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Flk-1 Specific Kinase Inhibitor (SU5416) Inhibited the Growth of GS-9L Glioma in Rat Brain and Prolonged the Survival

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Abbreviation: Flk-1 (fetal liver kinase-1)

[Background] Accumulating evidences suggest that tumor growth and metastasis depend on angiogenesis. At present, plenty of efforts are made to discover a chemical compound that specifically inhibits tumor angiogenesis either by reducing pro-angiogenic factor or increasing anti-angiogenic factors. [Object] SU5416, a novel, synthetic, potential inhibitor of angiogenesis specifically blocks the Flk-1/KDR tyrosine kinase activity. In vivo effect of SU5416 in the treatment of intracranial tumors has not been previously described. [Methods] We transplanted GS-9L cells into the right caudate nucleus of male Fisher 344 rats and administrated SU5416 intraperitoneally (i.p.) to investigate the impact of SU5416 on tumor angiogenesis and growth in vivo. Starting on Day 1 or Day 8, forty-two animals were treated with SU5416 at three different doses (e.g. 12.5, 25.0 and 50.0 mg/kg body weight) via i.p. injection every day until the end-point. As a control, seven animals received no treatment and after implant fourteen animals were treated with vehicle (DMSO) only. [Results] SU5416 prolonged the survival in the treated groups without any significant systemic adverse effect. Median survival in the treated group started on Day.1 was statistically longer compared to that in the control groups ($p<0.01$). Histological analysis of the treated tumors showed an increase in necroses and reduced in vascularity compared to the control tumors. Furthermore, the number of apoptotic cells increased in the treated tumors on a TUNEL stain. [Conclusion] Small molecular compounds, such as SU5416 may be useful therapeutics that specifically inhibits the enzymatic activity of Flk-1 kinase and downstream events of tumor angiogenesis.

Among the wide spectrum of endothelial growth factors, vascular endothelial growth factor (VEGF) has been shown to be a central mediator of tumor angiogenesis. VEGF binds to its tyrosine kinase receptors (Flt-1 and KDR/Flk-1 expressed mainly on the surface of tumor endothelial cells) and regulates angiogenesis during the development of brain tumors, such as astrocytomas and glioblastomas (9,10). The expression of VEGF in these tumors is remarkably up-regulated and restricted to hypoxic areas (peri-necrotic palisading cells) (7,11,18). Recently, several observations strongly support the major role of VEGF/VEGF receptor system in tumor angiogenesis and the paracrine mechanism in the development of solid tumors. This paracrine loop of VEGF produced by tumor cells and its receptors

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expressed on adjacent endothelial cells plays a primary role in malignant transformation, neovascularization, and invasiveness of malignant gliomas (1). The restricted expression of Flk-1/KDR to the endothelial cells strongly suggests that it plays a major role in tumor angiogenesis (6,11). This is also supported by several animal experiments, where VEGF/Flk-1 paracrine mechanism was inhibited in athymic mice by dominant-negative Flk-1 mutants (7,8), anti-VEGF monoclonal antibodies (4), anti-Flk-1 monoclonal antibodies (12), recombinant soluble VEGF receptor (3), Flk-1 specific kinase inhibitors (2,16) or anti-VEGF antisense (13,14). These therapeutical approaches revealed a significant inhibition of tumor angiogenesis and tumor growth in vivo. Therefore, the inhibition of VEGF-Flk-1/KDR signal transduction might be promising for the treatment of highly vascularized tumors, such as malignant gliomas.

Recently, SU5416, a new specific inhibitor of Flk-1 tyrosine kinase activity, was developed by SUGEN Inc. (USA). Flk-1, a tyrosine kinase receptor, is autophosphorylated with VEGF followed by downstream signal transduction and promotes angiogenesis. SU5416 that can specifically reduce tyrosine autophospholylation of Flk-1 interrupts this signaling for angiogenesis. In a tissue factor induction assay, SU5416 inhibits the proliferation of HUVEC (Human umbilical vein endothelial cell) promptly and completely at 70nM of IC_{50}. Testing of SU5416 using NIH3T3 cells overexpressing various receptors revealed an inhibiting activity with an IC_{50} of 1.04±0.53μM in Flk-1 overexpressing NIH3T3. In contrast, NIH3T3 cells overexpressing EGFR, insulin receptor and FGFR showed a complete lack of activity (IC_{50}>100μM) (2). However, SU5416 have no anti-tumor effect in vitro, since it does not directly exert on tumor cells. In contrast, intravenous or intraperitneal injection of SU5416 showed high anti-tumor effect on various subcutaneous xenograft models accompanied by a remarkable decrease in tumor vascurature (2). Since SU5416 can be available by oral intake, clinical trials (phase I/II) against lung cancer, colon cancer, breast cancer and prostartor cancer are presently under evaluation in USA and Europe.

SU5416 represents the first synthetic inhibitor of VEGF receptor function to enter clinical studies. It has been shown to inhibit tumor growth in several extracranial solid tumor models, such as subcutaneous and intracolonic xenograft models. However, in vivo effect of SU5416 on the treatment of intracranial tumors has not previously been described. Accordingly, we transplanted syngeneic GS-9L cells into the right caudate nucleus of male Fisher 344 rats and treated with intraperitneal administration of SU5416 to investigate the impact of SU5416 on tumor angiogenesis in vivo and survival of tumor-bearing animals. This is the first study of angiogenic inhibition by SU5416 in rat brain tumor.

MATERIALS AND METHODS

Synthesis of Compounds. SU5416 is 3 -[(2, 4 –dimethylpyrrol –5 –yl) methyllidenyl] –indolin –2 -one (Fig.1). Characterization of this compounds was performed by standards analytical methods (nuclear magnetic resonance and spectrometry). SU5416 was prepared from commercially available 3,5-dimethylpyrrol-2 calboxaldehyde by aldocondensation with indolin-2-one in ethanol in the presence of piperidine (17).

Cell line and cell culture. GS9L (chemically induced gliosarcoma) cells were grown in RPMI 1640 (Gibco) supplement with 10% heat-inactivated fetal bovine serum (FBS). All cultures contained 100 units of penicillin and 0.1mg of streptomycin per milliliter of medium. Incubations were carried out in an atmosphere of humidified 5% CO_2 at 37^{o}C.

Animals and tumor models. Male Fisher 344 rat (150-200g) were used for syngeneic GS9L cell implants. For intracerebral implantation, animals were anesthetized with a i.p. injection of Ketanest (100mg/kg body weight) and Rompum (10mg/kg body weight).

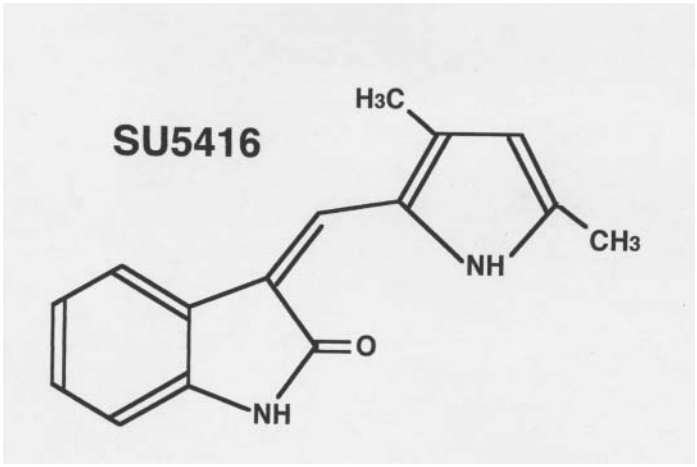


FIG. 1. SU5416.

Animals were fixed in a stereotaxic apparatus, a midline skin incision was made and a burr hole was opened 1mm anterior and 3mm lateral of the bregma. $5x10^4$ GS9L cells (suspended in a volume of 5µl serum-free medium) were injected slowly into right caudate nucleus 5mm deep from brain surface using a Hamilton syringe.

Anti-angiogenic therapy. Flk-1 specific kinase inhibitor (SU5416) was kindly supplied by Fong A. (Sugen Redwood City, CA94063, USA). SU5416 was dissolved in Dimethyl sulphoxide (DMSO: Sigma Chemical Co.) at three different doses: 12.5mg/kg bw/100µl DMSO, 25.0 mg/kg bw/100µl DMSO, 50.0 mg/kg bw/100µl DMSO. Sixty three rats were stereotactically implanted with $5x10^4$ GS-9L cells in the brain and were divided into five groups: Group 1; wild-type non-treated control group, Group 2; DMSO treated group, Group 3; SU5416 treated group: 12.5 mg/kg bw/100µl, Group 4; SU5416 treated group: 25.0mg/kg bw/100µl, Group 5; SU5416 treated group: 50.0 mg/kg bw/100µl. Group 1 has seven rats, Group 2-5 has fourteen rats each. In the treated groups, anti-angiogenic therapy with daily i.p. administration of SU5416 was started on Day 1 (Group A) or Day 8 (Group B) when tumors reached measurable dimensions after implantation (average tumor volume at Day 8 was $35.3\pm10.9mm^3$) and discontinued at the survival end-point. Animals were checked daily for the development of symptoms associated with progression of implanted intracerebral tumors. In survival experiment, survival end point was the onset of hemiplegia, moribund state, or inability to feed or drink. At this occurrence, animals were euthanized with an overdose of anesthesic and difference between control and treated groups was evaluated. All animals experiments were conducted in accordance with the Kobe University guidelines for the care of laboratory animals. Statistical significance was analyzed using a paired Student's t-test. All data in this study are expressed as means ± SD. A probability values of <0.05 was considered to indicate a significant difference.

Histological analysis. At the end of the treatment period, tumors were harvested. Part of the specimen was fixed in 4% paraformaldehyde, embedded in paraffin, cut with a microtome to 5µm sections and stained with Hematoxylin & Eosin or analyzed with immunohistochemistry.

Immunohistochemistry (CD31 staining). Paraffin-embedded tissue sections (5µm thick) were dewaxed in xylene, rehydrated and washed in PBS. Slides were incubated for 1 hour at 37^oC with 0.05% protainase K in PBS, washed in PBS and incubated in 0.1% H_2O_2 in methanol for 30 minutes. Paraffin sections were then washed in PBS and incubated in 20%

normal goat serum in PBS for 30 minutes. Sections were then incubated with a primary antibody (mouse anti-rat CD31, Serotec Ltd. Oxford, England, 1:500 diluted) overnight at 4°C, washed with PBS, incubated with a second antibody (biotinated goat anti-mouse IgG, 1:500 diluted) for 1 hour at room temperature, and finally incubated with a peroixydase-streptoavidin complex (Vector) according to the manufacturers instructions. Immunoreactivity was visualized with 3,3 diaminobenzidine (Sigma Chemical Co.) dissolved at 0.06% (w/v) and 0.03% H_2O_2 in PBS. Slides were counterstained with Hematoxylin, dehydrated, cleared in xylene and mounted.

Microvascular density. To evaluate the quantitative change in tumor vasculature, microvascular density (MVD) was estimated on CD31 immunostained sections. The number of microvessels was counted under microscope in several fields and the mean MVD was calculated to the average number of microvessels.

In Situ Apoptotic Cell Detection Assay. To detect apoptotic cells, paraffin embedded sections were processed using the MEBSTAIN Apoptosis Kit II (MBL) according to the manufactures manual. The apoptotic index was estimated by the percentage of positive staining cells visualized under fluorescent microscopy at high power magnification field (x200).

RESULTS

Survival analysis. With intracerebral inoculation of GS-9L cells alone (5 x 10^4 cells), tumors formed in all rats. In Group 1, all animals showed signs of raised intracranial pressure 16.3±0.5 days after grafting and were sacrificed. In the Day 1 started group (Group A), the median survival of SU5416 treated group was 16.8±2.3 (Group 2, NS), 24.3±5.7 (Group 3, $p<0.05$), 25.2±2.6 (Group 4, $p<0.0005$), 24.5±5.4 (Group 5, $p<0.05$) days, respectively. The survival was significantly prolonged by the treatment of SU5416 (Fig.2). In Day 8 started group (Group B), the median survival of SU5416 treated group was 14.3±0.8 (Group 2, $p<0.00001$), 17.9±1.4 (Group 3, $p<0.01$), 16.6±1.3 (Group 4, NS), 17.0±2.2 (Group 5, NS) days, respectively. Only the survival of Group 3 was significantly prolonged compared with the control group. However, comparing to the Group 2 (treated with carrier DMSO), all groups treated with SU5416 had a significant prolongation independent of dose escalation (Group 3; $p<0.0001$, Group 4; $p<0.001$, Group 5; $p<0.01$) (Fig.3).

Histological analysis. Untreated tumors (Group 1) revealed a homogeneous mass of viable cells, which were very well vascularized with only a few small necrosis. In contrast, tumors treated with SU5416 displayed a large number of necrosis surrounded by a dense layer of viable tumor cells deficient in capillaries and other blood vessels (Fig.4). These findings suggest the anti-angiogenic effect of SU5416 on tumor tissue.

To investigate further the inhibition of tumor angiogenesis, immunohistological analysis of tissue samples with anti-CD31 antibodies was performed. In GS-9L gliomas, few small vessels were sparsely distributed throughout the tumor, while in the treated groups, few vessels around the necrosis were observed.

To exclude the participation of host immune reaction in the anti-tumor effect, infiltration of T-lymphocytes was investigated by immunohistochemistry for CD3. However, immunostaining of tissue samples revealed no significant infiltration of CD3 positive T-cells (data not shown).

Microvascular density (MVD). The mean MVD was significantly reduced on the SU5416 treated groups (21.5±7.5 / field x 200, $p<0.001$) compared with control groups (87.4±4. 4 / field x 200). In addition, Group A, where the treatment of SU5416 was started on Day 1, showed higher reduction of MVD than Group B where the treatment was initiated on Day 8 (38.2±6.6 vs 80.3±7.1 / field x 200, $p<0.001$).

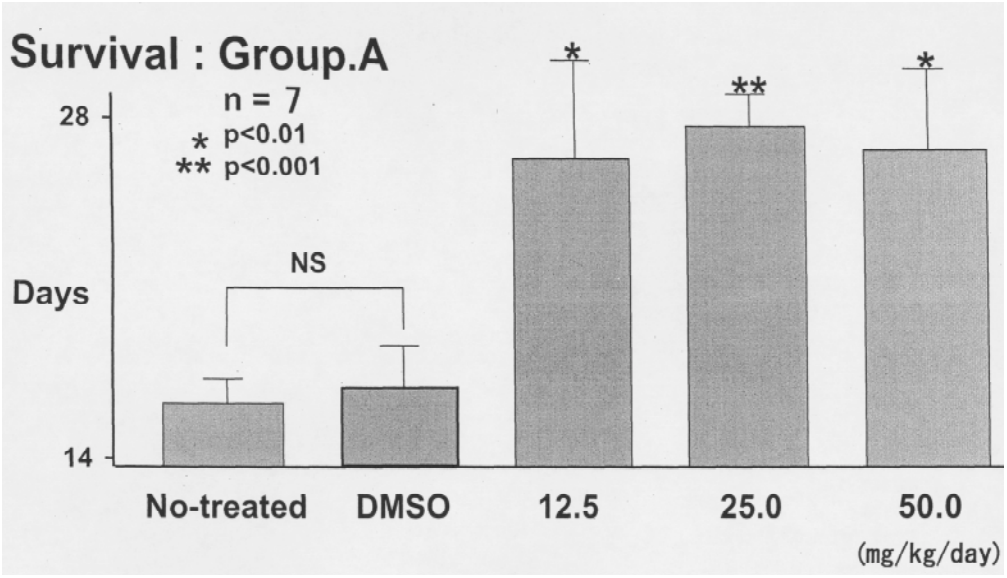


FIG. 2. Survival of rats bearing GS-9L treated with SU5416 (Day 1 start).

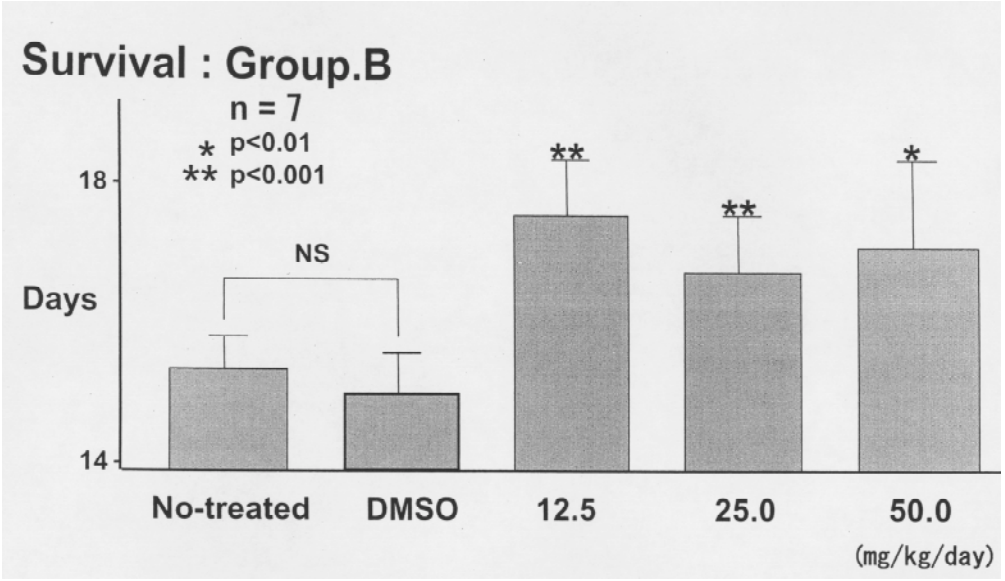


FIG. 3. Survival of rats bearing GS-9L treated with SU5416 (Day 8 start).

Apoptosis Index. Apoptosis Index of SU5416 treated group (Group 5, Day 1 start) was significantly increased (25.4%, $p<0.01$), compared with non-treated negative control (12.1%) (Fig.5). These findings suggest that apoptosis is involved in the anti-tumor effect of SU5416.

Toxicity study. There was no evidence of weight loss and neuronal toxicity, such as white matter changes, hemorrhages or hydrocephalus. The ventricular size appeared normal and there was no demyelination or neuronal loss in the striatum and corpus callosum adjacent to the mass. Flk-1/KDR plays an important role in the development of fetal vascular network, however, after birth, its constitutive expression is restricted only a few organs, such as choroid plexus in a brain, glomerulus in a kidney. In this study, no apparent damage was identified on these organs.

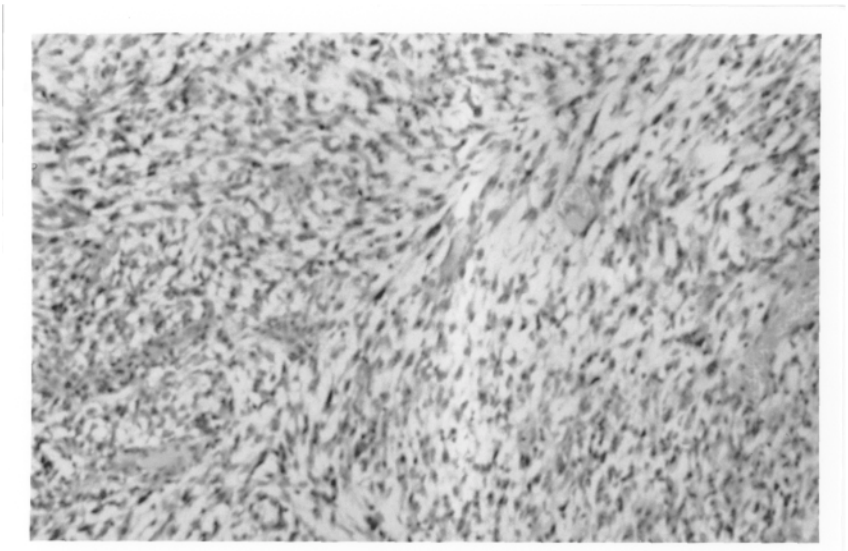
DISCUSSION

Conventional chemotherapeutics have direct cytotoxic effect on tumor cells, but induce high incidence of drug resistance and serious systemic adverse effects as well. In contrast, recent anti-angiogenic substances, such as small molecule inhibitors are less likely to develop drug resistance and serious side effects since they target only activated endothelial cells on a molecular basis instead of diverse tumor cells. Although the effect is temporary and cytostatic, they inhibit angiogenesis and lead to small avascular tumors maintained in a domant state. This is especially important for the patients need for a long-time control of residual tumors and a prevention of tumor recurrence. A number of inhibitors of angiogenesis have been reported to be capable of inhibiting the growth of experimental brain tumors. Clinical trials using at least 20 angiogenesis inhibitor are in progress in USA and Europe.

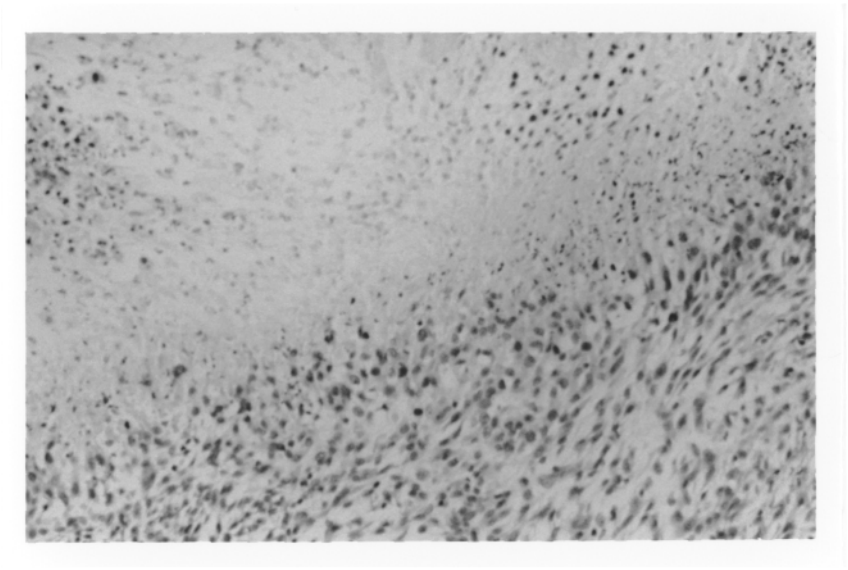
In the present study, we demonstrated on anti-tumor effect of SU5416 caused by the inhibition of tumor angiogenesis in vivo. We showed the effect of SU5416 on the prevention of tumor growth and on the treatment of established tumors. A large number of necrosis, increase the number of apoptotic cells, and no sign of immune reaction, suggest the anti-angiogenesis effect of SU5416 on gliomas. SU5416 inhibited the proliferation of endothelial cells followed by the reduction of tumor vessels and depletion of various survival factors for tumor and endothelial cells. Depletion of these survival factors induced the regression of endothelial cells and collapse of tumor vessels, resulting tumor necrosis and secondary apoptosis.

In Day 1 started groups (Group A), there was statistical significance between the treated and non-treated groups, whereas in a Day 8 started groups (Group B), there was partial difference between them. This implicates the importance of initiating time of this treatment. This fact is also supported by the results of MVD. On Day 8, implanted tumor grows up approximately $35.3\pm10.9mm^3$. At this stage, the tumors are too large to be supplied enough amount of SU5416 and consequently grow more rapidly comparing with Day 1 started groups.

In our experiment, even continuous administration of SU5416, all animals finally died of tumor development at the end of this study, although histological analysis showed so much necroses in tumor tissues. There may be several potential explanation for the lack of a persistent anti-tumor effect of SU5416 in the GS-9L glioma. Firstly, the concentration of SU5416 in tumor tissue was too low? The interval of administration was too short? The half-life is somewhat dose dependent following i.p. administration. It varies from about 0.5hr to 2hrs. In experiments from with a similar compound, plasma level of up to 30μg/ml is observed 1 hr following a i.p. dose of 50mg/kg in DMSO and decreases by almost 50% in about 2 hrs (2). In another experiment, SU5416 maintained a long-lasting effect (at least 72 hrs) even after removal of the compound in spite of a short plasma half-life and twice a week

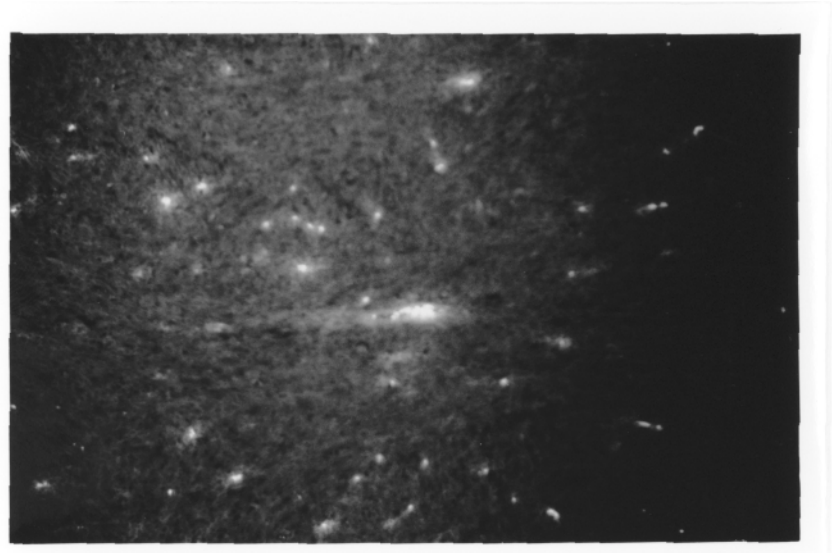


(a)

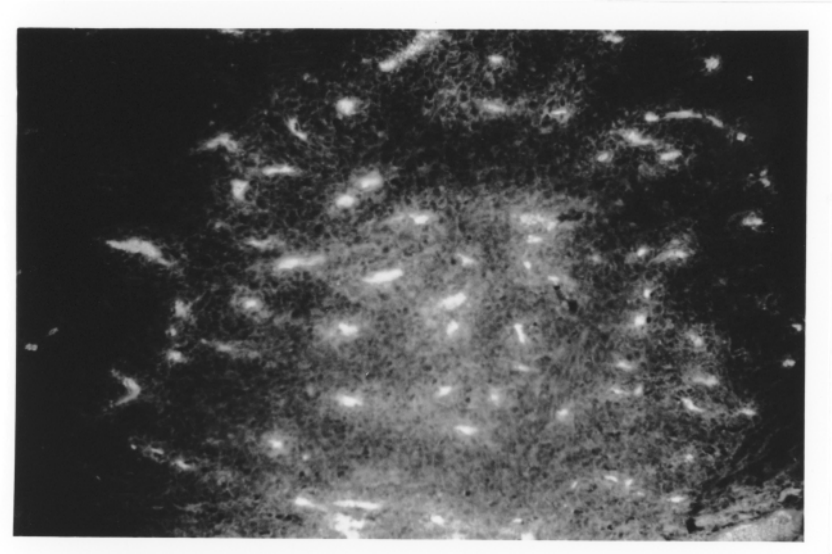


(b)

FIG. 4. Histological analysis of tumors from GS-9L cells treated with SU5416 (H&E); (a) vector only control. (b) treated with SU5416 (Day 1 start, 50mg/kg/day).



(a)



(b)

FIG. 5. In situ detection of tumor cell apoptosis by TUNEL method; (a) vector only control. (b) treated with SU5416 (Day 1 start, 50mg/kg/day).

was recommended as an administration interval. Therefore, our dosage and interval were within an effective range. In our study, the inhibitory effect of SU5416 did not show a dose-dependent manner. This may suggest that 12.5mg/kg is an upper limit of maximum response. (i.e. Flk-1 receptors are fully inactivated.) An important problem in the treatment of CNS disorders is the insufficient delivery of substances through B.B.B.. Though SU5416 is a small molecule (Mw=232), hydrophobic and expected of high penetration of B.B.B., there is no reliable data about the penetration rate of this agent in the brain tumor models. In addition, intracranial GS-9L xenograft is a good model for human glioblastoma, where B.B.B. is extensively broken. Then a higher and selective uptake of SU5416 is expected into tumor tissue rather than normal brain. If the permeability of this compound through B.B.B. is insufficient, trans-arterial administration of this compound may increase the anti-tumor effect. Secondly, GS-9L glioma xenografts produced angiogenic factors or used angiogenic pathways that were not blocked by SU5416. When one angiogenic pathway, such as VEGF / VEGF receptor system is blocked with SU5416, other angiogenic pathway, including bFGF, PDGF, TGF-β, Tie-2 signaling may compensate for it (15). It was recently proposed that VEGF is essential only for the initial phase of tumor growth, and when the tumor grows up to a certain size, other factors, such as bFGF, may substitute for VEGF (19). In such case, SU5416 may be ineffective in a large tumors. Thirdly, the efficacy of SU5416 may depend on the growth rate of tumors and the requirement of new vessels for tumor growth. GS-9L gliomas are highly proliferative and grow up more than 1000 mm^2 within 2 weeks after implantation. In these fast-growing tumors, the turn over of Flk-1 may exceed the inhibitory effect of SU5416. Taken together, the efficacy of SU5416 may depend on many factors including the extent of VEGF or VEGF receptor expression in tissue, the type of tumor, size of tumor, timing of administration, relation with other angiogenic pathways, and other pharmacological factors.

As described above, the effect of this kind of compounds is basically cytostatic and temporary. Then it may be difficult to control fast-growing tumors with only one compound rather a combined therapies with operation, radiotherapy, chemotherapy or immunotherapy are in realistic. These combined therapies implicates “two compartment therapy” that has two targets e.g., tumor cells and endothelial cells. At nontoxic dose, any temporally weigh loss, glomerular or choroids plexus dysfunction where Flk-1 receptors are exceptionally and constitutively expressed, could not be identified in our study. However, in other study, an average mortality rate 2.5% was reported in subcutaneous xenograft model (2). As for a safety consideration, more prolonged observation is needed to know the long term adverse effect, because this type of therapeutics should be continued as long as tumors exist.

CONCLUSION

We presented the anti-tumor effect of a novel synthetic inhibitor of Flk-1 tyrosine kinase, SU5416. Which induced the reduction of tumor vasculature and prolongation of survival on rat brain tumor model. Small molecular compound, such as SU5416, may be useful therapeutics that specifically inhibit the enzymatic activity of Flk-1 kinase and downstream events for the treatment of malignant brain tumors without significant adverse effect.

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