Enhanced Susceptibility of Irradiated Tumor Vessels to Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibition

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Abstract

Previous experiments with PTK787/ZK222584, a specific inhibitor of vascular endothelial growth factor receptor (VEGFR) tyrosine kinases, using irradiated human FaDu squamous cell carcinoma in nude mice, suggested that radiation-damaged tumor vessels are more sensitive to VEGFR inhibition. To test this hypothesis, the tumor transplantation site (i.e., the right hind leg of nude mice) was irradiated 10 days before transplantation of FaDu to induce radiation damage in the host tissue. FaDu tumors vascularized by radiation-damaged blood vessels appeared later, grew at a slower rate, and showed more necrosis and a smaller vessel area per central tumor section than controls. PTK787/ZK222584 at a daily dose of 50 mg/kg body weight had no impact on growth of control tumors. In contrast, tumors vascularized by radiation-damaged vessels responded to PTK787/ZK222584 with longer latency and slower growth rate than controls, and a trend toward further increase in necrosis, indicating that irradiated tumor vessels are more susceptible to VEGFR inhibition than unirradiated vessels. Although not proving causality, expression analysis of VEGF and VEGFR2 shows that enhanced sensitivity of irradiated vessels to a specific inhibitor of VEGFR tyrosine kinases correlates with increased expression of the molecular target.

Introduction

Vascular endothelial growth factors (VEGF) are the key regulators of tumor angiogenesis (1–4). Extensive experimental and early clinical data show that specific inhibitors of VEGF-dependent angiogenesis and endothelial cell survival can reduce the growth rate of tumors and incidence of metastasis (5–8). In preclinical studies, administration of VEGF/VEGFR receptor (VEGFR) inhibitors combined with irradiation results in longer tumor growth delay (9–18) and higher local tumor control (13, 19) than either treatment alone. Many of the experimental data suggest a greater than additive effect of VEGF/VEGFR inhibitors on radiation response. Several mechanisms might explain the greater than additive effect of simultaneously administered inhibitors of the VEGF pathway on tumor radiation response, including decreased tumor hypoxia (20–22) and radiosensitization of endothelial cells (9, 23, 24).

Materials and Methods

Animals and tumor. Female and male and NMRI (nu/nu) mice, 7 to 14 weeks old, were obtained from the specific pathogen-free animal breeding facility at the Experimental Center of the Medical Faculty Carl Gustav Carus, University of Technology, Dresden, Germany. The animal research ethics committees of the University of Dresden and the Regierungspräsidium Dresden approved the animal facilities and the experiments according to institutional guidelines and the German animal welfare regulations. To immunosuppress the mice further, they were whole-body irradiated 2 days before transplantation with 4 Gy (200 kV X-rays, 0.5 mm Cu filter, dose rate of ~1 Gy/min). FaDu is an established human hypopharyngeal squamous cell carcinoma line, kept in high passage by the American Type Culture Collection (Rockville, MD). In nude mice, FaDu grows as a poorly differentiated, nonkeratinizing carcinoma. Following a standardized protocol, small tumor chunks were transplanted s.c. into the right hind leg of the recipient mice. Lactate dehydrogenase electrophoresis of the xenografts showed a typical human isoenzyme pattern.

Irradiation of the tumor transplantation site. Ten days before tumor transplantation, a single dose of 12.5 Gy was given to the right hind leg of the animals. This radiation dose has been shown to induce a clear-cut tumor bed effect in FaDu tumors growing s.c. in nude mice (33).

Recent experiments on human squamous cell carcinomas in nude mice suggested a more than additive effect of PTK787/ZK222584 (PTK/ZK), a specific inhibitor of VEGFR tyrosine kinases (VEGFR TKI), when given adjuvantly over 45 to 75 days after irradiation had been completed (18, 25). Prolonged growth delay of tumors treated with PTK/ZK after a short-term fractionated (18) or after a more clinically relevant fractionated irradiation over 6 weeks (25) was observed. As the VEGFR TKI in these experiments was given after irradiation, increased radiosensitivity was not the underlying mechanism of this phenomenon. As single radiation doses as well as fractionated irradiation can impair tumor vascularization (26, 27), we hypothesized that radiation-damaged tumor vessels are more sensitive to VEGFR TKI than unirradiated tumor vessels. To test this hypothesis in vivo, we investigated FaDu tumors growing in s.c. tissues that were irradiated before tumor transplantation to induce the so-called tumor bed effect (28). This effect is considered to be caused by radiation damage to the host vasculature, resulting in a reduced tumor growth rate compared with tumors growing in unirradiated tissues (29–32). Whereas in the previous experiments (18, 25), both the tumor and the stromal cells were irradiated before treatment with PTK/ZK, the present study was designed to explore the effects of PTK/ZK on growth of unirradiated tumors cells vascularized by radiation-damaged vessels. To identify possible mechanisms of action, necrosis, vasculature, hypoxia, and expression levels of VEGF and VEGFR2 were evaluated.
developed by Schering AG (Berlin, Germany) and Novartis Pharma (Basel, Switzerland). For the experiments reported here, the compound was obtained from Schering. Using a stock solution of 10 mg/mL [suspended in 5% DMSO/ethanol (1 + 1) and 95% m/5-saline (0.85 g/L)], PTK/ZK was given p.o. by gavage daily (50 mg/kg body weight). Treatment started the day after tumor transplantation and was continued until the end of the experiment (i.e., when a tumor diameter exceeded 15 mm).

**Experimental design.** Animals were randomly allocated to four different experimental groups. Tumors were transplanted either into unirradiated (group A and B) or into preirradiated a.c. tissues (group C and D). Animals of groups B and D were treated with PTK/ZK. Vehicle was given to animals of groups A and C.

**Evaluation of tumor growth data.** Tumor diameters were measured every other day using calipers. Tumor volumes (V) were determined by the formula of a rotational ellipsoid

\[ V = \frac{\pi}{6} \times a \times b^2 \]  

(A)

where \( a \) is the longer and \( b \) is the perpendicular shorter tumor axis. To account for systematic errors coming from application of this formula, a correction factor determined previously for FaDu tumors by a calibration curve based on excision weights according to ref. (34) was used. Growth curves for the individual tumors were fitted by a Gompertz function (35)

\[ V(t) = V_0 \times \exp(c/d(1 - \exp(-c/d \times t))) \]  

(B)

where \( c \) and \( d \) are constants and \( V_0 \) represents the tumor volume at start of tumor growth. Only data after start of tumor growth were included. Data were omitted if tumor ulceration occurred. Tumor growth rate was calculated by the derivative of Eq. B:

\[ \frac{dV}{dt} = V_0 \times c \times \exp(c/d) \times \exp(-c/d \times \exp(-d \times t)) \times \exp(-d \times t) \]  

(C)

Tumor volume doubling time (VDT(t)) was then determined by

\[ \text{VDT}(t) = \ln 2 \times V(t)/(dV/dt) \]  

(D)

To compare tumor growth data from different experimental groups, VDT at start of tumor growth (VDTs), and at tumor volumes of 100 mm\(^3\) (VDT100), 200 mm\(^3\) (VDT200), and 400 mm\(^3\) (VDT400) were calculated. Latency was defined as the time from tumor transplantation to first detection of tumor growth.

**Histology.** Histologic analysis of the micromilieu was done as previously described (33). Briefly, tumor-harbouring animals were injected i.p. with the hypoxic cell marker pimonidazole (Natural Pharmacia International, Inc., Research Triangle Park, NC; 0.1 mg/g body weight, dissolved at 10 mg/mL in NaCl) 1 hour before tumor excision. Tumors at a median volume of 255 mm\(^3\) (95% confidence interval, 240-270) were excised immediately after the start of tumor growth (VDT0), and at tumor volumes of 100 mm\(^3\) (VDT100), 200 mm\(^3\) (VDT200), and 400 mm\(^3\) (VDT400) were calculated. Latency was defined as the time from tumor transplantation to first detection of tumor growth.

**Reverse transcription-PCR of vascular endothelial growth factor receptor.** Reverse transcription-PCR (RT-PCR) of total RNA was used to detect transcription of human VEGFR1 (forward primer: 5'-GCTTTGGCCCAATAATGCAGA-3', reverse primer: 5'-ACACGACCTGTGATGTCGAT-3'), human VEGFR2 (forward primer: 5'-GCTTTGGCCCAATAATGCAGA-3', reverse primer: 5'-TGCTCCACAGAAGAACCATGC-3'), mouse VEGFR1 (forward primer: 5'-CTTTCACCTGAGAAGAAGGAGG-3', reverse primer: 5'-TCATGGCAAGAGTTGTTG-3'), and mouse VEGFR2 (forward primer: 5'-AGATCCACCTTCATCGCCCTC-3', reverse primer: 5'-TCTCCGGCAGAAGACCATGC-3') in human FaDu xenograft tumors growing in nude mice. Biopsy material from a human tumor and mouse tissues were used as controls.

**Cell proliferation assay.** FaDu cells grown in DMEM medium with 10% FCS, 1 mmol/L sodium pyruvate, 20 mmol/L HEPES, 1% (v/v) nonessential amino acids, and 1% (v/v) penicillin/streptomycin solution (Biochrome, Berlin, Germany) were plated at densities of 2.5 \times 10^5, 5 \times 10^5, and 10 \times 10^5 cells per well in 96-well plates (Nunc, Roskilde, Denmark). After 24 hours, the medium of each well was replaced by medium with different concentrations of PTK/ZK (triplicates). Proliferation was assayed 72 hours later by adding 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, Deisenhofen, Germany) and incubation for 4 hours. The absorbance was measured after lysis of cells in acidic isopropanol with 1% SDS at 590 nm using a Thermomax microtiter plate spectrophotometer (Molecular Devices Corp., Menlo Park, CA). The concentration of PTK/ZK to reduce the absorbance by 50% compared with untreated controls (IC\(_{50}\)) was calculated using the reader software package.

**ELISA analysis of human vascular endothelial growth factor and murine VEGFR2.** Tumor pieces of ~100 mg were homogenized and ultracentrifuged (100,000 \times g, 60 minutes). The cytosolic fraction was aliquoted for VEGF analysis. For VEGFR2 analysis, the pellet was resuspended and incubated with solubilization buffer [1% NP40, 10% glycerol, 20 mmol/L Tris-Cl, 137 mmol/L NaCl (pH 8)]. Levels of human VEGF\(_{165}\) and murine VEGFR2 were quantified by ELISA according to manufacturer's protocol (R&D Systems, Wiesbaden, Germany).

**Statistical analysis.** Medians, their 95% confidence intervals, and SEs were determined according to Sachs (36) and compared using the Mann-Whitney U test. Data fitting and statistical tests were done using commercially available software (STATA 7.0, STATA Corporation, College Station, TX; GraphPad Prism version 3.03 for Windows, GraphPad Software, San Diego, CA). P values <0.05 were considered significant and P values 0.05 < P ≤ 0.10 were interpreted as a statistical trend.

**Results.** Vascular endothelial growth factor receptor gene expression in FaDu xenografts. RT-PCR analysis of human FaDu tumors growing in nude mice revealed transcription of murine VEGFR
genes (murine VEGFR1/flt, murine VEGFR2/flk), but not of the human homologues VEGFR1 (flt-1) or VEGFR2 (KDR/flk; Fig. 1).

**Effect of PTK/ZK on FaDu cells in vitro.** Incubation of FaDu cells over 3 days with PTK/ZK at different numbers of seeded cells per flask 2,500, 5,000, and 10,000, resulted in IC50 values of 2.7, 9.7, and 11.0 μmol/L, respectively.

**Effects of preirradiation of the transplantation site and PTK/ZK on tumor growth.** After preirradiation of the transplantation site, tumor growth started later and tumors grew at a slower rate than controls (Table 1; Fig. 2). FaDu tumors growing in unirradiated s.c. tissues did not respond to PTK/ZK at a daily dose of 50 mg/kg body weight. When tumors were transplanted into preirradiated s.c. tissues, administration of PTK/ZK resulted in significantly prolonged latency and increased tumor VDT (Fig. 2; Table 1). The effect was statistically significant at first comparison with controls (P = 0.08). Preirradiation of the transplantation site resulted in a trend toward increased tumor necrosis (P = 0.08) and unchanged areas for vessels and hypoxia (P = 0.92, P = 0.12) compared with tumors in preirradiated tissues treated with vehicle. Compared to controls, preirradiation and administration of PTK/ZK resulted in significantly increased necrosis (P = 0.006), decreased vessel area (P = 0.04), and a trend toward increased hypoxia (P = 0.07).

**ELISA analysis of human vascular endothelial growth factor.** Neither PTK/ZK nor preirradiation had an impact on human VEGF expression levels (P = 0.77, P = 0.17; Fig. 4A). Tumors growing in preirradiated s.c. tissues and treated with PTK/ZK showed a trend toward increased human VEGF levels in comparison with controls (P = 0.08).

**ELISA analysis of murine VEGFR2.** No differences in murine VEGFR2 levels between the experimental groups were observed when the data were related to tumor mass. Assuming that in FaDu xenografts murine VEGFR2 is expressed preferentially by tumor vascular endothelial cells, murine VEGFR2 concentration for each individual tumor was analyzed in relation to tumor mass normalized to the vessel area per central tumor section determined by histologic analysis. Using this estimation of the vessel mass in the tumor, Fig. 4B shows that PTK/ZK did not alter mVEGFR2 expression levels in controls or after preirradiation (P = 0.37, P = 0.25). After preirradiation, murine VEGFR2 expression was higher with and without PTK/ZK (P = 0.04 and P = 0.07) than in tumors growing in unirradiated tissues.

**Discussion**

Recent studies on human squamous cell carcinoma in nude mice suggested a greater than additive effect of long-term adjuvant treatment with PTK/ZK on growth of irradiated tumors (18, 25). Radiosensitization of tumor or endothelial cells by PTK/ZK cannot explain this effect because the compound was administered adjuvantly (i.e., after fractionated irradiation had been completed). From this observation, we hypothesized that irradiated tumor vessels are more susceptible to VEGFR TKI.

PTK/ZK is a p.o. bioavailable, specific VEGFR TKI that has entered clinical trials (37–39). In experimental studies, PTK/ZK inhibited VEGF-induced angiogenesis and retarded the growth of several different tumor lines and metastasis when given alone (12, 37, 40) or with fractionated irradiation (12, 18, 25). RT-PCR analysis of human FaDu squamous cell carcinoma growing in nude mice revealed that VEGFR is expressed in mouse tissue but not in tumor cells (Fig. 1). Given that VEGFRs are preferentially expressed in endothelial cells (41), it can be concluded that in FaDu tumor xenografts the host vasculature represents the target of PTK/ZK. This interpretation is supported by the IC50 values for PTK/ZK determined for FaDu cells in vitro. These values are 10 to 40 times higher than IC50 values for murine VEGFR2 tyrosine kinase (37). As in our previous study (18), PTK/ZK at a daily dose of 50 mg/kg body weight had no significant impact on growth, necrosis, vessel area, or hypoxia in FaDu tumors growing in unirradiated tissues (Figs. 2 and 3; Table 1). This suggests that although VEGFR2 and VEGF are expressed (Figs. 1 and 4), either the VEGF pathway is not essential or the dose of PTK/ZK is not sufficient to inhibit angiogenesis in FaDu tumors.

To examine the effect of PTK/ZK on irradiated tumor vessels, we transplanted tumor chunks into tissues that were irradiated...
damage to the vessels cannot be answered directly by our data. Fractionated scheme of preirradiation would result in a similar dose to induce vascular damage. Whether a more conventional potential limitation of our study is the use of a single radiation necrotic cell loss at a constant cell production rate (33). A decrease of the viable tumor cell compartment due to increased this tumor showing that the tumor bed effect is caused by radiation-induced damage to the host vasculature resulting in an impaired tumor angiogenesis (29–32). In line with these observations, we found a decreased vessel area for FaDu tumors growing in preirradiated tissues (Fig. 3). In addition, histologic analysis of tumors growing in preirradiated tissues revealed an increase in necrotic cell loss. This is in line with the results from previous studies on growing in preirradiated tissues (29). Angiogenesis is not sufficient to induce a detectable tumor bed effect (47) but may stimulate VEGF expression (9).

In contrast to the controls, FaDu tumors growing in preirradiated tissues (i.e., supplied by a radiation-damaged vascular network) showed a clear-cut response to PTK/ZK (Figs. 2 and 3; Table 1). As the tumor cells were not irradiated and do not express the target of PTK/ZK, this indicates that irradiated tumor vessels are more susceptible to VEGFR TKI than unirradiated vessels. The trend toward a further increase in necrosis (Fig. 3) suggests that increased necrotic cell loss is the mechanism by which PTK/ZK delayed growth in FaDu tumors supplied with radiation-damaged blood vessels. Comparison of the VDT at different tumor volumes shows that the effect of PTK/ZK is more pronounced at smaller volumes (Table 1). This is in line with the observation that VEGF appeared to be less important for angiogenesis in larger tumors (48). This might be due to a smaller fraction of immature blood vessels devoid of pericytes in larger tumors. Pericytes seem to render endothelial cells resistant to VEGF withdrawal (49).

The finding of enhanced sensitivity of irradiated tumor vessels to VEGFR TKI is a further biological rationale for adjuvant inhibition of VEGFR after radiotherapy has been completed. This concept is supported by results from preclinical studies using different inhibitors of VEGF-dependent angiogenesis (13, 14, 16, 18, 19, 25). Although the radiosensitizing potential of VEGF or VEGFR inhibitors is not exploited by this approach, adjuvant administration of these compounds seems to be a promising strategy to reduce tumor growth rate after fractionated irradiation. First, new formation of blood vessels is essential for the regrowth of recurrent tumors because not only tumor cells but also tumor blood vessels are reduced after irradiation (26). Second, angiogenesis after irradiation precedes regrowth of recurrent tumors (50). Third, after

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**Table 1.** Median time from tumor transplantation to first detection of tumor growth (latency) and VDTs at different tumor volumes (VDT0, VDT100, VDT200, VDT400)

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 17)</th>
<th>Drug (n = 15)</th>
<th>TBE-control (n = 13)</th>
<th>TBE-drug (n = 16)</th>
<th>P* for control vs drug</th>
<th>P* for control vs TBE-control</th>
<th>P* for TBE-control vs TBE-drug</th>
</tr>
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<tbody>
<tr>
<td>Latency (d)</td>
<td>6 (4-6)</td>
<td>6 (4-8)</td>
<td>8 (6-10)</td>
<td>13 (8-16)</td>
<td>0.88</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>VDT&lt;sub&gt;0&lt;/sub&gt; (d)</td>
<td>1.9 (1.3-2.1)</td>
<td>2.4 (1.5-3.0)</td>
<td>2.4 (1.7-2.8)</td>
<td>3.5 (3.3-3.8)</td>
<td>0.25</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>VDT&lt;sub&gt;100&lt;/sub&gt; (d)</td>
<td>2.9 (2.4-3.3)</td>
<td>3.5 (2.5-4.0)</td>
<td>4.6 (3.7-5.0)</td>
<td>6.0 (5.4-6.3)</td>
<td>0.18</td>
<td>&lt;0.001</td>
<td>0.008</td>
</tr>
<tr>
<td>VDT&lt;sub&gt;200&lt;/sub&gt; (d)</td>
<td>3.6 (3.3-3.9)</td>
<td>4.1 (3.4-4.5)</td>
<td>5.7 (4.9-7.0)</td>
<td>7.7 (6.2-8.4)</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>0.06</td>
</tr>
<tr>
<td>VDT&lt;sub&gt;400&lt;/sub&gt; (d)</td>
<td>4.9 (4.1-5.4)</td>
<td>5.3 (4.6-6.2)</td>
<td>9.0 (5.1-10.4)</td>
<td>10.6 (6.3-11.5)</td>
<td>0.29</td>
<td>&lt;0.001</td>
<td>0.29</td>
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NOTE: Values in parentheses represent the 95% confidence intervals. FaDu tumors were transplanted into unirradiated (control) or into preirradiated (TBE) s.c. tissues. Animals of both groups were treated with either carrier (control, TBE-control) or 50 mg/ kg body weight PTK787/ZK222584 (drug, TBE-drug).

* Mann-Whitney U test.

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Figure 2. Median tumor volume as a function of time after tumor transplantation. Tumors were transplanted into unirradiated (circles) or preirradiated (squares) s.c. tissues. Animals were treated either with vehicle (●, ■) or with PTK/ZK (○, □). In each group, 13 to 17 tumors were treated. Data were fitted by a Gompertz function. Bars, SE.
administration of potential curative radiation doses, the tumor shrinks and only a few tumor cells survive. This may possibly resemble an early tumor stage with a high susceptibility to anti-VEGF agents (51). Moreover, enhanced susceptibility of radiation-damaged tumor vessels may possibly already occur during a course of fractionated irradiation, which in clinical practice is typically administered over a period of 5 to 7 weeks. If so, this might contribute to the effects of concurrently administered inhibitors of the VEGF pathway. Such concurrent applications during fractionated irradiation have been shown to radiosensitize tumors (9–17).

ELISA analysis of murine VEGFR2 shows that an increased expression of the molecular target of PTK/ZK correlates with enhanced sensitivity of irradiated tumor vessels (Fig. 4B), although this does not prove causality. Up-regulation of VEGFR2 after exposure of endothelial cells to ionizing irradiation in vitro has also been observed by others (23, 52). Under the assumption that up-regulation of VEGFR2 is the underlying mechanism for the response of irradiated vessels in FaDu tumors to PTK/ZK, it is conceivable that expression levels of VEGFR2 may be predictive for response to VEGFR TKI and may account for the intertumoral heterogeneity in response to VEGFR TKI.

In contrast to VEGFR2, expression of human VEGF in FaDu tumors was not altered by preirradiation of the transplantation site (Fig. 4A). VEGF levels in tumors are regulated by numerous factors such as metabolic stress, hypoxia, mechanical stress, cytokines and hormones, immune response, and genetic alterations, e.g., activated oncogenes or inactivated tumor suppressor genes (3, 53). The similar level of VEGF in FaDu tumors growing in preirradiated tissues and in controls is in line with a stable tumor microenvironment measured by pimonidazole binding in vital tumor areas (Fig. 3) and previously shown by functional radiobiological assay (33).

In summary, our results show that FaDu tumors transplanted into unirradiated tissues did not respond to the VEGFR inhibitor PTK/ZK at a daily dose of 50 mg/kg body weight. In contrast, FaDu tumors growing in preirradiated tissues (i.e., supplied by a radiation-damaged vascular network) appeared later and grew at a slower rate when treated with PTK/ZK. This indicates an enhanced susceptibility of irradiated tumor vessels to VEGFR TKI and supports results from other experiments suggesting a greater than additive effect of adjuvant VEGFR TKI on the growth of irradiated tumors. Although our data do not prove causality, up-regulation of the molecular target in irradiated vessels correlates with the enhanced susceptibility to VEGFR TKI. Taken together, our data provide further biological rationale for adjuvant administration of VEGFR TKI after fractionated irradiation.

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