Simultaneous Inhibition of the Receptor Kinase Activity of Vascular Endothelial, Fibroblast, and Platelet-derived Growth Factors Suppresses Tumor Growth and Enhances Tumor Radiation Response

Robert J. Griffin, Brent W. Williams, Robert Wild, Julie M. Cherrington, Heonjoo Park, and Chang W. Song

Department of Therapeutic Radiology-Radiation Oncology, University of Minnesota Medical School, Minneapolis, Minnesota 55455 [R. J. G., B. W. W., H. P., C. W. S.], and Sugen, Inc, South San Francisco, California 94080 [R. W., J. M. C.]

ABSTRACT

We have investigated the effect of simultaneous inhibition of multiple angiogenic growth factor signaling pathways on tumor growth, tumor blood perfusion, and radiation-induced tumor-growth delay using SU6668, an inhibitor of the receptor-tyrosine kinase activity of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). The SCK mammary carcinoma, FSaII fibrosarcoma, and CFPAC human pancreatic carcinoma were grown s.c. in the hind leg of A/J mice, C3H mice, and Balb/cAnNCrl-nuBr nude mice, respectively. Daily i.p. injection of 100 mg/kg of SU6668 markedly suppressed the growth of these three tumor types. SU6668 also markedly prolonged the survival time of host mice bearing SCK tumors, which appeared to be caused by a reduction of metastatic tumor growth in the lung. There was little or no change in normal tissue blood perfusion, whereas in SCK tumors the perfusion decreased by 50% at 1 h after a single i.p. injection of SU6668, slightly recovered at 4 h, and completely recovered by 8 h. Interestingly, the tumor blood flow was significantly increased above the baseline level 24 h after SU6668 injection. After extended daily i.p. injections of SU6668, the tumor blood flow in all of the three tumor types studied was markedly decreased compared with control. The observed effects of this drug on tumor blood perfusion may partially explain the effectiveness of the compound in suppressing tumor growth and extending survival of tumor-bearing mice. We also observed that daily SU6668 administration and a single dose of 15 Gy of X-irradiation was significantly more effective than either treatment alone in suppressing tumor growth. Our results suggest that SU6668 increased the radiosensitivity of tumor blood vessels. We conclude that SU6668 is a potent therapeutic agent potentially useful to suppress tumor growth and enhance the response of tumors to radiotherapy.

INTRODUCTION

Among several dozen angiogenic growth factors thus far identified, VEGF, FGF, and PDGF are perhaps the most studied (1). The stimulation of endothelial cell proliferation by VEGF is mediated by interaction between VEGF and high-affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed on the surface of endothelial cells (2–6). FGF is a potent pleiotropic heparin-binding mitogen for vascular endothelial cells and tumor cells, and it synergistically acts with VEGF in stimulating new vessel growth (7–10). PDGF stimulates angiogenesis by up-regulating VEGF production and modulating the proliferation of pericytes (11, 12) and fibroblast-like cells surrounding the endothelium (13).

It has been repeatedly demonstrated that angiogenic treatment is potentially useful to halt tumor growth. Among a number of emerging strategies that aim at nullifying tumor angiogenesis, blocking the interaction of growth factors with their receptors has been demonstrated to be potentially useful (5, 6, 14). For example, significant antitumor effects have been observed with the use of antibodies raised against VEGFRs (5, 14). Recent studies have also demonstrated that the inhibition of the kinase activity of VEGFRs suppresses tumor growth by inhibiting angiogenesis. SU5416, 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin-2-one, is a synthetic small molecule designed to inhibit VEGFR-2 (Flk-1/KDR) tyrosine kinase activity, and it causes marked growth inhibition in a variety of experimental tumors (15–20). Phase III clinical trials to evaluate this drug are presently in progress (21).

In light of the important role of VEGF, FGF, and PDGF and their receptors in tumor angiogenesis, it is reasonable to expect that simultaneously antagonizing the VEGF, FGF, and PDGF signaling pathways may be more effective than antagonizing VEGF signal transduction alone. SU6668, (Z)-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-1H-pyrrol-3-yl-propionic acid, is a multipurpose small synthetic molecule designed to inhibit the kinase activity of FGF and PDGF receptors as well as VEGFRs (22). The angiogenic and antitumor effects of SU6668 have recently been demonstrated in a variety of experimental tumors (20, 22, 23).

Importantly, various angiogenic agents have been demonstrated to enhance the response of murine tumors to chemotherapy or radiotherapy. Although the mechanism is not yet completely understood, these studies may represent what is becoming a paradigm shift in the type of adjuvant treatment to be used with traditional chemotherapy or radiotherapy (14, 24–28). In this connection, it has been reported that the inhibition of VEGFR tyrosine kinase activity using SU5416 markedly enhanced the radioresponse of experimental tumors (19). We have investigated the effect of SU6668, which inhibits VEGF, FGF, and PDGF receptor kinases, on the response of tumors to radiotherapy in the present study. To our knowledge this is one of the first studies to elucidate the effects of simultaneous inhibition of multiple angiogenic growth factors on the response of tumors to ionizing radiation. In addition, we have for the first time quantified the effect of SU6668 on tumor blood perfusion to better understand the antitumor effects of this intriguing compound alone and as an adjuvant therapy.

MATERIALS AND METHODS

Tumors

SCK Tumor. This mammary carcinoma spontaneously arose in a female A/J mouse (Jackson Laboratories) in our laboratory. Cells from an early generation are stored in liquid nitrogen, and new cultures are established every 2–3 months.

FSaII Tumor. This fibrosarcoma of C3H mice (Jackson Laboratories) was originally obtained from the laboratory of Dr. Herman Suit (Massachusetts General Hospital, Boston, MA). Stock cells are stored in liquid nitrogen and new cultures are established every 2–3 months. The SCK and FSaII tumor cells grow well in RPMI 1640 supplemented with 10% calf serum.

CFPAC Human Pancreatic Carcinoma. This human pancreatic carcinoma (American Type Culture Collection, Manassas, VA) is grown in Balb/cAnNCrl-nuBr nude mice (Charles River Labs, Wilmington, MA). Cells stored...
in liquid nitrogen are grown in Iscove’s modified Dulbecco’s medium with 10% FCS (Life Technologies, Inc.).

Tumor Induction

The SCK and FSaII tumor cells in exponential growth phase in culture were harvested using 0.25% trypsin and were washed and counted. About $2 \times 10^5$ cells in 0.05 ml of serum-free medium were injected s.c. into the hind thigh of male A/J mice for SCK tumors and female C3H mice for FSaII tumors. The SCK tumors and FSaII tumors grow to 250 mm$^3$ in about 8 days and 12 days, respectively. The CFPAC tumor cells were harvested using a 0.05% trypsin-EDTA mixture, washed, suspended in Dulbecco’s PBS, and counted. About $1 \times 10^6$ cells in 0.05 ml of PBS were injected s.c. into the hind thigh of female nude mice. The CFPAC tumors grew to 250 mm$^3$ in ~21 days. The tumor size was determined using a caliper, and the tumor volume was calculated using the formula $a^2b/2$, where $a$ and $b$ are the shorter and longer diameters of the tumor, respectively.

Drug Treatments

Starting on the day when the tumors had grown to the desired volume, depending on the purpose of the experiment, the tumor-bearing mice received i.p. once-a-day injections of 100 mg/kg SU6668 (Sugen Inc., South San Francisco, CA) dissolved in 0.05 ml of DMSO. Control animals received injections of the same volume of DMSO.

Irradiation of Tumors

The A/J mice bearing SCK tumors were treated with 100 mg/kg SU6668 once a day for two days. Two h after the second SU6668 treatment, the host mice were anesthetized with an i.p. injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. About 15 min later, the mice were covered with a 4 mm thick lead shield and the tumor-bearing legs were gently extended into the radiation field and exposed to X-rays at a dose rate of 1.4 Gy/min. A Philips 250 kV orthovoltage machine (Philips Medical Systems, Brookfield, WI) was used for the irradiation.

Blood Perfusion Determination

The blood perfusion in tumors and several normal tissues was measured with the $^{86}$RbCl uptake method (29). The mice were anesthetized as described above, 5 $\mu$Ci of $^{86}$RbCl in 0.1 ml of PBS was injected through the lateral tail vein, and the mice were killed 60 s later by cervical dislocation. The tissues were removed, weighed, and the radioactivity was counted with a well-type gamma counter (1282 Compu gamma; Pharmacia LKB Wallac, Turku, Finland). From the radioactivity in the tissue sample and that in the reference, the percentage of injected $^{86}$RbCl per gram of tissue was calculated.

Statistical Analysis

Data sets were analyzed using a commercially available software package (InStat 2.03, Graphpad Software, Inc.). A two-tailed Student’s $t$ test was used to determine the validity of the differences between control and treatment data sets. A $P$ of 0.05 or less was considered significant.

RESULTS

Tumor Growth and Animal Survival. Fig. 1 shows that daily i.p. injection of SU6668 at 100 mg/kg significantly suppressed the growth of CFPAC tumors, FSaII tumors, and SCK tumors. The treatment of CFPAC tumors with SU6668 was begun when the tumor volume reached 250–300 mm$^3$. The volume doubling time of control CFPAC tumors was 8.9 ± 0.9 days, whereas daily treatment with SU6668 increased the tumor volume doubling time to 18.8 ± 0.7 days ($P < 0.0001$). Control FSaII tumors that averaged 250 mm$^3$ grew to 1000 mm$^3$, a 4-fold increase, in 9.4 ± 0.7 days, whereas the volume of tumors treated with SU6668 increased four times in 13.4 ± 0.8 days ($P < 0.006$). The growth of SCK tumors was also significantly suppressed by SU6668 treatment. To reveal whether the efficacy of SU6668 on tumor growth depends on the tumor size at the start of treatment, we began treating SCK-bearing host mice with SU6668 when the tumor volume reached either 125 mm$^3$ or 250 mm$^3$. SU6668 treatment was effective in suppressing the growth of both 125 mm$^3$ and 250 mm$^3$ tumors. The large and small control tumors grew 4-fold in volume in 6.4 ± 0.3 and 5.0 ± 0.8 days, respectively, whereas large and small tumors treated with SU6668 grew 4-fold in volume in 9.5 ± 0.3 days and 7.9 ± 1.5 days, respectively ($P \leq 0.007$). The difference between the growth delay of large (3.1 days) and small (2.9 days) tumors caused by SU6668 treatment was not statistically significant.

Fig. 2 shows the effect of daily treatment of mice bearing SCK tumors with SU6668 or vehicle (DMSO) on the survival time of the host mice. The treatment was started when the tumors reached 125 mm$^3$ (~4–5 days after tumor cell inoculation) and the day of death represents the number of days after treatments were started. The control mice treated with only the vehicle started to die on day 4, and all of the control animals died by day 12, when the primary tumor volume had reached an average of 1575 ± 197 mm$^3$. On the other hand, the mice treated with SU6668 began to die on day 10, and some tumor-bearing mice survived until day 18, when the average primary tumor volume was 1878 ± 105 mm$^3$. The median survival time of host mice treated with only the vehicle was 8 days, whereas that of mice treated with SU6668 was 14 days after the start of treatment.
Blood Perfusion. The effect of single or multiple injections of SU6668 on tumor blood perfusion was determined with $^{86}$Rb uptake as shown in Fig. 3. The $^{86}$Rb uptake in control SCK tumors was $2.1 \pm 0.2\%$ of the total injected radioactivity per gram. As shown in Fig. 3A, when host mice received an injection of a single dose of 100 mg/kg of SU6668, the $^{86}$Rb uptake in SCK tumors decreased to about one-half of that in control mice within the 1st hour after drug administration ($P < 0.008$). The perfusion recovered to control level by 8 h and increased to about 40% above the corresponding control value by 24 h after injection ($P = 0.03$). At 2 h after a second dose of 100 mg/kg SU6668, given 24 h after the first dose, the blood perfusion again declined to 75% of the control value ($P = 0.05$). The perfusion in the skin, muscle, and kidney of A/J mice was measured concomitantly with the tumor blood perfusion. In contrast to the effects of SU6668 on tumor perfusion, there was no significant difference in normal tissue perfusion found between control and drug-treated mice except for the kidney perfusion at 1 h after SU6668 injection, which decreased to 60% of control level and recovered completely by 4 h (data not shown).

Fig. 3B shows the effect of daily administrations of SU6668 on tumor blood perfusion determined by measuring $^{86}$Rb uptake in the FSaII and CFPAC tumors that were used to study the effect of SU6668 on tumor growth (shown in Fig. 1). The $^{86}$Rb uptake in control FSaII tumors was $2.8 \pm 0.3\%/g$ and $1.3 \pm 0.3\%/g$, respectively. At 24 h after the last of 17 daily injections with SU6668, the $^{86}$Rb uptake in CFPAC and FSaII tumors was reduced to $1.9 \pm 0.3\%/g$ and $0.5 \pm 0.1\%/g$, respectively ($P \leq 0.05$). The $^{86}$Rb uptake in control SCK tumors was $2.1 \pm 0.2\%/g$, as stated above, but 24 h after 7 daily injections of 100 mg/kg SU6668, the $^{86}$Rb uptake was reduced to $1.4 \pm 0.3\%/g$ ($P = 0.01$).

Enhancement of Tumor Radiation Response. After SCK tumors grew to about 125 mm$^3$, we began daily treatment of the host mice with 100 mg/kg of SU6668. Tumors were irradiated with 15 Gy of X-rays 2 h after the second drug treatment, and daily treatment of host mice with SU6668 was continued after tumor irradiation until the end of the experiment. Fig. 4 shows that the suppression of tumor growth caused by daily 100 mg/kg SU6668 was slightly greater than that caused by 15 Gy of radiation, and the combination of SU6668 treatment and radiation was far more effective than either treatment alone. In Table 1 the number of days required for the tumors to grow four times in volume after various treatments are compared. The tumor-growth delay caused by daily 100 mg/kg SU6668 treatment in combination with 15-Gy irradiation was 15.3 $\pm$ 1.6 days which was roughly triple that caused by either of these treatments alone.

| Table 1 Response of SCK tumors to SU6668 treatment and local irradiation |
|-----------------------------|----------------------|--------------------------|
| Treatment                  | Days required for tumors to grow four times the original volume ($\pm$ SE) | Growth delay (days) |
| Control                    | 5.0 $\pm$ 0.8         | 5.3                      |
| 15 Gy                      | 10.3 $\pm$ 0.8        | 4.3                      |
| SU6668                     | 9.3 $\pm$ 0.6         | 5.3                      |
| SU6668 + 15 Gy             | 20.3 $\pm$ 1.6        | 15.3                     |

Fig. 4. Radiation-induced growth delay of SCK tumors. The mice were treated with either 50 $\mu$L of DMSO or 100 mg/kg SU6668 for 2 days; 2 h after the second drug treatment, the tumors were irradiated with 15 Gy. The tumor-bearing host mice were continuously treated with daily injections of drug or vehicle until the end of the experiment. Each group contained 7–10 mice; error bars, $\pm$ 1 SE.
(P ≤ 0.0003). There was also a synergistic effect of SU6668 and tumor irradiation on animal survival. The mice in the X-irradiation-alone group and SU6668-treatment-alone group began to die on day 6 and 10, respectively, whereas none of the host mice that received the combined treatments died before day 26 (data not shown).

**DISCUSSION**

Our results with two different murine tumor models and a human pancreatic carcinoma xenograft model confirm previous reports that the inhibition of angiogenesis-related receptor tyrosine kinase activity suppresses tumor growth (15–20, 22–23). The suppression of tumor growth caused by daily treatment with SU6668 (Fig. 1) was apparently related to the marked decline in tumor blood perfusion caused by SU6668 (Fig. 3). Microscopic observation of vascular density in histological preparations of tumors and changes in vascular patterns in small tumors grown in window chambers are commonly used as a measure of antiangiogenesis. Although these methods are useful to investigate morphological and structural changes in blood vessels, we believe that measuring actual tumor blood perfusion, an important and novel part of the present study, is an invaluable indicator of the action of antiangiogenic agents on existing tumor vasculature.

A question that remains is what amount of the reduction of tumor blood perfusion after multiple daily treatments with SU6668 (Fig. 3B) was attributable to the inhibition of new blood vessel formation as opposed to damage to existing tumor blood vessels. It has been reported that VEGF, FGF, and PDGF may be required not only for angiogenesis but also for survival of existing endothelial cells (20). Therefore, it is probable that the marked reduction of tumor blood flow caused by extended treatment with SU6668 in the present study was caused, in part, by damage of existing tumor vasculature. In this connection, the early response of tumor microvasculature to SU6668 treatment has been recently reported (30). Here, SU6668 induced apoptosis in tumor vessels within 6–12 h after drug administration. Moreover, SU6668 treatment resulted in a decrease in tumor mitotic index and dramatically reduced the functional microvessel density in the tumor. These data suggest that the antitumor action of SU6668 is, at least in part, dependent on a significant degree of damage to existing vessels.

Interestingly, although the tumor blood perfusion decreased in the 1st hour after a single i.p. injection of SU6668, it increased by 24 h after the drug administration (Fig. 3A). This observation may be related to the findings of Laird et al. that when the tumor endothelium was undergoing breakdown several hours after SU6668 treatment, VEGF transcript levels in RNA isolated from the tumor were elevated 2- to 3-fold and then returned to near normal levels by 24 h (30). One interpretation of these data are that the rapid vessel perturbation and the accompanying reduction in tumor perfusion caused by SU6668 resulted in hypoxic stress in the tumor tissue leading to induction of VEGF transcription. VEGF is known to induce nitric oxide production leading to increases in vascular permeability and vasodilation (31), which may explain why blood perfusion was transiently elevated after the single dose of SU6668. Ultimately, repeated administration of SU6668 may render many vessels nonfunctional because of a massive loss of endothelial cells. Clearly, the complete mechanism of the dynamic changes in tumor perfusion after single or multiple injections of SU6668 needs further elucidation.

The ability of SU6668 to significantly prolong the survival time of tumor-bearing host mice (Fig. 2) is potentially important and echoes the results of other recent studies (20, 23). In our studies, we treated A/J mice bearing SCK tumors, which normally metastasize to the lung, with SU6668 at 100 mg/kg/day beginning when the tumors were about 125 mm³ in size. An example of lung preparations of tumor-bearing A/J mice treated for 7 days with either DMSO or 100 mg/kg SU6668 is shown in Fig. 5. In a preliminary histological study, we observed that in lung sections from DMSO-treated tumor-bearing mice, there were 9.8 ± 3.7 metastatic loci/section, whereas only 2.3 ± 0.3 metastatic loci/section were observed in the lungs of tumor-bearing mice treated daily with SU6668. In addition, the size of the metastases in the lungs of mice treated with SU6668 was noticeably smaller than that in DMSO-treated control mice. The significant decline in the number and size of metastatic nodules in the lungs seems to reflect the prolonged survival time caused by SU6668. Our future studies aim at determining the mechanism of the survival benefit afforded to tumor-bearing mice by SU6668 treatment.

Our observation that SU6668 significantly enhances radiation-induced tumor-growth delay supports the growing body of data indicating that antiangiogenic agents may be used to improve the efficacy of radiotherapy or chemotherapy. The list of antiangiogenic agents that have been demonstrated to enhance the antitumor effect of radiation includes angiostatin (26), anti-VEGF or VEGFR antibodies (14, 27, 28), isocoumarin (24), TNP-470 (25), and VEGF-toxin constructs. The mechanisms underlying the enhancement of radiation response caused by antiangiogenic agents is unclear. To date, a time-dependent increase in tumor pO₂ after treatment with antiangiogenic agents (14, 28) or an inhibition of VEGF-induced protection against, and/or repair of, radiation damage in endothelial cells (27, 32) have been suggested. In our study of the combination of SU6668 and radiation, tumors were irradiated 2 h after the second daily administration of the drug when tumor blood perfusion was at 75% of control level (Fig. 3A). Therefore, the increase in radiation response of the tumors by exposure to SU6668 did not appear to have resulted from an increase in tumor oxygenation via an increase in tumor blood perfusion. We cannot rule out, however, the possibility that SU6668 alters the oxygen consumption rate in the tumor endothelium, the tumor cells, or both. Although significant, our results lean toward an additive rather than an interactive effect of the combination of SU6668 and radiation. We are currently working to understand the optimal combined treatment scheme to produce a greater-than-additive antitumor response. A recent study has demonstrated that SU5416, an inhibitor of VEGF receptor kinase, increased the radiation-induced apoptosis in endothelial cells (19). It has also been reported that SU6668 caused apoptosis in endothelial cells in vivo (23). Taken together, these data suggest that enhancement of tumor response to radiation by SU6668 may occur most readily by increasing the radiosensitivity of tumor blood vessels.

In conclusion, this study indicates that the multipotent angiogenesis inhibitor SU6668 is effective in perturbing existing tumor vascular function and further uncovers the mechanism(s) by which growth

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4 R. Wild et al., unpublished observations.
factor inhibition alters tumor growth. Our data shows that SU6668, currently in Phase I clinical trials (21), has potential in combating tumor progression and may be particularly effective as an adjuvant to radiotherapy of human tumors.

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REFERENCES

9. de Jong, J. S., van Diest, P. J., van der Valk, P., and Baak, J. P. Expression of growth factor inhibition alters tumor growth. Our data shows that SU6668, currently in Phase I clinical trials (21), has potential in combating tumor progression and may be particularly effective as an adjuvant to radiotherapy of human tumors.

SU6668 AS AN ADJUVANT TO TUMOR RADIOTHERAPY

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