Sulfoglycolipids as candidate antiangiogenic radiosensitizers

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Angiogenesis is considered an essential process for the growth of solid tumors and, accordingly, angiogenesis has been a focus of attention for cancer therapy. Although various antiangiogenic agents have been developed, adverse effects and limitations associated with antitumor therapies have recently become apparent. To overcome these problems, combining such agents with chemotherapy or radiotherapy is now strongly recommended in clinical practice. Provided such combination treatment, from the onset of therapy, different strategies in developing antiangiogenic agents should be used to enhance any combinatory effects and reduce adverse effects. By applying the concept of radiosensitizers, a new class of antiangiogenic treatments should now be possible. We recently developed sulfoglycolipids that possess such properties. In this review, we discuss the properties of antiangiogenic radiosensitizers and their potential usefulness. ***Anti-Cancer Drugs*** **18:1–5 © 2007 Lippincott Williams & Wilkins.**

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History of antiangiogenic agents

Angiogenesis is an essential process for the growth of solid tumors, and is a complex process consisting of the migration, invasion and cell growth of vascular endothelial cells [1–3]. Folkman [4] proposed in 1971 that the inhibition of angiogenesis would be a useful strategy to treat cancer. After two decades, his group found a fragment of plasminogen, angiostatin, which possessed strong antiangiogenic activity [5]. This endogenous factor explained a clinically known phenomenon in which tumors in the metastatic region grow vigorously after the excision of primary tumors. More specifically, angiostatin produced by primary tumors inhibited tumor growth in such regions. Endostatin, a fragment of collagen XVIII, is another antiangiogenic factor also found by Folkman's group [6]. The preclinical results regarding the antitumor effects of angiostatin and endostatin were dramatic: tumors disappeared or became dormant only after several doses and the dormant tumors did not grow without drug administration [7]. Drug resistance was not detected because the target was not tumor cells, but rather the vascular endothelial cells, which have extremely low genetic instability. Furthermore, adverse effects were thought not to occur because these drugs are specific to vascular endothelial cells during angiogenesis, which is present primarily in tumor tissues. Therefore, clinical applications were enthusiastically anticipated. However, they have not yet been approved; instead, a rationally designed humanized antibody targeting vascular endothelial growth factor (VEGF), bevacizumab (Avastin; Genentech, South San Francisco, California, USA), was first approved by the Food and Drug Administration. Avastin was effective against metastatic colon cancer and non-small cell lung cancer when combined with chemotherapy [8,9]. Contrary to the preclinical studies, it seems that a single administration is insufficient for complete eradication of disease and even after prolonged administration, tumors recur if the treatment is stopped. Adverse effects, including hypertension, bleeding and arterial thrombosis, were also observed [10], despite the anticipation that antiangiogenic agents would not induce adverse effects. Antiangiogenic agents are now considered unlikely to be the anticancer panacea they were once predicted to become, and a combination of antiangiogenic agents and other modalities is thus recommended in clinical practice.

Mechanism of the combined effects with radiation

To our knowledge, the first paper reporting that the combination of radiation and antiangiogenic agents was effective in animal studies was by Weichselbaum's group [11]. The antiangiogenic agent was angiostatin, and the

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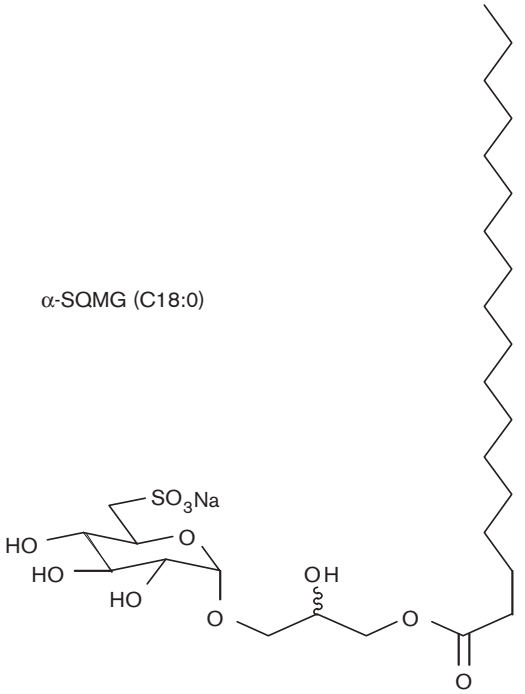
combined effect was not remarkable and nearly additive, but the report was noteworthy at the time because antiangiogenic agents were believed to possess no side-effects or drug resistance. The paper did not describe the mechanism of the combined effect except to note that the induction of apoptosis was not enhanced in endothelial cells after the combined therapy. Thereafter, other groups reported the combination of other antiangiogenic agents with radiation, most of which exhibited additive effects, although some were synergistic [12,13]. Still, little was known about the mechanism of the combined effect. Considering that radiation induces damage of both tumor cells and vascular endothelial cells, the apparent radioresponsiveness of solid tumors reflects direct tumor cell killing and indirect tumor growth inhibition or cell death mediated by the antiangiogenic effects of the damaged vascular endothelial cells. On the other hand, some researchers reported that radiation actually activates angiogenesis via the production of proangiogenic and antiapoptotic factors, such as VEGF, from irradiated tumor cells [14,15]. Thus, it remains unclear as to whether radiation inhibits or activates angiogenesis. In any event, it is conceivable that combining antiangiogenic agents with radiation serves to enhance any antitumor effects. This is, however, paradoxical. Radiobiological considerations raise the issue that the inhibition of angiogenesis may increase hypoxic fractions, thereby mitigating any radiation effects. Hypoxia in solid tumors has been a major cause of the failure of radiotherapy, which induces extreme radioresistance of tumor cells [16]. Indeed, several papers have reported that the combination of antiangiogenic agents and radiation reduced the radiation effect [17,18]. In an effort to better understand these issues, Jain's group proposed a model of 'vascular normalization' [19]. Tumor blood vessels are generally leaky, tortuous and dilated. The endothelial cells lining these vessels have an aberrant morphology and pericytes are either loosely attached or absent. These vascular abnormalities lead to abnormal tumor microenvironment characteristics, including interstitial hypertension, hypoxia and acidosis. Interestingly, Jain's group showed that such abnormal vasculature is normalized after treatment with antiangiogenic agents. The treatment decreases the interstitial fluid pressure via the reduction of vascular permeability, leading to the efficient delivery of oxygen or therapeutics to solid tumors.

Mild hyperthermia reportedly increases vessel blood flow and the vascular permeability of tumor tissues, which is considered to increase the oxygen tension of tumor tissues and eventually leads to radiosensitization when combined with radiotherapy [20]. Interestingly, the complete opposite occurs between vascular normalization and mild hyperthermia in terms of vascular permeability, although reoxygenation occurs in both cases. The increase in oxygen tension after vascular normalization is never induced when blood flow is severely slowed by the inhibition of angiogenesis [19], so appropriate levels of angiogenesis inhibition are required. To this end, adjusting the optimal timing between irradiation and vascular normalization would be difficult. It is still unclear whether the antiangiogenesis and reoxygenation processes are necessarily coupled. If we can elucidate the exact molecular mechanism of the reoxygenation process associated with decreased interstitial fluid pressure, the agents targeting only this specific mechanism may prove to be a novel type of radiosensitizer.

Concept of antiangiogenic radiosensitizers

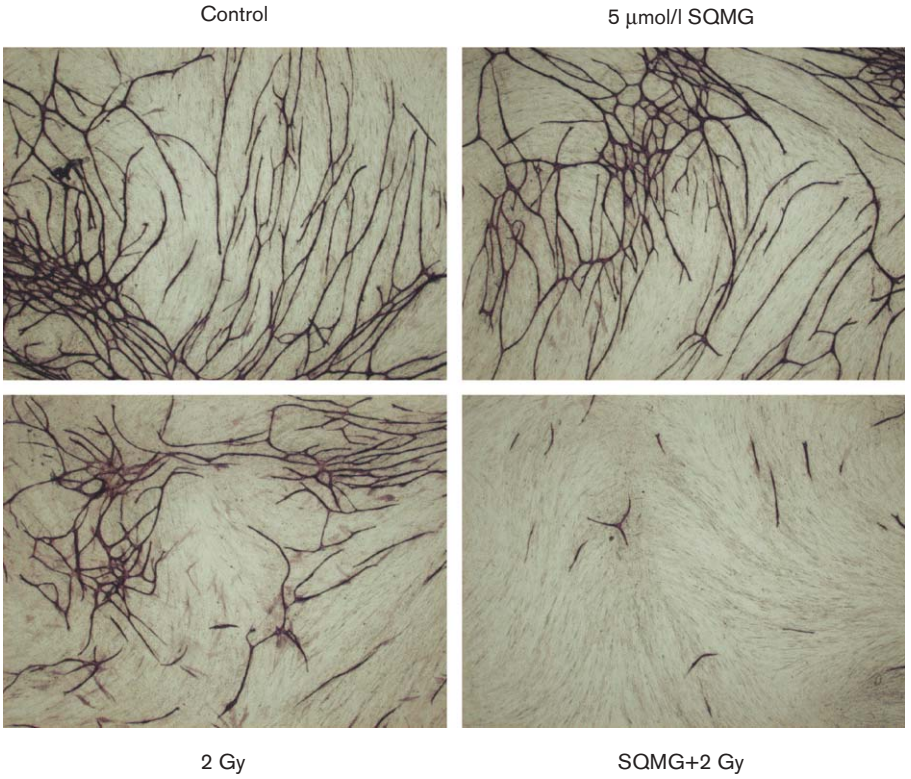
The vascular normalization model was the first to implicate synergistic interaction mechanisms between the effects of radiation and the antiangiogenic agents of reoxygenation. This encouraged us to seek agents with efficient synergistic actions in combination and led us to develop the novel concept of 'antiangiogenic radiosensitizers', which exhibit synergistic antiangiogenesis only when combined with radiation. Radiosensitizers by themselves have no antitumor effects, but synergistically enhance them when combined with radiation [21]. Although radiosensitizers generally target tumor cells, this concept can also be applied to vascular endothelial cells. These agents are harmless in the absence of irradiation, which minimizes systemic side-effects.

Fig. 1



Schematic chemical structure of α-sulfoquinovosylmonoacylglycerol (SQMG). C18, the carbon number of the fatty acid chains; 0, saturated fatty acids.

Fig. 2



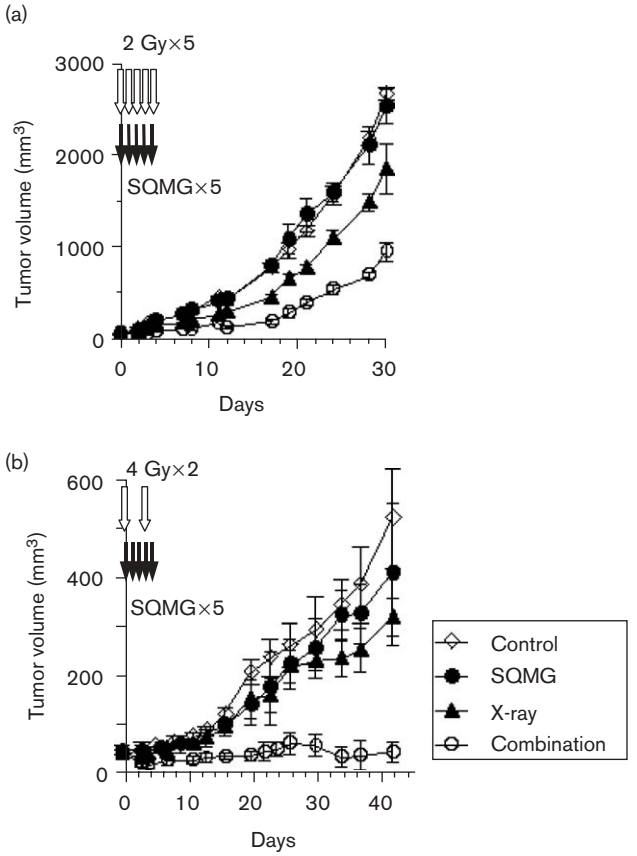
Tube formation of human umbilical vein endothelial cells (HUVECs) in HUVEC–fibroblast cocultures after combined treatment.

Radiotherapeutic techniques make it possible to focus radiation on tumor tissues. Therefore, such agents synergistically inhibit angiogenesis only within tumor tissues when combined with radiation, subsequently inducing vascular normalization and eventually leading to the radiosensitization of tumor cells via reoxygenation after the next properly timed irradiation.

Sulfoquinovosylmonoacylglycerol as a candidate antiangiogenic radiosensitizer

We recently found a group of candidate antiangiogenic radiosensitizers. Sulfoquinovosylacylglycerols (SQAGs) are sulfoglycolipids originally derived from sea urchins [22], and are also found naturally in higher plants and sea algae [23]. They fall into two groups according to the number of fatty acids, monoacyl forms (sulfoquinovosylmonoacylglycerol, SQMG) (Fig. 1) and diacyl forms (sulfoquinovosyldiacylglycerol). Two isomers (α and β) are structurally possible, but natural products include only the α-isoforms. We can now chemically synthesize most types of SQAGs. Sahara *et al.* [24] reported that SQMGs inhibit the growth of the human lung adenocarcinoma cell line A549 after subcutaneous transplantation into nude mice. Interestingly, this activity was apparent under in-vivo conditions but not in in-vitro cell culture. We hypothesized that, *in vivo*, SQMGs may adversely affect angiogenesis. Using a human umbilical vein endothelial cell–fibroblast coculture assay, we found that this was the case. We then examined whether the agents possessed the properties of antiangiogenic radiosensitizers. Interestingly, all assays concerning capillary-like formation on a Matrigel layer, invasion through a Matrigel layer and tube-like formation by human umbilical vein endothelial cell–fibroblast coculture assay (Fig. 2) revealed synergistic inhibition after combining SQMGs with radiation at doses presenting no effects when either was used alone [25]. When several human tumors transplanted into nude mice were treated with a combination of radiation and SQMGs, a synergistic delay of tumor growth was observed at doses presenting no antitumor effects of SQMG used alone [25] (Fig. 3). Furthermore, the vascular density was significantly lower in tumors that received the combined treatment than in those treated with radiation alone [25]. We are now investigating the kinetics of the fluctuation of oxygen tension after treatment, which would determine the optimal timing of the next irradiation, assuming vascular normalization. These results demonstrate that agents with the characteristics of antiangiogenic radiosensitizers do exist.

Fig. 3



Tumor growth delay in *in vivo* after combined treatment. (a) SAS (a human tongue squamous cell carcinoma cell line) and (b) A549 (a human lung adenocarcinoma cell line).

Induction of a senescence-like phenotype after combination treatment with sulfoquinovosylmonoacylglycerol

In the course of examining the radiosensitivity of vascular endothelial cells, we noticed some interesting properties of the combination therapy of radiation and SQMGs. Colony-forming ability was not affected at doses up to 25 μmol/l SQMG incubated for 24 h. The surviving fraction, however, significantly decreased when radiation was combined with 5–10 μmol/l SQMG. Surprisingly, apoptosis was not significantly detected even after the combined treatment. The colony morphology revealed that the induction of a senescence-like phenotype was enhanced after the combination treatment; the surviving cells became large and extended, staining positive in a β-galactosidase assay, which is a representative marker of senescence. We speculate that senescence-like growth arrest would be a strong angiogenesis inhibitor [25]. Garcia-Barros *et al.* [26] reported that the apoptotic activity in vascular endothelial cells after irradiation was the primary determinant of tumor response. Our data may shed light on the role of senescence in endothelial cells in tumor radioresponsiveness.

We recently found that radiation alone (~8 Gy) induces a high-frequency (>90%) senescence-like phenotype in vascular endothelial cells and that such growth arrest could result in the strong inhibition of angiogenesis (Igarashi *et al.* 2006, in preparation). The results explain the fact that the synergistic effect of SQMGs on the surviving fraction becomes lower as the radiation dose increases. This indicates, therefore, that SQMGs enhance the radiation-induced senescence-like phenotype that leads to the dysfunction of vascular endothelial cells, suggesting that surviving endothelial cells that express a senescence-like phenotype could also contribute to the inhibition of angiogenesis in addition to inducing apoptosis. Although the mechanism of the radiation-induced senescence-like phenotype is still unclear, the action of SQMGs could help uncover more mechanistic details.

Molecular targets of sulfoquinovosylacylglycerols

Avastin is a rationally designed humanized antibody against VEGF, so its molecular target is clear. The molecular targets of SQAGs, on the other hand, are unknown, although these agents are strong inhibitors of DNA polymerases α and β [27,28]. Indeed, SQAGs inhibit DNA polymerases in a cell-free system, although DNA replication in living cells is not efficiently inhibited because SQAGs are membrane-impermeable. Considering that the SQMG dose at which angiogenesis is strongly inhibited did not inhibit DNA replication [25], the molecular targets of SQAGs should exist on cell membranes or as extracellular proteins, such as growth factors or cytokines. To identify the molecular targets, we used a phage display screen. By screening a phage library, we found several candidates including VEGF, platelet-derived growth factor, Tie-1, and Tie-2, which are growth factors or endothelial cell-specific membrane-bound receptors. SQAGs bound to the extracellular domains of these receptors (unpublished data). Further screening is underway, and the discovery of actual targets would help clarify the combined mechanism of SQAGs and radiation.

Radiation induces VEGF production by tumor cells [29], so radiation may enhance the accumulation of SQAGs in tumor tissues if SQAGs also bind VEGF *in vivo*. Furthermore, we can form SQAG vesicles by taking advantage of their lipidic properties [30]. By utilizing these properties of SQAGs, many new therapeutic possibilities may become available, including drug delivery systems and imaging techniques in addition to those of radiosensitizers.

Conclusion

In this review, we have described antiangiogenic radiosensitizers and introduced novel radiosensitizers with similar properties. Availability of SQAGs may open new possibilities in the development of multimodal combination therapies against cancer.

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