Therapeutic Synergy of TNP-470 and Ionizing Radiation: Effects on Tumor Growth, Vessel Morphology, and Angiogenesis in Human Glioblastoma Multiforme Xenografts

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ABSTRACT
We examined the effect on tumor growth, vessel morphology, and expression of angiogenic factors of combining radiotherapy and antiangiogenesis in the human glioblastoma line U87 grown in the flank or intracranially in the nude mouse. The antiangiogenic agent TNP-470 was given 6.7 mg/kg s.c. daily on day 1–7 starting 1 week after transplantation. Irradiation (IR), 10 Gy × 1, was administered on day 7. A series of tumors were excised 8 and 48 h after the end of treatment. The vascular morphology was evaluated in CD31 immunostained cryosections and by electron microscopy, and the pattern of expression of angiogenic factors (mRNA and protein) was quantitatively analyzed by phosphorimaging of Northern blots and Western blots. Significant inhibition of s.c. flank tumor growth relative to untreated controls was achieved by monotherapy with both TNP-470 (P < 0.001) and IR (P < 0.001). A significant enhancement of this effect was obtained by combining TNP-470 and IR (P < 0.05). We saw no effect of TNP-470 either alone or in addition to the effect of IR on the survival of mice with intracranial tumors. CD31 immunostaining of s.c. tumors showed acute endothelial swelling and luminal protrusion in irradiated tumor vessels but never in tumors pretreated with TNP-470, and not in the untreated controls. The vessel density (Chalkley point counts) was unchanged by TNP-470 therapy. In the TNP-470-treated tumors, we observed a distinct broadening of the endothelial basement membrane by an approximately 400–700-nm-thick electron-dense yet uncharacterized fibrillar material. TNP-470 treated tumors +/− IR also had a significantly increased mRNA expression of angioptoinet-1, whereas angioptoinet-2, vascular endothelial growth factor and basic fibroblast growth factor mRNA were unchanged by the treatments. In conclusion, TNP-470 significantly enhanced the tumor effect of ionizing IR, and our findings strongly indicate that acute microvascular damage after IR is effectively prevented by concurrent TNP-470 treatment. A significant up-regulation of angiopoietin-1 seems to play a role in this protective mechanism, which as yet is not fully elucidated.

INTRODUCTION
Angiogenesis is considered essential for tumors to grow beyond a few millimeters in size (1). Angiogenic processes are regulated by the balance between proangiogenic and antiangiogenic factors in the microenvironment. VEGF3 and angiopoietin-1 and angiopoietin-2 are more specific to endothelium than bFGF, whose receptors are present in numerous tissues. Glioblastoma multiforme, the most common primary malignant brain tumor in humans, is microscopically characterized by certain vascular patterns produced by endothelial hyperproliferation. Cultured glioblastoma cells or tissues also express large amounts of angiogenic factors, mostly VEGF (2, 3). Therefore, antiangiogenesis is likely to contribute to the outcome of the overall management of this disease entity, in which cranial radiotherapy plays a pivotal role.

The antiangiogenic compound TNP-470 is an inhibitor of endothelial cells (4). The mechanism of action is not fully elucidated, but the compound is believed to act by inhibiting methionyl aminopeptidase-2, an intracellular enzyme related to protein myristoylation (5). Accordingly, the effects of TNP-470 are mediated by the resulting inhibition of membrane proteins, such as nitric oxide synthase, which are translocated to the cell surface membrane by myristoylation (6). TNP-470 has been shown to inhibit the growth of gliomas and other brain tumors in a number of animal studies (7–11).

The combination of radiotherapy and TNP-470 has been investigated in murine mammary and lung carcinomas but not in human glioblastoma multiforme. Murata et al. (12) found that TNP-470 administered before IR did not induce tumor hypoxia, whereas TNP-470 given during fractionated radiotherapy did decrease the radiocurability of murine mammary carcinoma. In studies on murine mammary carcinoma and Lewis lung carcinoma, Teicher et al. (13, 14) found that TNP-470 in combination with other antiangiogenic agents, given during fractionated radiotherapy, increased the inhibitory effect of radiation on tumor growth. Angiostatin, another potent antiangiogenic agent, has been used in combination with fractionated radiotherapy

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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against human glioma on nude mice with a greater than additive effect on tumor-growth inhibition (15).

On this basis, we evaluated the effect of combination therapy with TNP-470 and ionizing radiation on human glioblastoma xenografts in the s.c. space and intracranially in the nude mouse. In addition to the tumor response evaluation, we studied the effects on vascular morphology and on the expression pattern of a number of relevant angiogenic factors.

By this approach, the present study provides information of relevance to the introduction of antiangiogenic therapy in effective combinations with other treatment modalities.

MATERIALS AND METHODS

Animals. A total of 120 male 8-week-old athymic nude mice (NMRI-nu/nu) obtained from M&B (Ry, Denmark) were used. The mice were kept in laminar air flow benches. They received sterile food pellets and water ad libitum. Institutional guidelines for animal welfare and experimental conduct were followed.

Tumor. The human glioblastoma cell line U 87 MG was originally purchased from American Type Culture Collection (Rockville, MD). Xenografts were established by s.c. injection of cells and maintained by serial transplantation. Prior to transplantation, the mice were anesthetized by a s.c. injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) in 0.9% NaCl solution. Through a 1-cm incision in the dorsal skin, 1-mm³ cells were injected. The excised tumor tissue was processed for morphology and expression of angiogenic factors.

Three parallel series of experiments were performed. In each experiment the mice were divided into four groups, given: (a) TNP-470 (6.7 mg/kg/d); (b) IR 10-Gy single dose; (c) TNP-470 + IR in the same doses; or (e) no treatment. TNP-470 was administered for 1 week immediately before IR. In all of the experiments, the IR was applied 1–2 h after the last TNP-470 dose.

In a separate experiment with s.c. tumor transplants, treatment was initiated when an average tumor diameter of 7 mm was reached, and tumors were excised 8 and 48 h after treatment. The excised tumor tissue was processed for morphology studies and expression of angiogenic factors.

Effects on tumor growth were determined in two types of experiments: (a) in s.c. tumors, in which tumor growth was measured; and (b) with intracranial tumor implants using survival as end point.

Growth Calculations. The s.c. tumor size was measured five times per week. Two measurements in two perpendicular dimensions (d₁ and d₂) were recorded using a sliding gauge, and tumor volume [V(t)] curves were obtained according to the following formula:

\[ V(t) = 0.35(d_1(t) \times d_2(t))^{3/2} \]

To evaluate the effect of treatment on total tumor growth, we calculated the time from the start of TNP-470 treatment until a tumor volume of 600 mm³ was reached.

Table 1  Time from treatment start (day 8) until a total tumor volume of 600 mm³ is reached

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>( T_{600} ) days</th>
<th>Median (range)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>13</td>
<td>13 (8–20)</td>
<td></td>
</tr>
<tr>
<td>Combination (IR + TNP-470)</td>
<td>28.5</td>
<td>28.5 (7–61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vs. controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs. TNP-470</td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>vs. IR</td>
<td></td>
<td></td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>TNP-470</td>
<td>23</td>
<td>23 (12–36)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vs. controls</td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IR</td>
<td>23.5</td>
<td>23.5 (5–42)</td>
<td></td>
</tr>
</tbody>
</table>

\( T_{600} \) is the median time from the start of treatment until a tumor volume of 600 mm³ is reached. The two-sample Wilcoxon test was applied.

A tumor volume of 600 mm³ was chosen as a representative size of a large tumor in exponential growth (see Fig. 1).

Treatment. Single-dose 10 Gy tumor X-radiation was given antero-posteriorly to flank tumors and, as two opposing lateral fields including the whole brain, to mice with brain tumors. The X-radiation was given during anesthesia with ketamine/xylazine under aseptic conditions using a Stabilipan (Siemens) therapeutic unit that yields 4.58 Gy/min at 300 kV.

TNP-470 (O-chloroacetyl carbamoyl fumagillol) was generously provided by Leo Pharmaceutical Products (Ballerup, Denmark). A stock solution of 70% ethanol and TNP-470, 10 mg/ml, was prepared and stored at 4°C. Immediately prior to treatment, the stock solution was diluted with 0.9% NaCl to obtain a dosing concentration of 1 mg/ml. TNP-470 was administered as a daily s.c. injection, 6.7 mg/kg/d. This treatment regimen was found in previous experiments to be efficient, with relatively low toxicity (11).
Nonparametric tests were used for statistics because q-q plots of the growth data clearly demonstrated a non-Gaussian distribution.

**Immunohistochemistry.** Tissue was frozen in cooled isopentane, and frozen sections were fixed in formalin and postfixed in ethanol:acetic acid (2:1). CD31 immunostaining was performed on sections from tumors in each group. Sections were washed in PBS and TBS and incubated with 10% normal rabbit serum for 30 min. They were then incubated with a mixture of two monoclonal rat antimouse CD31 antibodies at a dilution of 20 mg/ml overnight at 4°C. The antibodies used were clone 390 (Serotec Ltd.) and MEC 13.3 (PharMingen). Rat IgG2a (Serotec Ltd.) was used as a negative control. Sections were incubated with biotin-conjugated rabbit antirat immunoglobulin (DAKO), at a dilution of 1:600 (2.3 mg/ml) for 30 min, washed, and incubated with alkaline phosphatase-conjugated streptavidin (DAKO) at a dilution of 1:200 (1.5 mg/ml) for 30 min. As substrate for the alkaline phosphatase reaction, we used freshly prepared Fast Red Substrate System (DAKO), followed by a 10-min wash in tap water. After this, sections were counterstained with hematoxylin and mounted with aqueous mounting media.

**Vessel Density.** Vessel density was recorded as the number of point counts of CD31-positive vessels per field, at ×200, viewed through an ocular Chalkley Point Array (Graticules Limited, Tonbridge, United Kingdom). Ten fields per section, randomly selected from nonnecrotic areas of tumors from control and TNP-470 groups, were examined with a Leica DMRB microscope.

**Preparation for Electron Microscopy.** After a brief fixation in 70% Karnovsky’s fixative (3% paraformaldehyde and 3.5% glutaraldehyde in 0.1 M cacodylate buffer) each tumor was cut into tissue slices of 760 μm with an EMS Automatic Oscillating Tissue Slicer (Electron Microscopy Sciences, Fort Washington, PA). The slices were immersed in Karnovsky’s fixative for 1 h and subsequently washed and stored in 0.1 M cacodylate buffer. Six to ten small tissue blocks were randomly sampled from tumor slices from each group using a biopsy needle with a diameter of 1 mm. The tissue blocks were further fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in ethanol followed by 1,2-propylenoxide, and embedded in Epon. Ultrathin (70-80 nm) sections were cut on a RMC, MT 6000-XL ultramicrotome. Sections were mounted on copper grids and contrasted with 4% uranyl-acetate (30 min) and Reynolds lead-citrate (2.5 min). Ultrathin sections were examined in a JEOL electron microscope.

**Northern Blotting.** Tumor tissue blocks of approximately 0.25 g were frozen in liquid nitrogen. Polyadenylated RNA was extracted and isolated using the guanidinium thiocyanate-based QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). The mRNA concentration was measured by spectrophotometry. Three μg of denatured mRNA were separated on 1% agarose gels containing 6.6% formaldehyde and were subsequently transferred by capillary electrophoresis in 10× SSC buffer to a nylon membrane (GeneScreenPlus, NEN Research Products). The mRNA was cross-linked to the membranes with 1200 J UV light in a UV stratalinker 1800.
Membranes were prehybridized for 3 h in 5% dextran sulfate, 50% formamide, 1% SDS, 1 M NaCl, and 100 μg/ml salmon sperm DNA at 42°C and hybridized with [32P]-labeled cDNA probes overnight at 42°C. The mRNA expression was visualized on a phosphorimager (STORM 840, Molecular Dynamics Inc.), quantified by the software program Image Quant version 5.0 (Molecular Dynamics Inc.), and the VEGF, bFGF, angiopoietin-1, and angiopoietin-2 expression was adjusted to the GAPDH expression. To compare data from different membranes, mRNA from U87 cells in vitro was loaded on all of the gels as a common standard. We used cDNA probes encoding VEGF, bFGF, angiopoietin-1 and angiopoietin-2, and GAPDH (Clontech).

Immunoblotting. Tissue blocks were thawed on ice and homogenized in lysis buffer with four short bursts on a Vibra Cell 50 (Sonics & Materials Inc.). The protein concentration was determined with a BCA Protein Assay (Pierce Inc.) and 20/40 μg of protein from each tumor were separated on 1.0-mm NuPage 10% Bis-Tris gels (NOVEX) and transferred onto PVDF membranes (NOVEX) by semi-dry blotting.

Membranes were blocked in Tris-buffered saline (pH 7.4), 10% skim milk powder, and 0.1% Tween 20, and washed in Tris-buffered saline (pH 7.4). Incubation with primary and secondary antibodies for 1–16 h was followed by washing in Tris-buffered saline (pH 7.4) and 0.1% Tween 20. The protein expression was visualized with ECL+ Plus (Amersham) and quantified by chemiluminescence scanning on a STORM 840 (Molecular Dynamics Inc.). Expression of VEGF protein was adjusted to the α-tubulin expression. The following antibodies and concentrations were used: (a) monoclonal mouse-anti-human VEGF (1 μg/ml, PharMingen); and (b) polyclonal goat-anti-human bFGF (0.2 μg/ml; R&D Systems). Monoclonal mouse-anti-α-tubulin (1:10,000) was obtained from Sigma Chemical Co. Horseradish peroxidase-conjugated polyclonal goat-anti-mouse-immunoglobulin (1:3000 and 1:5000) and horseradish peroxidase-conjugated polyclonal rabbit-anti-goat-immunoglobulin (1:2000) was obtained from DAKO.

RESULTS

Effect of Ionizing Radiation and TNP-470 on Tumor Growth. Both IR and TNP-470 significantly inhibited the growth of s.c. U87 tumors, and a significantly enhanced effect was obtained by the combination of treatments (Fig. 1; Tables 1 and 2). All of the tumors eventually regrew, but some tumors in the combination group started growing very late after treatment.
The time for tumors in the combination group to reach a volume of 600 mm$^3$ ($T_{600}$, Table 1) was significant longer than in all of the other tumor groups. Intracranially implanted U87 tumors were treated with TNP-470, IR, or combination therapy in the same doses. In these mice, TNP-470 treatment did not affect survival (Fig. 2), whereas the expected effect of single-dose IR was observed.

**Effect of Ionizing Radiation and TNP-470 on Tumor Endothelium.** In tumors treated with ionizing radiation alone, a large fraction of the CD31-positive tumor vessels appeared irregular with swelling and luminal protrusion of endothelial cells at light microscopic examination 8 h after treatment. These changes were not found in tumors pretreated with TNP-470 nor in untreated controls (Fig. 3, A, B, and C).

Similar changes were found by electron microscopy. Here, a large fraction of the irradiated endothelial cells were seen to be rounded, protruding into the lumen of the vessel, and with loss of contact between cells and loss of normal cellular organelles (Fig. 4A). At the ultramorphological level, untreated tumor vessel endothelium in nonnecrotic areas was generally found to be continuous with tight junctions between endothelial cells (Fig. 4B). With light microscopy, an increased thickness of vessel walls was observed in TNP-470-treated tumors (Fig. 3C) and, correspondingly, by electron microscopy, an approximately 400–700-nm thick layer of electron-dense material was ubiquitously observed along the endothelial basement membrane (Fig. 4C). This material was found in TNP-470-treated tumors with and without additional radiotherapy but not in untreated controls nor in tumors treated with ionizing radiation only.

**Effect of TNP-470 Treatment on Vessel Density.** After 1 week of TNP-470 treatment, no significant difference in vascular density was seen between treated and control tumors (Fig. 3, D and E), despite a pronounced growth-retarding effect of TNP-470. The mean number of vessel counts (Chalkley point counts) per field in TNP-470-treated tumors was 5.0 (4.5–5.5) compared with 4.6 (4.2–5.0) in control tumors (parentheses indicate 95% confidence intervals). A $t$ test for equality of means yielded no significant difference ($P = 0.13$).

**Effect of Treatment on Expression of Angiogenic Factors.** The expression of VEGF, bFGF, angiopoietin-1, and angiopoietin-2 at mRNA level in different treatment groups was examined using Northern blots. Four membranes, each comprising tumor-tissue mRNA from one of the mice from each treatment group, were analyzed. We found no significant difference in GAPDH-adjusted expression of VEGF, angiopoietin-1, and bFGF between groups (Table 3 and Fig. 5), whereas a significantly greater expression of angiopoietin-1 was observed at 48 h after withdrawal of the 1-week TNP-470 treatment (Table 3).

At the protein level we examined the expression of VEGF and bFGF with Western blotting. No difference in VEGF protein was found between groups. The bFGF expression was very low in all of the groups at both the mRNA and protein level.

The mRNA expression of VEGF, bFGF, angiopoietin-1, and angiopoietin-2 per total amount of mRNA was, without exception, greater in cultured U87 cells in vitro than in the corresponding U87 tumors grown in vivo (Fig. 5).

**Discussion**

Our findings document that pretreatment with TNP-470 significantly enhances the growth-retarding effect of ionizing IR on a s.c. transplanted human glioblastoma tumor line in nude mice. TNP-470 therapy alone also produced a significant delay relative to untreated controls. As a result of the pretreatment with TNP-470, the tumors in the combination-treatment group were smaller than the tumors in the IR group at the day of radiation. But this difference in size could not alone explain the difference in growth curves between these two groups because the time for each tumor to reach a 3-fold increase of its volume on the day of IR was longer in the combination-treatment group than in the IR group (Table 2).
In contrast, similar treatment schedules with TNP-470 had no effect on the survival of mice with intracranial tumors, given alone or in combination with IR. This discrepancy may reflect differences in tumor vessels due to differences in tumor micro-environment. In the present study, the TNP-470 treatment of intracranial tumors was applied while tumors were relatively small, i.e., from day 7 to 14 after the injection of tumor cells. Recently, Holash et al. (16) proposed a preangiogenic phase, in which small tumor implants are predominantly supported by cooption of existing brain vessels. They observed that intracranially injected glioma cells grew around existing vessels in the first 2 weeks, and that de novo angiogenesis did not start until later. This could explain the presumable lack of effect on the intracranial formation of tumor vessels in the present study.

### Table 3  mRNA expression of angiogenic factors

<table>
<thead>
<tr>
<th></th>
<th>+IR a</th>
<th>−IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+TNP-470</td>
<td>−TNP-470</td>
</tr>
<tr>
<td>VEGF 8 h</td>
<td>0.21 (0.11–0.24)</td>
<td>0.18 (0.09–0.77)</td>
</tr>
<tr>
<td>VEGF 48 h</td>
<td>0.23 (0.20–0.27)</td>
<td>0.14 (0.04–0.17)</td>
</tr>
<tr>
<td>bFGF 8 h</td>
<td>0.02 (0–0.03)</td>
<td>0.01 (0–0.04)</td>
</tr>
<tr>
<td>bFGF 48 h</td>
<td>0.03 (0–0.07)</td>
<td>0 (0–0.03)</td>
</tr>
<tr>
<td>Ang-1 b</td>
<td>0.38 (0–0.83)</td>
<td>0.14 (0–0.06)</td>
</tr>
<tr>
<td>Ang-1 c</td>
<td>0.65 (0.43–1.08)</td>
<td>0.26 (0–0.38)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.77 (0–0.62–1.63)</td>
<td>0.77 (0–0.59–1.32)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>1.03 (0–0.64–1.27)</td>
<td>0.65 (0–0.64–1.26)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.14 (0–0.06)</td>
<td>0.22 (0–0.01)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.26 (0–0.38)</td>
<td>0.49 (0–0.35–0.72)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.14–1.58 (0.06–0.49)</td>
<td>0.47 (0.16–0.38)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.53 (0–0.40)</td>
<td>0.77 (0–0.41–1.58)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.38–1.08 (0.16–0.38)</td>
<td>0.68 (0.35–0.72)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>0.72 (0–0.39–0.58)</td>
<td>0.53 (0–0.77)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>1.08 (0–0.40)</td>
<td>0.58 (0–0.10–0.75)</td>
</tr>
<tr>
<td>Ang-2 c</td>
<td>1.32 (0–0.64–1.27)</td>
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<td>Ang-2 c</td>
<td>1.27 (0–0.64–1.26)</td>
<td>1.26 (0–0.64–1.26)</td>
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<td>Ang-2 c</td>
<td>0.72 (0–0.39–0.58)</td>
<td>0.75 (0–0.41–1.58)</td>
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<tr>
<td>Ang-2 c</td>
<td>0.53 (0–0.39–0.58)</td>
<td>0.76 (0–0.41–1.58)</td>
</tr>
</tbody>
</table>

a Median values from 4 membranes; numbers in parentheses, range. Arbitrary units; mRNA from U87 in vitro was loaded on each membrane and the U87 in vitro value was set to 1.

### Western

**Northern**

- **VEGF**
- **bFGF**
- **Angiopoietin-1**
- **Angiopoietin-2**
- **GAPDH**

**Western**

**VEGF**

Fig. 5  Representative membrane showing expression of angiogenic factors at mRNA level 8 h and 48 h after treatment. Angiopoietin-1 mRNA expression is up-regulated 48 h after the withdrawal of TNP-470, both with and without IR. No significant difference in expression of VEGF and angiopoietin-2 is seen between different treatment groups. bFGF expression is low in both treated and untreated tumors, compared with in vitro expression. GAPDH is used as loading control. Bottom, no difference in VEGF protein expression is found.
because a much smaller tumor volume is lethal in the brain. To what extent the blood-brain barrier also plays a role for the delivery of TNP-470 to endothelial cells in brain tumors is uncertain. The microenvironment of the brain stimulates formation of a blood-brain barrier, and the U87 tumor environment may disrupt the vascular barriers through release of VEGF and other cytokines. According to the competitive environment hypothesis, the outcome of the competition between these environmental factors determines the intracranial tumor vessel permeability (17). Previous reports on TNP-470 treatment of intracranial gliomas are equivocal. Takamiya et al. (18) treated mice with U87 tumors with TNP-470 from day 5 after implantation until death and found prolonged survival compared with controls. Wilson and Penar (19) found no effect of TNP-470 on survival in mice with intracranial gliosarcomas.

We observed specific morphological effects of IR on tumor endothelium that apparently were avoided by pretreatment with TNP-470. Single-dose radiation in the same doses as used here has been shown to induce an immediate increase in vascular permeability (20). On this basis, we suggest that TNP-470 inhibits the acute endothelial cell membrane damage and edema that are normally seen after irradiation. We, therefore, propose a potential clinical synergetic of the combination of TNP-470 and radiotherapy because TNP-470 apparently prevents an acute side effect of ionizing IR while adding to its beneficial effects.

The extensive accumulation of basement membrane material in vessels of tumors treated with TNP-470 is, to our knowledge, a new observation. Perivascular amorphous matrices not organized as a conventional basal lamina have been observed after gentamicin and cisplatin treatment in normal brain and in some malignant tumors and benign lesions (21). The composition and nature of the basement membrane-related material found in TNP-470-treated tumors are not defined at present. Angiopoietin-1 is considered a regulator of vascular remodeling subsequent to the formation of proliferating vascular sprouts by interaction with pericytes through pathways distinct from those of VEGF (22, 23) and also in concerted action with VEGF (16). The increased expression of this proangiogenic factor after termination of TNP-470 treatment may be related to the morphological changes discussed above. This potential relationship deserves further experimental validation.

The tumor vessel endothelium in the nonirradiated tumors in this study was generally continuous with tight junctions between endothelial cells. Fenestrations were rarely seen. Others have found that U87 tumors on nude mice have fenestrations in 37% of vessels, with 1–44 fenestrations per vessel profile (24). The reason for this difference is not clear. One explanation may be the passage number used because they (24) used U87 tumors passed more than 7 times on nude mice to select for aggressive, faster growing, and more angiogenic tumors. We used passage 3 for our morphological studies.

There is a theoretical problem with combining radiotherapy and antiangiogenesis because the biological effect of ionizing irradiation is sensitive to hypoxia. Antiangiogenic agents, by their inhibition of vessel formation, are sometimes believed to decrease vessel density, and, as a consequence, tumor hypoxia would increase. Assuming that angiogenesis is a growth-limiting step in tumor growth—which is why antiangiogenic therapy works—the ratio of tumor cells to vessels, i.e., vessel density, must remain relatively constant in an individual tumor unless its nutritive demands are changed. Concordantly, we observed no significant change in Chalkley counts of tumor vessels after 1 week of TNP-470 treatment despite a significant growth inhibition. Also, with a 6-week TNP-470 schedule in human glioblastoma xenographs, no changes in vascular density were reported (7), whereas others have actually found a decrease in vessel density after TNP-470 treatment of brain tumors (8–10).

TNP-470 in combination with another antiangiogenic compound, minocycline, has been shown to actually decrease tumor hypoxia (25). We found no change in the VEGF expression, and, because VEGF is known to be up-regulated by hypoxia (26–28), this further supports our notion that TNP-470 does not induce hypoxia.

In summary, pretreatment with TNP-470 significantly enhanced the growth-retarding effect of ionizing IR, while preventing radiation-induced microvascular damage. A broadening of the endothelial basement membrane and an increased expression of angiopoietin-1 seem to be involved in this protective mechanism. The vessel density was found unaltered by the antiangiogenic treatment, which further adds to the evidence that vessel counts are not reliable parameters for the evaluation of antiangiogenic effect.

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REFERENCES


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