Effects of PTK787/ZK 222584, a Specific Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases, on Primary Tumor, Metastasis, Vessel Density, and Blood Flow in a Murine Renal Cell Carcinoma Model

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ABSTRACT

Antiangiogenic therapy is a promising new strategy to inhibit tumor growth and formation of metastases. Vascular endothelial growth factor (VEGF) and its receptors, VEGF-receptor 1 (VEGF-R1; FLT-1) and VEGF-R2 (KDR), have been shown to play a major role in tumor angiogenesis. PTK787/ZK 222584, a specific inhibitor of both VEGF-receptor tyrosine kinases, was investigated for its antitumoral and antiangiogenic activity in a murine renal cell carcinoma model. After intrarenal application of the renal carcinoma cells, mice develop a primary tumor and metastases to the lung and to the abdominal lymph nodes. Daily oral therapy with PTK787/ZK 222584 at a dose of 50 mg/kg resulted in a significant decrease of 61 and 67% in primary tumors after 14 and 21 days, respectively. The occurrence of lung metastases was significantly inhibited at both time points (98% reduction and 78% reduction, respectively). After 14 days, no lymph node metastases developed in the PTK787/ZK 222584-treated group, whereas after 21 days of treatment, the lymph node metastases were reduced by 87%. Vessel density in tumor tissues, detected by immunohistochemistry with an anti-CD31 antibody, was significantly decreased by PTK787/ZK 222584. Using color Doppler imaging ultrasound, significant changes in blood flow in the tumor feeding renal artery were found under treatment with PTK787/ZK 222584. Blood flow changes correlated with changes in vessel density but not with tumor volume. The compound was well tolerated in all in vivo experiments and had no significant effects on body weight or general well-being of the animals. This was in contrast to the animals treated with the antiangiogenic agent TNP-470, s.c. therapy with 30 mg/kg TNP-470 every other day had to be discontinued after 13 days because of animal weight loss (>20%) and ataxia.

These results demonstrate that PTK787/ZK 222584 is a potent inhibitor of tumor growth, metastases formation, and tumor vasculization in murine renal cell carcinoma. Furthermore, we have been able to demonstrate that color Doppler imaging ultrasound can be used to measure blood flow to a tumor and that flow correlates with vessel density. Thus, this may be a valuable noninvasive method for monitoring the effects of antiangiogenic agents such as PTK787/ZK 222584 on tumor vasculature.

INTRODUCTION

Renal cell carcinoma is characterized by a lack of early warning signs, which results in a high proportion of patients with distant metastases at the time of diagnosis. Primary tumors are well vascularized, and hematogenous metastases are found especially in lung, bones, and lymph nodes. Renal cell carcinoma is resistant to radiotherapy, chemotherapy, or other systemic therapies, and surgery is currently the only therapeutic option. However, a minority of patients show complete or partial response to IFN, interleukin, or Tamoxifen (1). Besides their known antitumor mechanisms, all three agents are also known to have antiangiogenic activity (2). Therefore, antiangiogenic therapy may be a promising therapeutic concept in renal cell carcinoma.

More than 25 years ago, Judah Folkman (3) proposed that the growth of solid tumors and the formation of metastases are dependent on the development of new blood vessels. Tumors originate from a single cell, which has been transformed by genetic events such as activation of oncogenes, e.g., ras and erbB2, inactivation of tumor suppressor genes, e.g., p53 and von Hippel-Lindau, or both. The transformed cells gain growth advantage and form tumor cell clones of a few millimeters in diameter before the supply of nutrients becomes limited. The tumor clone will remain in this stage until cells acquire an angiogenic phenotype, which means increased secretion of angiogenic factors and reduced production of negative regulators of angiogenesis. As a consequence, endothelial cells change their genetic program, produce proteolytic enzymes, migrate, proliferate, and form new vessels oriented toward and growing into the tumor cell clone (4). This process has been clarified by the discovery and molecular sequencing of various endothelial cell growth factors and endogenous angiogenesis inhibitors. Various angiogenic factors produced by solid tumors have been identified, and their role in the formation of new blood vessels has been elucidated. The VEGF is one of the most potent and endothelial cell specific angiogenic factors with a key role in tumor angiogenesis (5). VEGF is also known to induce vascular hyperpermeability. Its specificity is explained by the preferential expression of its two receptors, VEGF-R1 (FLT-1) and VEGF-R2 (KDR), on the surface of endothelial cells of the tumor vessels. Therefore, VEGF-Rs are promising targets for the inhibition of angiogenesis in growing tumors.

To investigate novel therapeutic strategies for the treatment of human renal cell carcinoma, such as adoptive immunotherapy or cytokine therapy, murine renal cell carcinoma has been a particularly suitable animal model for assessing novel therapeutic approaches (6, 7). In this model, primary kidney tumors are induced by subcapsular renal injection of renal carcinoma (RENCA) cells with subsequent development of metastases in the lungs, lymph nodes, and spleen (8).

Our aim was to study the antiangiogenic and antitumoral effects on murine renal cell carcinoma using the compound PTK787/ZK 222584 which is known to be a potent inhibitor of both VEGF-R-tyrosine kinases and which can be applied p.o. Its pharmacological properties have been described elsewhere (9). Here we describe the effect of PTK787/ZK 222584 on the primary tumor, metastasis, and vessel density in murine renal cell carcinoma. TNP-470, a compound known for its antiangiogenic and antitumor activity in the murine RENCA model, was used for comparison (10). The effect of treatment with PTK787/ZK 222584 on blood flow in afferent tumor vessels was monitored using the color Doppler imaging ultrasound technique.
MATERIALS AND METHODS

Compounds. PTK787/ZK 222584 was provided by Novartis Pharma AG (Basel, Switzerland) and Schering AG (Berlin, Germany). TNP-470 was obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan).

Cell Culture. Murine RENCA cells were originally obtained from a tumor that arose spontaneously in the kidney of BALB/c mice. Histologically, RENCA consists of granular cell type adenocarcinoma, which is pleomorphic with large nuclei. Monolayers of murine RENCA cells were grown in RPMI 1640 with phenol red supplemented with 10% FCS, 2 mm l-glutamine, 100 units penicillin/ml, and 100 μg of streptomycin/ml. RENCA cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Media were routinely changed every 3 days. Cells were released from the tissue flasks by treatment with 0.05% (versene) trypsin/EDTA, and viability was monitored using the cell analyzer system Casy 1 from Scharfe System (Reutlingen, Germany). For the experiments, cells were collected during logarithmic growth phase.

Animal Experiments. All experiments were carried out according to the guidelines of the Ethical Committee of the Regierungspärasium (Freiburg, Germany). Female BALB/c mice were housed under controlled conditions and were routinely used at 6–8 weeks of age (approximate weight, 20 g).

The injection of 10^6 RENCA cells in 0.2-ml aliquots into the subcapsular space of the left kidney was performed through a flank incision after the animals were anesthetized with 0.5–1.5% volume percent isoflurane, which is used in combination with an oxygen flow of 1.5 l/min. The subcapsular renal injection of 10^6 RENCA cells in a syngeneic BALB/c mouse is followed by the progressive development of a primary tumor mass in the left kidney. One week after application, the primary tumor is macroscopically visible; after 10 days, spontaneous lung metastases develop in the regional lymph nodes, in the liver, and the peritoneum, and the liver, allowing the RENCA model to be staged similarly to human renal cell carcinoma. The mean survival time of RENCA-bearing mice is ~32 days when 10^6 RENCA cells are injected.

Administration of Drugs. Drug treatments with PTK787/ZK 222584 or TNP-470 were initiated 1 day after tumor cell inoculation into the subcapsular space of the left kidney. Mice received either PTK787/ZK 222584 (50 mg/kg, p.o. once daily) or TNP-470 (30 mg/kg, s.c. every other day). Oral applications were performed using a gastric tube. The dose for TNP-470 has been reported previously to be effective in the RENCA model (10). The animals of the control groups received either the vehicle for PTK787/ZK 222584, which was dissolved in distilled water, or the vehicle for TNP-470, which had to be diluted in 1 ml of 0.9% NaCl with 3% ethanol and 5% gum arabicum. The vehicles were applied in the same way as the drug formulations. Animal weights were taken every other day.

Evaluation of Tumors. Two or 3 weeks after starting treatment, 6 or 10 mice, respectively, were sacrificed in each group for determination of weight and volume of primary tumors, weight, and number of metastasis of the lungs and metastasis formation in the abdominal lymph nodes. Volumes of primary tumors were taken macroscopically by taking and multiplying the distances of all three dimensions. Number of metastases in the lung and abdominal lymph nodes were counted using a dissection microscope. In the abdominal cavity, all visible lymph nodes were counted for detection of metastasis, knowing that in healthy animals visible lymph nodes are usually absent. Random lymph nodes were inspected microscopically and confirmed tumor-bearing material.

Immunohistochemistry. For histological examination of the tumor vascularature, the tumor tissues and lungs of all groups were quickly frozen in liquid nitrogen. Cryosections of the tissues at a thickness of 5–10 μm were taken from both treatment groups and control groups. For the visualization of the blood vessels, immunohistochemical staining for CD31 (PECAM-1 and MECl3.3; PharMingen, San Diego, CA) was performed, and vessels were counted microscopically using a defined magnification. For all sections, counting was done in three different parts of the sections, with each section being counted twice. The evaluation was performed by two persons, without knowledge of the animal treatment.

Color Doppler Imaging Studies. To determine whether PTK787/ZK 222584 influences blood flow in afferent vessels to the primary tumor, color Doppler imaging ultrasound technique was used in the RENCA model. Drug treatment was initiated 1 day after tumor cell inoculation into the subcapsular space of the left kidney. Mice received either PTK787/ZK 222584 (50 mg/kg, p.o. once daily) or vehicle. Ten animals were used for each group. Twenty days after tumor cell inoculation, primary tumors were located in all animals using a sonograph (Kranzbühler GE, Logic 700, München, Germany) with direct contact, high-frequency ultrasound transducer. By color imaging, the left renal artery was detected, and measurements on systolic and diastolic blood flow and resistance index were performed. To rule out a systemic effect of PTK787/ZK 222584, blood flow was also determined in the abdominal aorta. Measurements of the heart rate were also performed.

Statistical Analysis. For statistical evaluation of the data, the Mann-Whitney U test was significant at a level of P < 0.05. To determine whether the detected changes in blood flow in the left renal artery of RENCA mice treated with PTK787/ZK 222584 correlated with the changes in primary tumor volume or the changes in microvessel density of primary tumors, the Spearman’s coefficient of rank correlation [r, = 1 – 6 C D2/n (n^3 - n)] was calculated. A correlation coefficient >0.514 was considered significant.

RESULTS

Antitumoral Activity of PTK787/ZK 222584. To determine whether PTK787/ZK 222584 is effective in inhibiting tumor growth and development of metastases in the immune competent RENCA model, treatment was started 1 day after inoculation of the RENCA cells into the lower pole of the left kidney. The compound was given p.o. at a dose of 50 mg/kg daily. The antitumoral activity was detected at two time points, 14 and 21 days after inoculation of RENCA cells, respectively.

PTK787/ZK 222584 was well tolerated in all of the experiments and had no significant effects on body weight or general well-being of the animals (data not shown). One of six animals in the group treated with PTK787/ZK 222584 for 14 days and one of six animals in the group receiving vehicle for 14 days died because of tumor burden. In the groups planned to be sacrificed 21 days after inoculation of the cells, 8 of 10 animals in each group reached the end point. Two animals in both groups had to be sacrificed earlier because of tumor burden.

The primary tumor volume in the left kidney of the PTK787/ZK 222584 treatment group was compared with the volume of primary tumors in the control group. Treatment with PTK787/ZK 222584 for 14 days resulted in 61% (P < 0.01) inhibition of primary tumor growth; this effect reached statistical significance after 21 days of treatment by showing a reduction of 67% (P < 0.01; Fig. 1). The effect of PTK787/ZK 222584 on lung metastases was assessed by comparing the number of lung metastases in treated animals with those in untreated animals. Here, treatment with PTK787/ZK 222584 resulted in 98% (P < 0.0001) decrease of the number of lung metastases after 14 days and 78% (P < 0.007) decrease after 21 days showing high significance (Fig. 1). Visible lymph nodes were detectable after 14 days in untreated animals only. Only one of six animals that had been treated with PTK787/ZK 222584 developed visible lymph nodes. In the group of animals sacrificed 21 days after inoculation of the RENCA cells, treatment with PTK787/ZK 222584 resulted in a highly significant reduction in lymph node metastases of 87.4% (P < 0.01; Fig. 1).

Fig. 2 shows the macroscopic pictures of examples of the different tumor responses under treatment with PTK787/ZK 222584. Fig. 2 A represents the group of untreated animals, whereas Fig. 2 B represents the group of animals treated with PTK787/ZK 222584. In Fig. 2 B, the tumor with lowest response is located on top, and the tumor with best response is located at the bottom. From these macroscopic pictures, it is obvious that the tumors of the control group were very homogeneous, whereas the treatment group shows substantial variation. In Fig. 3, the macroscopic pictures of examples of lung metastases are shown. The high amount of metastases in the control group (Fig. 3 A) resulted in lung edemas, which were obviously avoided in the group of animals treated with PTK787/ZK 222584 (Fig. 3 B).
and 21 days after inoculation of RENCA cells. Administration of TNP-470 had to be discontinued after 13 days of treatment because of side effects such as weight loss >20% and ataxia (data not shown). The side effects were reversible. Two of six animals in the group treated with vehicle for 14 days died earlier because of tumor burden. In both groups that were planned to be sacrificed 21 days after inoculation of the cells, all animals reached the end point.

In the experiments with TNP-470, a reduction in primary tumor volume of 58% (P < 0.1) after 14 days and 66% (P < 0.3) after 21 days of RENCA cell inoculation was not significant (Fig. 4). The effect of TNP-470 on lung metastases was assessed by comparing the number of lung metastases in treated animals with those in untreated animals. Treatment with TNP-470 resulted in a significant decrease of 70% (P < 0.05) after 14 days and a decrease of 35% (P < 0.2) after 21 days, which was not significant (Fig. 4). The 58% (P < 0.1) reduction of visible lymph nodes in the treated animals at the 14-day time point was not significant. In the group of animals sacrificed 21 days after inoculation of the RENCA cells, the number of visible lymph nodes was significantly reduced by 71% (P < 0.02; Fig. 4).

**Antiangiogenic Activity of PTK787/ZK 222584.** For histological examination of the tumor vasculature, tumor tissues and lung metastases of all groups were stained for CD31. Examinations of all tissue sections at low magnification were performed, showing a necrotic and avascular area located in the bed of the left kidney, whereas the vessel density in the surrounding tumor tissue was surprisingly homogeneous with a lack of hot spots. Comparing vessel densities in primary tumors of untreated animals with those of PTK787/ZK 222584-treated animals, a dramatic difference becomes apparent, reflecting the decreased vessel density under therapy (Fig. 5). Evaluation of the histological pictures revealed a significant decrease of vessel density of 61% (P < 0.007) in primary tumors of animals treated for 14 days with PTK787/ZK 222584 and of 38% (P < 0.02) in the group of animals treated for 21 days (Fig. 6). In two of six animals treated with PTK787/ZK 222584 for 14 days, no lung metastases could be detected. Histological examination of blood vessels in the lung metastases of the remaining four animals revealed a significant reduction of vessel density of 36% (P < 0.02; Fig. 6). Lung metastases in the group for 21 days showed also a significant reduction of vessel density of 60% (P < 0.05; Fig. 6). It is noteworthy that in mice treated with vehicle only, fewer vessels in primary tumors could be detected after 3 weeks as compared with 2 weeks of tumor growth (Fig. 6). The vessel density decreased significantly by 48% (P < 0.01).

**Antiangiogenic Activity of TNP-470.** Vessels were counted in primary tumors and in any visible lung metastasis of animals treated with TNP-470 and of the respective control animals. After 14 days of treatment with TNP-470, a significant decrease in vessel density of 37% (P < 0.05) was found in primary tumors. Animals sacrificed after 21 days showed a 55% (P < 0.05) decrease in vessel density of primary tumors. In only one of six animals treated with TNP-470 for 14 days, lung metastases could be detected properly. Therefore, no statement for vessel density changes in lung metastases can be made for this time point. In the treated group of animals sacrificed after 21 days, vessel density in lung metastases was reduced by 58% (P < 0.05).
Blood Flow Changes under PTK787/ZK 222584 Treatment. To evaluate whether the decrease in vessel density induced by PTK787/ZK 222584 affected blood flow in afferent tumor vessels, the color Doppler imaging ultrasound technique was used. Twenty days after tumor cell inoculation, primary tumors could be located by sonography in all animals (n = 20; Fig. 7A). The resolution of the sonographic image allowed a distinction between the kidney, which showed a hydronephrosis, and the surrounding primary tumor. Color imaging was used to locate the left renal artery, which could be detected reproducibly in all animals (Fig. 7B). The measurements of blood flow and resistance index in the left renal artery were performed by Doppler technique, resulting in a flow-time curve (Fig. 7C), which gives information on systolic blood flow (cm/s), diastolic blood flow (cm/s), resistance index, and heart rate (beats/min). A highly significant difference between control group (n = 10) and PTK787/ZK 222584-treated animals (n = 10) could be detected for systolic (inhibition of 60%; P ≤ 0.01) and diastolic (inhibition of 58.7%; P < 0.006) blood flow (Fig. 8). The resistance index and the heart rate were not affected by PTK787/ZK 222584 (data not shown).

The Spearman’s coefficient of rank correlation was used to correlate the changes on blood flow with tumor volume and vessel density. There was a significant correlation between blood flow and vessel density (correlation coefficient = 0.582). Surprisingly, no correlation between blood flow and tumor volume (correlation coefficient = 0.319) could be detected. To rule out a systemic effect caused by changes in blood pressure, measurements on blood flow and resistance index were also performed in the abdominal aorta as a control vessel. No changes in blood flow and resistance index attributable to the medication occurred (data not shown).

DISCUSSION
Currently, almost 50% of cancer patients can be cured by surgery or radiotherapy. However, the metastatic stage of cancer is still difficult to treat and caused a total mortality rate of 166.9/100,000 in the population of the United States in 1996 (11). In this palliative situation, treatment options like chemotherapy, radiotherapy, and hormone therapy are characterized by low response rates and quality of life-reducing side effects. In particular for renal cell cancer patients with metastatic disease, efficient therapeutic options are lacking. At diagnosis, 30–40% of patients already are have metastatic disease, but chemotherapy and radiotherapy are essentially not effective. Drugs showing some efficacy such as IFN, interleukin, and Tamoxifen, with response rates between 10 and 20%, are also known to have antiangiogenic activity (2). Antiangiogenesis, a new concept in cancer treatment, may offer a therapy with likely fewer side effects and absence of drug resistance. The most important and selective factor in tumor angiogenesis is VEGF. Therefore, the aim of our study was to investigate PTK787/ZK 222584, a selective inhibitor of the VEGF-R1
and VEGF-R2 tyrosine kinases, for its antitumor and antiangiogenic effects in the VEGF-dependent murine renal cell carcinoma model (12).

Because of its excellent bioavailability, this low molecular synthetic molecule was administered p.o. at a dose of 50 mg/kg body weight (13). This dose was shown previously to be active in a VEGF-driven in vivo angiogenesis model (A431 alginate beads model), as well as in rodent tumor models like A431 epithelial carcinoma, LS174T colon carcinoma, HT-29 colon carcinoma, PC-3 prostate carcinoma, DU145 prostate carcinoma, and CWR-22 prostate carcinoma (9). In all our experiments, daily treatment was initiated 1 day after inoculation of the RENCA cells to the lower pole of the left kidney and resulted in a significant reduction of primary tumor growth of 61% after 14 days and 67% after 21 days. An even more pronounced inhibition was observed for the development of metastases to the lung (98% and 79%, respectively) and to the abdominal lymph nodes (97.4%) after 21 days. The antitumoral effects of PTK787/ZK 222584 are very likely attributable to the inhibition of blood vessel formation because a significant decrease of vessel density in primary tumors as well as in metastases of the lung was observed. The application of PTK787/ZK 222584 was well tolerated in all animals. Control animals with high tumor burden, treated with vehicle only, lost weight. This effect could be prevented by treatment with PTK787/ZK 222584. In contrast, treatment with TNP-470 resulted in severe weight loss and ataxia, as described previously in other studies (14, 15) and in clinical trials (16–18). Therefore, application of TNP-470 had to be discontinued after 13 days of treatment, which probably accounts for the poor significance in reduction in primary tumor volume and number of metastases.

Interestingly, in all untreated animals we found a lower vessel density in tumors 3 weeks after inoculation as compared with 2 weeks after inoculation. This may be because of the high proliferation rate of murine tumor cells. We hypothesize that the proliferation rate of RENCA cells is superior to the proliferation rate of endothelial cells, resulting in decreasing vessel density in this fast-growing tumor. Even greater antitumor efficacy with PTK787/ZK 222584 might be obtained with slower growing tumors, and future studies will address this question.

Our finding, that a well-tolerated, selective inhibitor of VEGF-receptors has potent antitumor and antiangiogenic activity after oral application, suggests the therapeutic properties of PTK787/ZK 222584. The selective inhibition of VEGF-driven angiogenesis avoids side effects of conventional chemotherapy. This needs to be confirmed in clinical trials. A murine renal cell carcinoma, which closely simulates the human disease, indicates that renal cell carcinoma will be an appropriate indication for clinical studies with PTK787/ZK 222584.

Currently, determination of vessel density in human tumors can only be done in biopsies or by angiography. Noninvasive methods to detect vessel density changes under therapy, like dynamic magnetic resonance imaging or sonography, are therefore needed for ongoing clinical studies with promising antiangiogenic substances (19). Therefore, we investigated whether a color Doppler imaging ultrasound technique could be used to monitor blood flow in the renal artery feeding the tumor, as a potential surrogate for vessel density. Comparisons of systolic and diastolic blood flow in vehicle-treated animals and PTK787/ZK 222584-treated animals revealed a highly significant decrease of blood flow in the treated animals. The blood flow was decreased by ~60% as compared with untreated animals. Resistance index and heart rate were not affected. A systemic effect of the compound is unlikely because blood flow and resistance index in the abdominal aorta remained unchanged. Changes in blood flow were found to significantly correlate with vessel density but not with tumor volume. Because our observation from the histological staining shows a homogeneous distribution of vessels throughout the tumor tissue with the lack of hot spots, these findings underline the above-made hypothesis that in fast-growing tumors such as RENCA, the proliferation rate of tumor cells is superior to the proliferation rate of endothelial cells, resulting in a partially angiogenesis-independent growth of tumor. Nonetheless, blood flow could be an indirect measure of vessel density in the tumor in the case of one major tumor feeding vessel. The use of blood flow measurements in primary breast tumors in patients to differentiate between benign and malignant lesions supports this hypotheses (20).

Our experiments show that selective inhibition of VEGF-Rs by PTK787/ZK 222584 leads to inhibition of primary tumor growth and development of metastases in murine renal cell carcinoma. This effect
correlates with a reduction of vessel density in the primary tumor as well as in metastases. In contrast to TNP-470, an antiangiogenic substance with an unknown mode of action, or to cytotoxic substances (21), PTK787/ZK 222584 caused no obvious side effects in the RENCA model. Furthermore, we demonstrated that changes in vessel density of tumor tissue correlate with blood flow in one major tumor feeding vessel. This suggests that color Doppler imaging might be an attractive, noninvasive method for indirect detection of vessel density in clinical studies.
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