as described previously (Nör et al., 2001a; 2001b). For details, see the APPENDIX.

Laser capture microdissection

For Laser Capture Microdissection (LCM), tissue sections were incubated with 1:100 polyclonal anti-human Factor VIII antibody (Lab Vision), followed by standard procedures for immunostaining. Approximately 2,500 endothelial cells and 500,000 perivascular tumoral cells were microdissected per condition for each sample using a Leica AS LMD Laser Microdissection Microscope (LMD6000, Leica Microsystems Inc, Wetzlar, Germany), as described (Kaneko *et al.*, 2007).

RT-PCR and Real-time PCR analyses

Bcl-2 and CXCL8 mRNA expression levels in from endothelial or tumor cells were evaluated by RT-PCR and by real-time PCR, as described (Kaneko *et al.*, 2007). For details, see the APPENDIX.

Data analyses

T-tests or one-way ANOVA were performed with SigmaStat 2.0 software (SPSS; Chicago, IL) for analysis of data presented here. The significance level was set at $p < 0.05$.

RESULTS

To understand the effect of the small molecule inhibitor of VEGF receptor signaling PTK/ZK on Bcl-2 expression in primary human endothelial cells, we performed a dose-dependency assay. Baseline Bcl-2 expression level in endothelial cells (HDMEC) is low. However, exposure to rhVEGF₁₆₅ induces Bcl-2 expression (Fig. 1A), as we have previously demonstrated (Nör *et al.*, 1999). Co-treatment of endothelial cells with VEGF and PTK/ZK resulted in a dose-dependent decrease in Bcl-2 expression. Notably, 100 nM PTK/ZK brought Bcl-2 levels back to baseline, and 1000 nM essentially abrogated Bcl-2 expression in these cells. These data guided our choice for the use of 100 nM PTK/ZK for the remainder *in vitro* experiments performed here.

To simulate *in vivo* conditions observed in the head and neck tumor microenvironment, we performed *in vitro* experiments using a non-contact co-culture method where endothelial cells were grown in the bottom chamber of 6-well plates while the tumor cells were grown in the upper chamber (*i.e.* Transwell). It is well known that head and neck tumor cells express high levels of VEGF (Chen *et al.*, 1999). Therefore, under co-culture conditions we observed that endothelial cell Bcl-2 expression in vehicle treated controls (Fig. 1B) that was consistently higher in single cultures of endothelial cells (Fig 1A). Notably, PTK/ZK treatment downregulated Bcl-2 expression in the endothelial cells co-cultured with UM-SCC-17B (Fig. 1B). In contrast, we did not observe a decrease in Bcl-2 protein expression in the UM-SCC-17B tumor cells *in vitro* (Fig. 1B). To evaluate if PTK/ZK-mediated inhibition of Bcl-2 and CXCL8 expression was regulated at the transcriptional level, we repeated the same experimental design as above and performed RT-PCR from either the endothelial cells or the head and neck tumor cells. We observed that Bcl-2 and CXCL8 message in endothelial cells was downregulated in PTK/ZK-treated co-cultures, as compared to vehicle-treated (Fig. 1C, E, F). We used a second squamous cell carcinoma cell line from the oral cavity (OSCC3) (Fig. 1D, E, F), and observed

that the effects of PTK/ZK were similar to those observed with UM-SCC-17B. These findings suggest that the effects of PTK/ZK reported here are not unique to one specific cell line.

To evaluate the effect of PTK/ZK on tumor angiogenesis and tumor progression *in vivo*, we engineered human tumors vascularized with human blood vessels using the SCID Mouse Model of Human Angiogenesis (Nör *et al.*, 2001a, 2001b). With this method, the HDMEC organize themselves into blood vessels that connect (anastomize) with the mouse vasculature and become functional (blood carrying) vessels (Nör *et al.*, 2001a). At the end of the experimental period, macroscopic evaluation of the tumors revealed that PTK/ZK treated tumors tended to be paler, as compared to vehicle treated controls (Fig. 2A). Indeed, a decrease in microvessel density ($p < 0.001$) in PTK/ZK-treated animals was observed in both UM-SCC-17B and OSCC3 tumors, as compared to vehicle treated controls (Fig. 2B and 2C). In addition, treatment of mice containing HDMEC-only scaffolds with PTK/ZK also resulted in a significant reduction in microvessel density as compared to controls (Fig. 2B and 2C). Tumor progression was evaluated over time to examine the anti-tumor effect of PTK/ZK administered as single treatment. We observed that daily administration of single agent PTK/ZK resulted in a trend for slower tumor progression (Fig. 3A), however the differences between treated and untreated tumors were not statistically significant ($P > 0.05$). Notably, mice did not show significant signs of toxicity with the daily administration of 50 mg/kg PTK/ZK, as demonstrated by the absence of weight loss during the experimental period of 21 days (Fig. 3B).

To quantify the effect of PTK/ZK treatment on Bcl-2 expression in tumor-associated endothelial cells *in vivo*, we retrieved endothelial cells by LCM. Factor VIII immunostaining was used to identify tumor associated endothelial cells (Fig. 4A), blood cells were eliminated from the lumen of the vessels (Fig. 4B), and endothelial cells were retrieved by LCM (Fig. 4C). And finally, tumor cells were retrieved by standard LCM procedures. We observed that PTK/ZK downregulated Bcl-2 expression in tumor-associated endothelial cells *in vivo* in both UM-SCC-17B (Fig. 4D) and OSCC3 (Fig. 4E) xenograft tumors. Quantification of Bcl-2 expression levels in endothelial cells of UM-SCC-17B xenografts with real time PCR, revealed a significant downregulation of Bcl-2 (approximately 5-fold) in PTK/ZK-treated animals, as compared to vehicle-treated controls (Fig. 4G). We observed that CXCL8 was downregulated in the endothelial cells of OSCC3 xenografts treated with PTK/ZK, as compared to vehicle-treated controls (Fig. 4E). CXCL8 was also downregulated in the tumor cells of xenografts treated with PTK/ZK, but could not be detected in the endothelial cells of the UM-SCC-17B xenografts (Fig. 4D, E). As an additional control, we implanted scaffolds that were seeded only with HDMEC (Fig. 4F). Endothelial cells collected from these scaffolds showed very low levels of Bcl-2 expression (Fig. 4F), presumably due to the absence of tumor cells as a source of VEGF. Notably, PTK/ZK was able to downregulate CXCL8 in the scaffolds seeded only with endothelial cells, as compared to vehicle-treated controls (Fig. 4F).

DISCUSSION

Despite significant advances in the treatment and care of patients with cancer, the survival of patients with head and neck tumors has not improved significantly over the last 30 years. Local recurrence, second primary tumors, and metastatic disease are the most common causes of treatment failure in these patients (Genden *et al.*, 2003). Therefore, the identification of patients at higher risk for recurrent or metastatic disease and establishment of a chemopreventive protocol could be particularly beneficial for head and neck cancer patients. Anti-angiogenic drugs tend to have less adverse effects than conventional chemotherapeutic drugs, and have been considered in chemopreventive schemes for prostate and head and neck cancer (Lingen, 1999; Singh and Agarwal, 2006; Noonan *et al.*, 2007). To begin to explore the potential of an

inhibitor of VEGFR signaling as a chemopreventive strategy for head and neck cancer, we evaluated here the effects of PTK/ZK in early stages of tumor angiogenesis.

PTK/ZK was selected for this study for several reasons: A) It can be given orally, which could be beneficial for chemopreventive regimens. B) PTK/ZK has been used extensively in preclinical studies and has reached Phase III clinical trials for treatment of cancer (Wood *et al.*, 2000; Solorzano *et al.*, 2001; Los *et al.*, 2007). C) PTK/ZK appears to have selectivity for the VEGF tyrosine kinase receptors (Hess-Stumpp *et al.*, 2005), which may explain the relatively mild nature of adverse effects observed with this drug. D) The molecular targets of PTK/ZK, *i.e.* VEGF receptors, appear to be suitable candidates for chemopreventive schemes. For example, therapeutic inhibition of the VEGF/VEGFR2 signaling axis has been shown to be associated with the anti-tumor and anti-angiogenic effect of the vitamin A analogue fenretinide (4HPR) in highly vascularized Kaposi's sarcomas (Ferrari *et al.*, 2003). Taken together, these features make an inhibitor of VEGF receptors such as PTK/ZK an exciting candidate drug for preventive inhibition of the angiogenic switch in patients with head and neck cancer.

We have previously demonstrated that VEGF enhance the survival and angiogenic potential of endothelial cells by inducing Bcl-2 and CXCL8 expression (Nör *et al.*, 1999; Karl *et al.*, 2005). We have also used a mathematical modeling approach and demonstrated that curtailing the synthesis of CXCL8 in early tumorigenesis significantly delay tumor progression and angiogenesis (Jain *et al.*, 2007). Furthermore, modeling showed that blocking Bcl-2 upregulation in endothelial cells at early stages of tumor development ensures that both microvascular and tumor cell density stabilize at low values, which can be translated as tumor growth control (Jain *et al.*, 2007). Here, we demonstrated that PTK/ZK-mediated anti-angiogenic effect is associated with inhibition of Bcl-2 and CXCL8 expression in the tumor-associated endothelial cells in two distinct xenograft head and neck tumor models. These results validated the putative downstream molecular targets of PTK/ZK *in vivo*. The ability to quantify gene expression in the endothelial cells of tumors by laser capture microdissection and quantitative RT-PCR allowed for an accurate analysis of the effectiveness of this anti-angiogenic drug in inhibiting its molecular targets. We believe that such experimental approach is ideally suited for the validation of the effect of anti-tumor drugs on angiogenic signaling pathways *in vivo*.

In conclusion, this study demonstrates that oral administration of PTK/ZK has anti-angiogenic in early stage head and neck tumors, which are accompanied by quantifiable inhibition of the VEGF-Bcl-2-CXCL8 signaling axis. Notably, effective blockade of the angiogenic switch with a chemopreventive strategy could potentially bring significant benefit to head and neck cancer patients after management of the primary tumor, since recurrent or metastatic disease is frequent and has a major impact on the clinical outcome of these patients.

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Abbreviations

HDMEC

human dermal microvascular endothelial cells

VEGF

vascular endothelial growth factor

CXCL8

CXC ligand-8

PTK/ZK

PTK787/ZK222584

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Figure 1.

PTK/ZK inhibits Bcl-2 and CXCL8 expression in endothelial cells. A, western blot to evaluate the effect of PTK/ZK on rhVEGF₁₆₅ induced Bcl-2 expression in human dermal microvascular endothelial cells (HDMEC). B–F, Non-contact culture of HDMEC (bottom chamber) and head and neck cancer cells (UM-SCC-17B or OSCC3) (upper chamber, Transwell). Cells were exposed to 100 nM PTK/ZK or vehicle control for 24 hours. B, Western blot analysis of the effect of PTK/ZK on Bcl-2 and CXCL8 expression in endothelial cells or head and neck tumor cells (UM-SCC-17B). C, D, RT-PCR analysis of the effect of PTK/ZK on Bcl-2 and CXCL8 expression in endothelial cells, or head and neck tumor cells, in co-cultures of HDMEC and UM-SCC-17B (C) or HDMEC and OSCC3 (D). Densities of the bands for Bcl-2 and CXCL8 were measured using the Image J software (NIH), normalized by the density of the respective GAPDH band, and represented numerically. The densities of the bands for vehicle-treated controls were set at “1” to facilitate interpretation of the data. E, F, Real-time PCR used to quantify Bcl-2 (E) and CXCL8 (F) expression in endothelial cells (HDMEC) in co-cultures of UM-SCC-17B and HDMEC, or OSCC3 and HDMEC, and exposed to either PTK/ZK or vehicle control. These data represent the expression of Bcl-2 or CXCL8 normalized by the expression of the endogenous control 18S in each sample. The average normalized Bcl-2 or CXCL8 expression in the vehicle-treated group was set at “1”. Statistical significance (asterisk) was determined at P 0.001 using respective vehicle treated groups as controls.

Figure 2.

PTK/ZK decreases the microvessel density of xenograft head and neck tumors. Tumors were generated by co-seeding HDMEC and UM-SCC-17B or OSCC3 cells in biodegradable scaffolds and implanting them bilaterally in the subcutaneous of SCID mice. As controls, we seeded scaffolds with HDMEC cells only. Mice were euthanized 21 days after implantation of scaffolds. A, Macroscopic view of tumors immediately after euthanasia. B, Representative photomicrographs (200x) of tissue sections immunostained for Factor VIII to depict tumor-associated blood vessels (red). C, Graph depicting the microvessel density of tumors treated with PTK/ZK, or vehicle control, after immunolocalization of blood vessels with factor VIII. Statistical significance (asterisk) was determined at $P < 0.05$, using the microvessel density of the correspondent vehicle treated groups as controls. Data represent mean values ($\pm$ s.d.) of 12 tumors per experimental condition.

Figure 3. Effect of oral administration of PTK/ZK on xenograft head and neck tumor progression. A, Graph depicting the volume of tumors generated by the co-implantation of OSCC3 cells and HDMEC in mice treated with PTK/ZK or in vehicle treated control animals. Data represent mean values ($\pm$ s.d.) of 12 tumors per experimental condition. B, Mouse weight during treatment with PTK/ZK or vehicle control. Data represent mean values ($\pm$ s.d.) of 6 animals per experimental condition.

Figure 4.

PTK/ZK inhibits Bcl-2 expression in the endothelial cells lining xenograft head and neck tumor blood vessels. A–C, Step-by-step characterization of the technique based on laser capture microdissection (LCM) that was used for retrieval of endothelial cells from paraffin embedded tissue sections. A, Factor VIII immunolocalization of endothelial cells. B, Removal of blood cells. C, Retrieval of Factor VIII positive endothelial cells. D, E, RT-PCR analysis of the effect of PTK/ZK on Bcl-2 and CXCL8 expression in endothelial cells, or head and neck tumor cells, in tumors generated by the co-implantation of HDMEC and UM-SCC-17B (D) or HDMEC and OSCC3 (E) in SCID mice. The expression of CXCL8 in the endothelial cells from tumors generated by co-implantation of HDMEC and UM-SCC-17B (D) was found to be below detectable levels, as measured by RT-PCR. F, RT-PCR analysis of the effect of PTK/ZK on Bcl-2 and CXCL8 expression in endothelial cells in scaffolds seeded only with HDMEC and implanted in SCID mice. D–F, Densities of the bands for Bcl-2 and CXCL8 were measured using the Image J software (NIH), normalized by the density of the respective GAPDH band, and represented numerically. The densities of the bands for vehicle-treated controls were set at “1” to facilitate interpretation of the data. G, Real-time PCR used to quantify Bcl-2 expression in endothelial cells retrieved from tumors generated by the co-implantation of UM-

SCC-17B and HDMEC in SCID mice that were either treated with PTK/ZK or with vehicle control. These data represent the expression of Bcl-2 normalized by the expression of the endogenous control 18S in each sample. The average normalized Bcl-2 expression in the vehicle-treated group was set at “1”. Statistical significance (asterisk) was determined at $P < 0.001$ using the vehicle treated group as control.