

Effect of Sunitinib Combined with Ionizing Radiation on Endothelial Cells

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The aims of present study were to evaluate the efficacy of combining sunitinib with ionizing radiation (IR) on endothelial cells *in vitro* and *in vivo*. Human umbilical vein endothelial cells (HUVECs) were exposed to IR with or without sunitinib pretreatment. Apoptosis assay and cell cycle distribution were analyzed by flow cytometry. Clonogenic survival assay at 3 Gy dose with or without sunitinib was performed. The activity of phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway was detected by Western immunoblot. Lewis lung carcinoma mouse model was built to examine the effect of combination therapy on endothelial cells *in vivo*. Microvasculature changes were detected by immunohistochemistry using anti-CD31 antibody. Our results showed combination therapy of sunitinib and IR significantly increased apoptosis of endothelial cells and inhibited colony formation compared to sunitinib or radiotherapy alone. It also resulted in cell cycle redistribution (decreasing cells in S phase and increasing cells in G2/M phase). The activity of PI3K/Akt signal pathway was inhibited, which could be the potential mechanisms that account for the enhanced radiation response induced by sunitinib. *In vivo* analysis showed that combination therapy significantly decreased microvasculature formation. The results demonstrated that combination therapy of sunitinib and IR has the potential to increase the cytotoxic effects on endothelial cells.

INTRODUCTION

Radiotherapy remains an important treatment modality for many human cancers. Approximately 50% or more of all cancer patients undergo radiotherapy at some point during the course of their disease.¹⁾ However, because of intrinsic or acquired radiation resistance of tumor, radiotherapy is often unsuccessful. More recently, a new paradigm has been introduced which suggested that tumor host component (e.g., the tumor microvasculature) may also be a target for the cytotoxic effects of IR. In particular, the tumor endothelium has received significant attention as a potential target for radiation sensitization.

The formation of new blood vessels inside a tumor is essential for cancer cell survival, local tumor growth, and the

development of distant metastases.²⁾ Tumor cells secrete angiogenic factors, which play the most significant role in neovascularization. Among several dozen angiogenic growth factors thus far identified, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) are perhaps the most studied.³⁾ 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.^{4,5)} 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.⁶⁾ PDGF stimulates angiogenesis by up-regulating VEGF production and modulating the proliferation of pericytes⁷⁾ and fibroblast-like cells surrounding the endothelium.⁸⁾ Furthermore, it has been demonstrated that VEGF, FGF and PDGF are all up-regulated in response to radiation⁹⁾ and they play important role in the resistance of tumors to radiotherapy effects. 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 in reducing new blood vessels formation than antagonizing VEGF signal transduction alone.

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Recent researches showed that endothelial cell survival could be enhanced through the ability of VEGF and demonstrated that VEGF was the critical factor determining tumor radiation response.^{10,11} Furthermore, in many preclinical researches, when narrow-spectrum inhibitors of VEGF (such as gefitinib, erlotinib, DC101 and SU5416)^{12–15} or broad-spectrum receptor tyrosine kinases (RTKs) inhibitors (such as PTK787, SU6668 and SU11248 {sunitinib})^{16–18} were combined with IR, the cytotoxic effects of combination therapy was predominantly enhanced in comparison with IR alone. These observations raised the possibility that blocking all these receptors and/or their ligands may theoretically provide additional antiangiogenic effects and increase radiotherapy effects. Therefore, a combined treatment approach using pharmacological inhibitors of angiogenesis and IR has a sound rationale for aiming at targets inside a solid tumor.

Sunitinib is a potent inhibitor of multiple RTKs, through which its antitumor and antiangiogenic activities are mediated. Target RTKs include PDGFR- α and - β , VEGF receptors (VEGFR-1, VEGFR-2 [FKL-1/KDR] and VEGFR-3), KIT, Fms-like tyrosine kinase-3 (FKT3), colony stimulating factor receptor type 1 (CSF-1R) and the glial cell line-derived neutrophilic factor receptor (RET). In in-vitro studies, sunitinib and its active metabolite (SU012662) have shown similar affinity for the RTKs PDGFR- α and - β , VEGFR-2 and KIT.¹⁹ Researchers demonstrated that using Sunitinib in combination with IR increased therapeutic effects in murine lung cancer model.¹⁸ In this study, using the same methods, we evaluated the effects of sunitinib in combination with IR on endothelial cells in vitro and in vivo.

MATERIALS AND METHODS

Cell culture

Lewis lung carcinoma (LLC) cells were maintained in DMEM medium supplemented with 10% FCS and 1% penicillin-streptomycin. Human umbilical vein endothelial cells (HUVECs) were maintained in EBM-2 medium. All cells were incubated in a humidified incubator with 5% CO₂ at 37°C.

HUVECs apoptosis assays

The number of HUVECs undergoing apoptosis was quantified by flow cytometry. HUVECs were divided into four groups: (1) vehicle control; (2) sunitinib (pure substance provided by Pfizer) alone; (3) IR alone; (4) sunitinib + IR. HUVECs (1×10^6 cells/well) were seeded in 6-well plates and allowed to attach overnight. 1 h after treatment with or without 1 μ M sunitinib, cells were irradiated with 3 Gy dose. 1 h after irradiation, the cell medium was replaced by fresh medium. HUVECs were incubated for 24 hours and then trypsinized to be examined by flow cytometry according to the manual. Briefly, cells were digested with trypsin and washed once in PBS, then fixed and permeabilized using the

Cytofix/Cytoperm™ Kit (BD Pharmingen) for 20 min at room temperature, then pelleted and washed with Perm/Wash™ buffer (BD Pharmingen). Cells were then stained with FITC labeled anti-caspase-3 active form (BD Pharmingen) for 60 min at room temperature in the dark. Following incubation with the antibody, cells were washed in Perm/Wash™ buffer, re-suspended in Perm/Wash™ buffer and analyzed by flow cytometry on Coulter Epics XL (Beckman Coulter, Inc., Fullerton, CA, USA) with System II software using the FL1 for FITC labeled Caspase-3 active form, and data analysis was performed by Win MDI software. Irradiation of cell cultures was carried out in 6-well plates using linear accelerator at 0.7 Gy/min. Delivered dose was verified by use of thermoluminescence detectors. From these numbers, the percentage of apoptotic cells for each group was determined.

DNA ladder assay

Approximately 5×10^6 HUVECs with different treatments (above mentioned) were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 mg/ml DNase-free RNase and incubated at 37°C for 1 h. The DNA was visualized on 1.5% agarose gel in the presence of 0.5 mg/ml ethidium bromide.

Clonogenic survival assay

Clonogenic survival assay was performed to evaluate the effectiveness of combination therapy of sunitinib with IR. Briefly, 5×10^3 HUVECs were plated in 100 mm Petri dishes and allowed to attach overnight. Culture plates were treated with 3 Gy radiation with or without 1 μ M sunitinib for 1 h before irradiation. The cell medium containing sunitinib or vehicle was replaced with fresh cell medium 1 h after irradiation and then incubated for 24 hours. Thereafter, cells were trypsinized and seeded in 100 mm dishes (500 cells per dish) for colony growth. After 14 days, cells were fixed with cold methanol and stained with 1% methylene blue. Colonies with > 50 cells were counted manually using a microscope, and the surviving fraction was determined.

Cell cycle analysis

HUVECs were treated as same as that in apoptosis analysis (above mentioned). After 24 h incubation, cells were trypsinized and washed with PBS, fixed with 75% ethanol overnight, then treated with 20 μ g/ml ribonuclease A (RNase A, Sigma) at 37°C for 30 min. Then, cells were stained with 50 μ g/ml propidium iodide (PI, Sigma) for 30 min and DNA content was analyzed by flow cytometry with Coulter Epics XL (Beckman Coulter, Inc., Fullerton, CA,

USA) using System II program (Beckman Coulter). Cell cycle distribution was analyzed by Multicycle V3.0 software.

Western immunoblots to detect PI3K/Akt pathway activity

HUVECs in culture were treated with 1 μ M sunitinib for 1 hour with or without irradiation with 3 Gy, as described above. HUVECs were counted and then washed twice with ice-cold PBS before the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium PPI, 1 mM phenylmethylsulfonyl fluoride, and leupeptin). Protein concentration was quantified by the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 8% SDS-PAGE gel, which was followed by transferring onto nitrocellulose membranes. Membranes were blocked by use of 10% nonfat dry milk in PBST for 2 hours at room temperature. The blots were then incubated with the rabbit anti-human (phosphor-AKT{Ser-473}, Cell Signaling) antibodies overnight at 4°C. Donkey anti-rabbit IgG secondary antibody (1:1000 Amersham) was incubated for 1 hour at room temperature. Immunoblots were developed by using the enhanced chemiluminescence detection system according to the manufacturer's protocol and autoradiography. Integrated density value was measured by using ImageTool 3.0 software.

Tumor model

Female C57BL/6 mice, 8 weeks old and weighting approximately 20 g were used. The experiment was carried out under both the Regulation for Animal Experiment of The Second Military Medical University and the Law of the Chinese government. The mice were housed four to six per cage and kept under standard laboratory conditions, during which food and water were supplied *ad libitum*. To establish tumors, LLC cells were injected s.c. into the right hind limb (1×10^6 cells in PBS) of C57BL/6 female mice. Treatment began when individual tumors reached a mean diameter of 8 mm.

Radiation schedule

Irradiated mice were immobilized in Lucite chambers and the entire body was shielded with lead except for the tumor-bearing hind limb. IR was administered within 30 min after sunitinib (40 mg/kg) intraperitoneally injected. A total dose of 6 Gy X-ray was administered to the appropriate mice using a 6 MV linear accelerator.

Histological examination

C57BL/6 mice bearing LLC tumors were treated with 6 Gy alone, sunitinib alone or sunitinib followed by 6 Gy radiation. Three mice were treated in each of the treatment groups. After 3 days, mice were sacrificed and tumors were taken off and fixed in 4% buffered formalin. Tumor sample

sections of 4 μ m embedded in paraffin were dewaxed with xylene, rehydrated with hematoxylin and eosin (H&E) to be examined histologically.

Immunohistochemistry

Tumor sections were dried, deparaffinized and rehydrated. After quenching endogenous peroxidase activity and blocking with bovine serum albumin, slides were incubated at 4°C overnight with the endothelial cell-specific anti-CD31 monoclonal antibody (PECAM-1, Dako), and were then incubated for 1 h with biotinylated sheep anti-rabbit immunoglobulin (Dako). Slides were counterstained with hematoxylin. 5 high-power fields (400 \times) were examined for each tumor section using a Nikon Microphot-FX microscope equipped with a Sony digital camera.

Statistical analysis

Statistical significance was determined using two-tailed Student's *t*-test (Fig. 1–3) or the Kruskal-Wallis test (Fig. 6B). *P*-value < 0.05 was considered statistically significant.

RESULTS

Sunitinib increased IR-induced HUVECs apoptosis

To determine whether broad spectrum RTK inhibitor enhanced the cytotoxic effects of radiation on vascular endothelium, sunitinib was added 1 h before irradiation. After treatment with 3 Gy radiation with or without 1 μ M sunitinib, we investigated the active form of caspase-3 which is the executor during apoptotic event. Figure 1 showed the percentage of HUVECs undergoing apoptosis after treatment with sunitinib alone, 3 Gy alone, or sunitinib followed by 3 Gy. Untreated control cells showed primarily negative ($1.1\% \pm 0.3\%$) for active caspase-3 as compared with $6.4\% \pm 0.6\%$ and $3.7\% \pm 0.4\%$ (mean \pm SE) of positive cells with active form of caspase-3 at 24 h after sunitinib treatment alone and IR alone, respectively. However, combination therapy of sunitinib and IR greatly increased apoptotic HUVECs percentage to $17.6\% \pm 2.1\%$. The differences in cell apoptosis percentage were all statistically significant ($P < 0.05$) compared to control, sunitinib treatment alone and IR alone, respectively. These results demonstrated that combined therapy with sunitinib and IR increased apoptosis in HUVECs, at least in part, by triggering caspase-3 pathway.

To confirm these results, we performed DNA ladder assay. In the present study, the formation of DNA fragmentation was observed only in combination therapy of sunitinib and IR (as shown in Fig. 2).

Combination therapy decreased HUVECs colony formation

To determine whether enhanced apoptotic response in endothelial cells treated with sunitinib combined with IR resulted in reduced clonogenic cell survival, HUVECs were

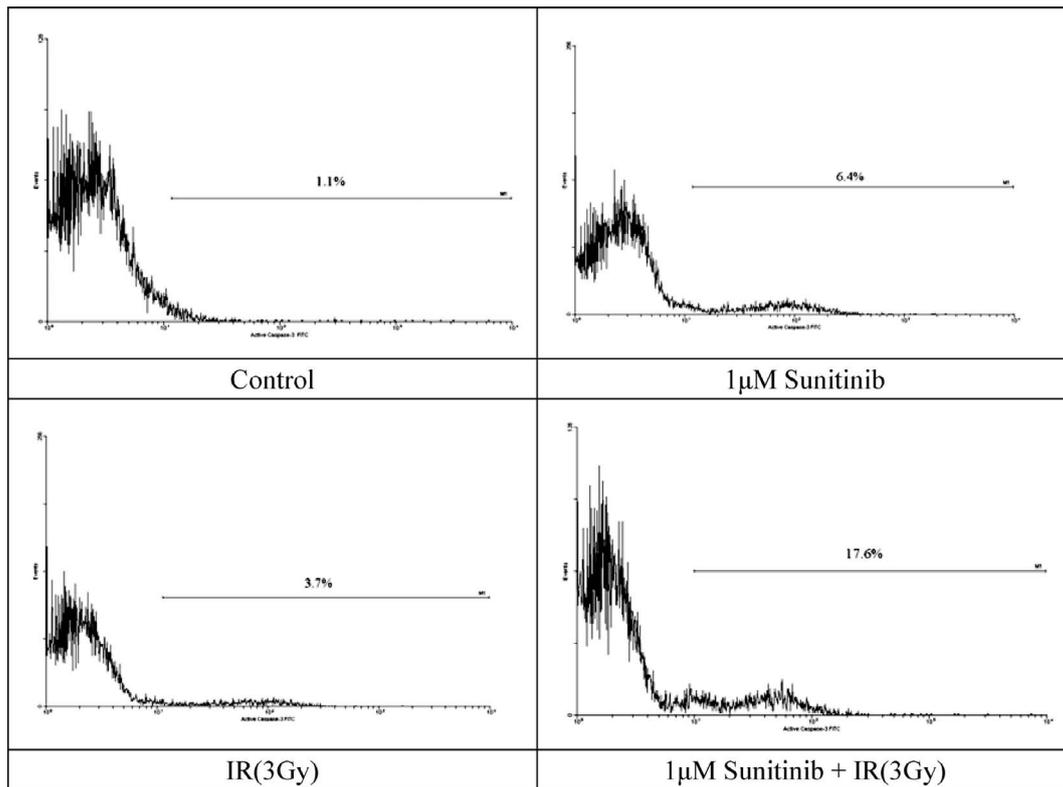


Fig. 1. Sunitinib increased IR-induced caspase-3 activity. The percentage of cells with the active form of caspase-3 was determined by flow cytometry. This is representative graphs of one set of triplicate experiments measuring the active caspase-3 by flow cytometry (Y axis: Events; X axis: Active caspase-3 FITC).

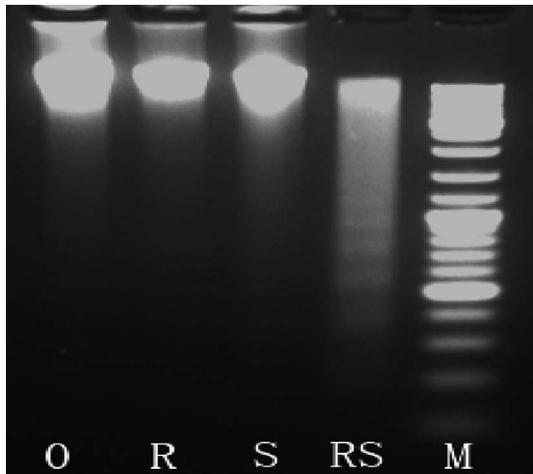


Fig. 2. Fragmentation of internucleosomal DNA. The DNA of HUVECs with different treatments was subjected to agarose gel electrophoresis (O: untreated blank; S: 1 μ M sunitinib; R: 3 Gy IR; RS: 1 μ M Sunitinib + 3 Gy IR; M: marker).

subcultured and colony formation was quantified. Figure 3 showed the surviving fraction of HUVECs treated with 3 Gy with or without 1 μ M sunitinib. HUVECs treated with

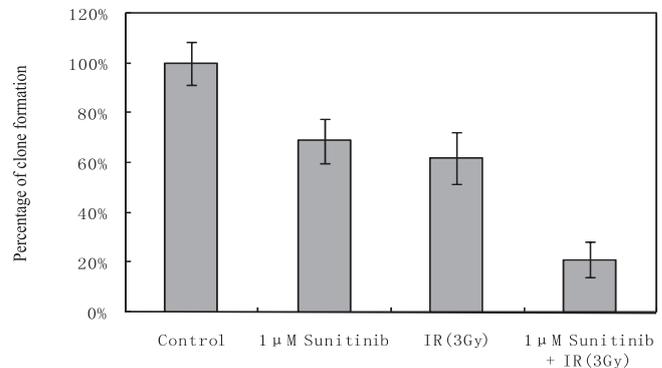


Fig. 3. Clonogenic survival analysis of HUVECs. Cells were treated with 3 Gy radiation with or without 1 μ M sunitinib. Colonies were stained with 1% methylene blue and counted under the light microscope after 14 days. Surviving fractions of HUVECs with different treatment are shown. Using Student's *t*-test, the colony formation was significantly decreased in combination therapy group ($P < 0.05$). The means \pm SD ($n = 12$) are shown.

sunitinib before irradiation showed a significant reduction in colony formation compared with radiation or sunitinib alone.

Combination therapy caused HUVECs cell cycle redistribution

Cell cycle was measured by flow cytometry after PI staining. As shown in Fig. 4, combination treatment with 1 μ M

sunitinib and IR lowered the percentage of cells in S phase and increased cells in G2/M phase compared to cells in vehicle control group and cells treated with radiation alone or sunitinib alone.

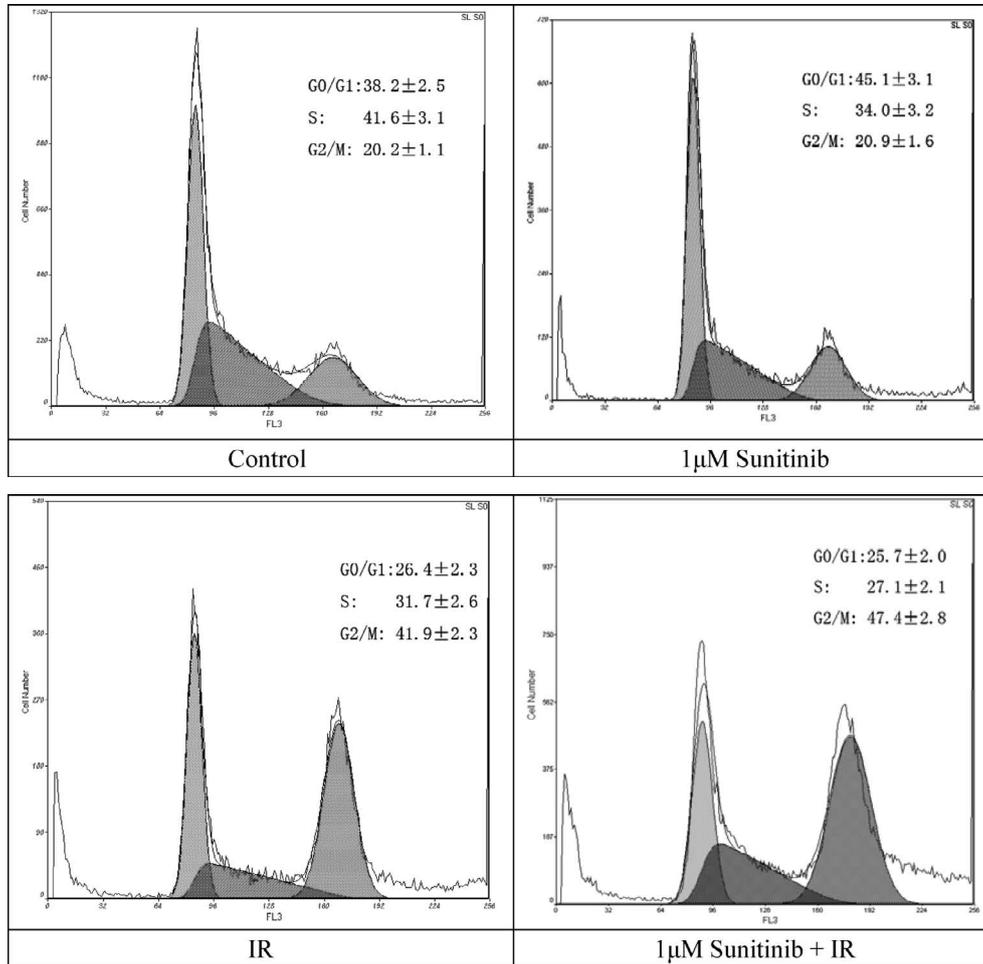


Fig. 4. Cell cycle redistribution in HUVECs. Cell cycle was determined by flow cytometry after PI staining. Data are presented as means \pm SD. (Using Student's *t*-test, Cells in S phase in combination therapy group were significantly decreased compared to sunitinib alone and IR alone group, $P < 0.05$. Cells in G2/M phase in combination therapy were significantly increased compared to sunitinib and IR group, $P < 0.01$ and $P < 0.05$, respectively) (Y axis: Cell number; X axis: DNA Content).

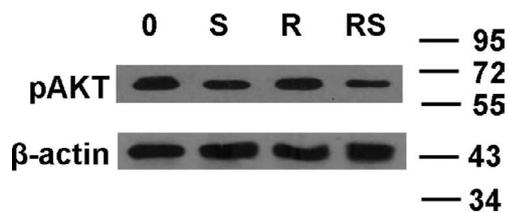


Fig. 5. Sunitinib attenuated radiation-induced activation of Akt. HUVECs were treated in the presence and the absence of sunitinib, with or without irradiation. Total cellular proteins were extracted after 3 Gy IR. The graph showed the results of Western immunoblots using antibody to phosphorylated Akt (0: untreated blank; S: 1 μ M sunitinib; R: 3 Gy IR; RS: 1 μ M Sunitinib + 3 Gy IR).

Table 1. Measurement of band integrated density

Band	Integrated Density
β -actin (0)	26448
β -actin (S)	28035
β -actin (R)	24840
β -actin (RS)	26952
pAKT (0)	22437
pAKT (S)	16184
pAKT (R)	21644
pAKT (RS)	12330

Combination therapy inhibited the PI3K/Akt pathway activation

In this study, we tested whether sunitinib blocks the Akt activation that was induced by irradiation. As shown in Fig. 5, the phosphorylated Akt was downregulated in the group of sunitinib treatment alone and combination therapy group. However, the inhibition of Akt phosphorylation was not complete, the residual Akt phosphorylation was still visible.

Then, the integrated density value of each band was measured by using ImageTool 3.0 software. The results were shown in Table 1.

Histological analysis and immunohistochemistry

Histopathological examination of control LLC tumor sections revealed a dense network of distended microvessels localized in the central part of the tumor. Moreover, a large

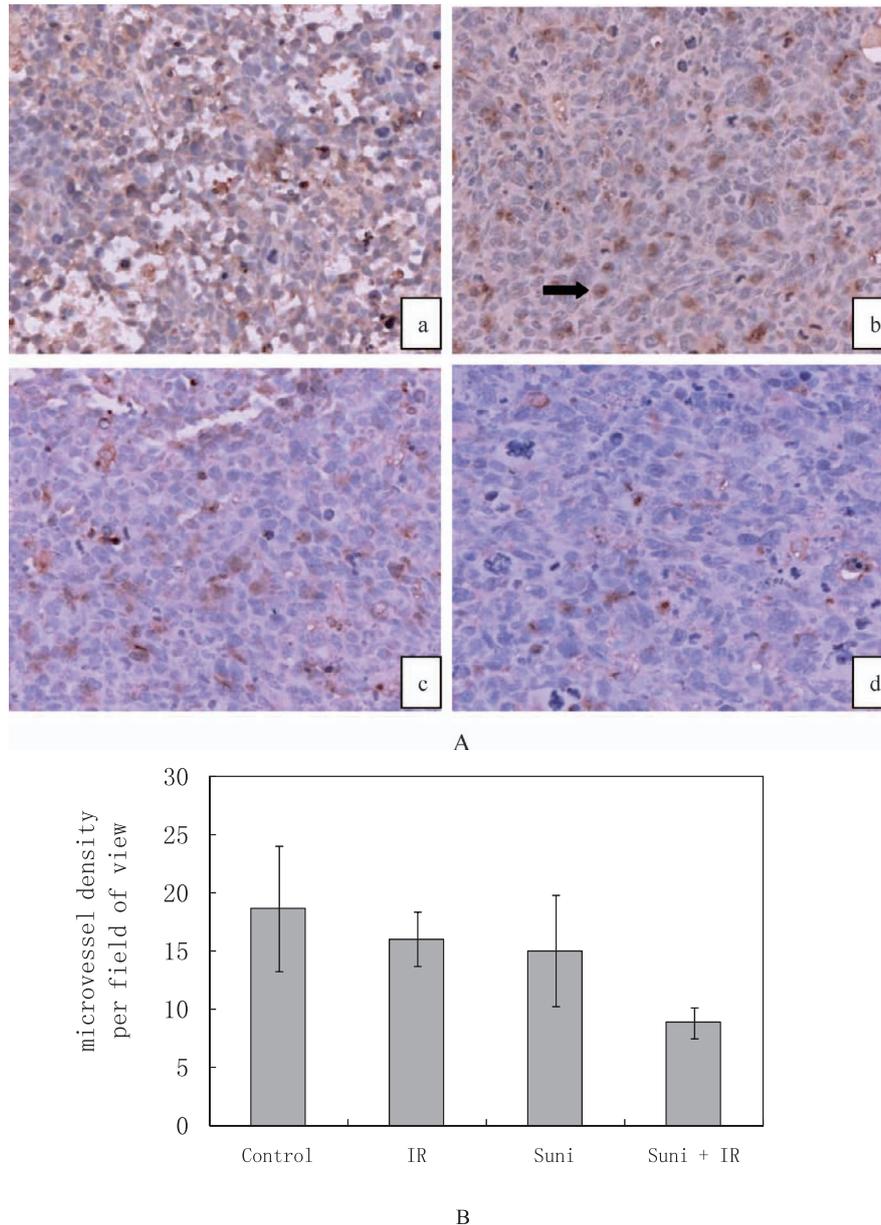


Fig. 6. A. Visualization of the tumor vasculature using anti-CD31 immunohistochemistry. Micrographs are of representative sections from LLC following treatment with sunitinib and IR. (a) Untreated control. (b) 6 Gy IR alone. (c) 40 mg/kg Sunitinib alone. (d) 40 mg/kg Sunitinib + 6 Gy IR. (the arrow points at the representative vessel stained with an endothelial cell-specific anti-CD 31 antibody). B. In Vivo microvessel density quantitative analysis. 5 high-power fields (400×) were examined for each tumor section. Data are presented as means ± SD (Microvessel numbers in combination therapy group were significantly decreased compared to sunitinib alone and IR alone group, $P < 0.05$).

number of vital microvessels were located at the border and locally in the necrotic and apoptotic tumor areas. In contrast, LLC tumor sections with the combined therapy displayed a dramatically decreased number of microvessels, often with only a very thin diameter located almost exclusively in the vital parts of the tumors (as shown in Fig. 6A). Tumor sections treated with sunitinib or IR alone showed comparable microvessel density to the control group. Immunohistochemistry assay demonstrated the number of LLC tumor vessels per high-power field was significantly decreased following exposure to combined treatment with sunitinib and IR compared with exposure to sunitinib or IR alone (as shown in Fig. 6B).

DISCUSSION

Solid tumors require angiogenesis for growth, and inhibition of angiogenesis is one promising strategy for cancer therapy. Endothelial cell is an important component during the course of angiogenesis. In this study, we examined the effects of combined treatment of sunitinib with IR on endothelial cells *in vitro* and *in vivo*.

Endothelial cells, although susceptible to apoptosis at high doses, are less sensitive to the lower doses of IR (2–3 Gy).²⁰ To explore whether cytotoxic effects could be enhanced when low dose of IR was combined with sunitinib, we studied apoptotic responses of endothelial cells by measuring caspase-3 activity using flow cytometry. Caspase-3 is a key protease of caspase family which plays an important role in apoptosis. In this study, although 1 μ M sunitinib treatment or 3 Gy IR alone had little effect on caspase-3 activity, when they were combined sequentially, the percentage of endothelial cells with active form of caspase-3 was significantly increased (as shown in Fig. 1) and DNA fragmentation was only observed in combination therapy group (as shown in Fig. 2). This data showed combination therapy increased cytotoxic effect of low dose of IR. To determine whether enhanced apoptotic response in endothelial cells treated with sunitinib and IR resulted in reduced clonogenic cell survival, we quantified HUVECs colony formation. Figure 3 showed combination therapy significantly inhibited colony formation compared to either of the treatment alone. These results demonstrated that sunitinib was a potent enhancer of radiotherapy in endothelial cells.

Then, we investigated whether combination therapy affected cell cycle distribution which may have influence on the effects of IR. Figure 4 showed that combination therapy decreased S phase cells and urged more cells to be arrested in G2/M phase compared to sunitinib alone and IR alone. It has been proved that cells in S phase are the least sensitive to IR-caused DNA damage and cells in G2/M phase are the most sensitive stage of cell cycle to IR.^{21,22} As we know, radiotherapy is a consecutive process and that redistribution of cell cycle put more cells into the radiosensitive phase at

the time of subsequent radiation, which maybe conduce to increase the cytotoxic effects of IR.

The activation of PI3K/Akt pathway by IR, either through paracrine effect, or via mechanism independent of growth-factor stimulus, was supposed to partly account for tumor vascular radioresistance.^{23,24} One of mechanisms of interaction between RTK antagonists and cytotoxic therapy is the attenuation of signal transduction through the PI3K/Akt pathway.²² Attenuation of this pathway not only alters the levels of Bcl gene products, resulting in increased susceptibility to apoptosis,²⁵ but increases radiosensitivity by affecting DNA repair.²⁶ Previous research demonstrated that using a PI3K inhibitor, wortmannin, could potentiate the antitumor effect of radiation *in vivo*, particularly on radioresistant murine tumors.²⁷ In the present study, to explore whether increased apoptosis of endothelial cells was related to the inhibition of PI3K/Akt pathway, we detected the phosphorylated-Akt activity by Western immunoblot. The result showed the combination therapy significantly decreased phosphorylated-Akt activity, although the inhibition was not complete. Measurement of band integrated density confirmed this result. The decreased phosphorylated-Akt activity contributed to endothelial cells into apoptosis procedure and increased caspase-3 activity. From Fig. 5, we also noticed that the pAkt band of IR treatment alone was not thicker than that of control, this phenomenon proved that the endothelial cells are less sensitive to lower dose of IR (2–3 Gy). These results were consistent with apoptosis analysis (as shown in Fig. 1). This data confirmed the inhibition of PI3K/Akt pathway was related to increased cytotoxic effects induced by combination therapy.

Tumors produce growth factors, including VEGF, PDGF and FGF, that could contribute to tumor resistance to cytotoxic therapy. Moreover, growth factors are all up-regulated in response to radiation.⁹ Sunitinib, an orally available indolinone-based synthetic molecule, was identified as a low-nM selective inhibitor of the angiogenic receptor tyrosine kinases Flk-1/KDR and PDGFR in both biochemical and cellular assays.¹⁹ Sunitinib blocks a broad spectrum of growth factors receptor, which could be advantageous in inhibiting angiogenesis. Promising antitumor effects were observed in the phase I clinical trial of concurrent sunitinib and radiotherapy followed by maintenance sunitinib for cancer patients with oligometastases.²⁸ In this study, in order to examine cytotoxic effects of combination therapy on tumor vascular endothelium *in vivo*, we built LLC mice model. To determine the effects of different treatments on tumor angiogenesis, we examined representative tissue sections from LLC tumor using anti-CD31 antibody and standard immunohistochemical techniques. Figure 6 showed the number of LLC tumor vessels per high-power field was reduced following exposure to combination therapy compared with those exposed to IR alone or sunitinib alone.

In summary, the current data suggested that sunitinib has

the potential to increase radiosensitivity in endothelial cells most likely by enhancing caspase activity and blocking PI3K/Akt pathway activation. The combination therapy also redistributed cell cycle of endothelial cells which is maybe helpful in consecutive IR. This study supported the concept that tumor microenvironment could be an effective target for radiation therapy. It warranted future investigation to evaluate the utility of sunitinib as radiosensitizer in radiotherapy.

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