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Research Paper

Comparison of the effects of marchantin C and fucoidan on sFlt-1 and angiogenesis in glioma microenvironment

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Abstract

Objectives This study aimed to examine the effects of marchantin C and fucoidan on angiogenesis induced by glioma cells and monocytes, and to elucidate the role of sFlt-1 in this process.
Methods T98G glioma cells and THP1 monocytes were pretreated with marchantin C or fucoidan, respectively. Conditioned media were used for endothelial cell tube formation assay and detection of sFlt-1 by ELISA. Depletion of sFlt-1 was achieved by a neutralizing antibody to assess its role in the process.
Key findings Marchantin C inhibited angiogenesis induced by T98G cells while fucoidan inhibited both T98G and THP1 cell-induced angiogenesis. In all three groups in which angiogenesis was inhibited, sFlt-1 level in the supernatants was elevated. Pretreatment of the conditioned media with sFlt-1 antibody restored the inhibited angiogenesis to a certain degree.
Conclusions This study suggested for the first time that marchantin C and fucoidan could significantly inhibit angiogenesis induced by glioma cells or monocytes. Up-regulation of sFlt-1 played an important role in this process.

Introduction

Malignant glioma is the most frequently occurring primary intracranial tumour in adults and also one of the most refractory tumours. Despite various therapeutic methods, such as surgical resection, radiotherapy and chemotherapy, no more than 3% of patients with glioblastoma can survive five years.[1] One of the main reasons for this recalcitrance is the excessive pathological angiogenesis in glioma tissue. Neovascularization is a hallmark of glioma biology and contributes to its malignancy. In astrocytoma, which is a subtype of glioma, high-grade tumours show higher microvessel density than low-grade tumours.[2] In the highly complicated process of angiogenesis, vascular endothelial growth factor (VEGF) has been studied extensively as a pro-angiogenic factor and is believed to occupy a central position.[3] Similar to neovascularization, VEGF concentration within glioma has been reported to correlate directly with its degree of malignancy.[4] In the microenvironment of glioma tissue, glioma cells and infiltrating myeloid cells are both important sources of VEGF. Although myeloid cells account for a much smaller fraction compared with tumour cells, they play an irreplaceable role in tumorigenic angiogenesis,[5] probably because of the unique effect of myeloid-derived VEGF-A on phosphorylation of VEGF receptor 2, which is the major mediator of VEGF-A's effects on endothelial cells. Over the past few decades, researchers have focused on anti-angiogenic therapy as an effective anti-tumour method and several products have shown promise in tumour treatment. The products, mainly targeting VEGF signal transduction, include VEGF antibody (e.g. bevacizumab), VEGF receptor tyrosine kinase inhibitors (e.g. sunitinib, cediranib) and decoy ligand (i.e. VEGF-TRAP). Anti-angiogenic agents influence tumour growth mainly in two ways. Firstly, they starve tumour cells by depriving them of their delivery of nutrients and oxygen. Secondly, they can transiently normalize the tortuous tumour vasculature and thus enhance the efficacy of concurrent radiotherapy and chemotherapy.[6,7] Despite evidence of their efficiency in improving patients' prognosis, the effects of the aforementioned products are still unsatisfactory and toxicity, such as hypertension, proteinuria and thromboembolic events, is frequently observed.[8–10] Therefore, a novel endogenous anti-angiogenic factor – soluble VEGF receptor 1 (sFlt-1) – has become the focus of much research.

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sFlt-1 was originally cloned in 1993 by Kendall *et al*.[11] and is an alternatively spliced variant of membrane-binding VEGF receptor 1.[12] Compared with membrane-binding VEGF receptors, sFlt-1 keeps the ligand-binding extracellular domain, but lacks transmembrane and intracellular domains.[13] As the affinity of sFlt-1 to VEGF-A is much stronger than that of VEGF receptor 2,[14] sFlt-1 competitively inhibits the binding of VEGF ligands to VEGF receptor 2. Another mechanism by which sFlt-1 inhibits VEGF-dependent signalling is to form heterodimers with membrane-binding receptors and inhibit VEGF signalling in a dominant negative way.[15] In human beings, sFlt-1 mainly comes from vascular endothelial cells, but other cells, such as glioma cells and monocytes, are also sources of sFlt-1.[16,17] With regard to gliomas, sFlt-1 was reported to correlate with VEGF level, microvessel density, tumour malignancy and prognosis.[18,19] Several studies have shown its efficacy in glioma treatment through gene delivery.[20–22]

Marchantin C, a family member of macrocyclic bisbibenzyls, is exclusively derived from liverworts.[23] This class of compound has shown a variety of biological functions (e.g. cytotoxicity, anti-tumour and anti-fungal activity).[24–26] Marchantin C was reported to inhibit proliferation and invasion of glioma cell lines U87 and T98G, and promote the apoptosis of A172 glioma cell line.[27,28] Fucoidan, a sulfated polysaccharide rich in L-fucose and ester groups, is extracted from brown seaweed and some marine invertebrates like sea urchins.[29,30] It is also called fucan, fucosan or sulfated fucan. Fucoidan has more complex biological functions than marchantin C, including anti-coagulant, anti-virus, anti-tumour, immunomodulatory, anti-oxidant, anti-complementary and anti-inflammatory activity.[31] In tumour necrosis factor-alpha (TNF-α)-stimulated and interferon-gamma (IFN-γ)-stimulated C6 glioma cells, fucoidan suppresses nitric oxide (NO) production and inducible NO synthase (iNOS) expression through p38 MAPK, JAK/STAT, AP-1 and IRF-1 pathways.[32] Up to now, no study of marchantin C or fucoidan on sFlt-1 production has been reported. In this study, we compared functions of marchantin C and fucoidan on sFlt-1 secretion from glioma cells and monocytes and subsequent angiogenesis.

Materials and Methods

Chemicals

The structure of marchantin C, isolated from *Dumortiera angust* was identified by interpretation of spectral data (MS, ^{1}H NMR, ^{13}C NMR, 2D NMR) as described previously.[33] Marchantin C was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at −20°C as stock solution. Suitable dilution was performed when used according to experimental requirements.

Fucoidan isolated from *Fucus vesiculosus* was obtained from Sigma-Aldrich (St Louis, USA). The drug was dissolved in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml and stored at −20°C as stock solution. Suitable dilution was performed according to experimental requirements when the drug was used.

Marchantin C and fucoidan were sterilized by a 0.2 μm syringe filter (Pall Life Sciences, Ann Arbor, USA).

Cell culture and treatment

Human malignant glioma cell line T98G cells, human monocyte cell line THP1 cells and human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (USA). T98G and THP1 cells were routinely incubated in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium (Hyclone, Logan, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Grand Island, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin. HUVECs were cultured in M199 medium (Gibco, Grand Island, USA) supplemented with 10% FBS, 5 mg/ml bFGF, 25 U/ml heparin, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a humidified atomosphere with 5% CO_2 at 37°C.

For drug treatment, T98G cells or THP1 cells were seeded in 24-well plates at a density of 1×10^5 cells/well. After incubation for 24 h, cells were treated with marchantin C (5 μM for T98G, 8 μM for THP1) or fucoidan (100 μg/ml) and incubated in growth medium for another 24 h. DMSO and PBS, which were the solvents of the two drugs, were used as controls at corresponding concentrations. Then conditioned media were collected and centrifuged at 5000 rpm for 5 min at 4°C to remove cell debris. Supernatants were immediately stored at −80°C for further ELISA detection and application in the tube formation assay.

Tube formation assay

A 96-well culture plate was coated with matrigel (60 μl/well) (BD Biosciences Pharmingen, San Diego, USA), which was then allowed to solidify at 37°C for 0.5 h. HUVECs were harvested using trypsin and suspended in mixed solution of equal amounts of M199 medium and conditioned medium to a final concentration of 5×10^5 cells/ml. A 200-μl volume of cell solution was added into each matrigel-coated well and the cells were incubated at 37°C in a 5% CO_2 atmosphere for 6 h. The resulting morphological changes in the endothelial cells were photographed at × 40 magnification and the level of tube formation was determined by counting branch points from tubes formed between discrete endothelial cells in three random fields per well. Unconditioned media added with corresponding amount of drugs right before tube formation assay were used as controls to eliminate direct anti-

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angiogenic effect of marchantin C or fucoidan. For antibody-treated groups, conditioned media were pretreated with 2 μg/ml sFlt-1 antibody (R&D Systems, Minneapolis, USA) for 0.5 h at 4°C.

Enzyme-linked immunosorbent assay

sFlt-1 in supernatants was detected using ELISA kit purchased from R&D Systems (Minneapolis, USA) according to the manufacturer's instruction. Recombinant human sFlt-1 were used to generate a standard curve from which sFlt-1 concentration was calculated.

Statistical analysis

Results were expressed as mean ±standard deviation (SD). Differences between groups were analysed by one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

Results

Marchantin C inhibits glioma cell-induced angiogenesis *in vitro*

The effects of marchantin C on angiogenesis induced by T98G or THP1 cells were determined by tube formation assay of HUVECs cultured with conditioned medium of marchantin C-treated cells. Figure 1a shows tubular structure formed by endothelial cells in each group. The level of tube formation was quantified by counting branch points from tubes formed between discrete endothelial cells. Compared with the control group, marchantin C reduced T98G cell-induced tube formation by 57% ± 7% while THP1 cell-induced tube formation was not significantly affected (Figure 1b).

Fucoidan inhibits both glioma cell-induced and monocyte-induced angiogenesis *in vitro*

The effects of fucoidan on T98G-induced or THP1-induced angiogenesis were also determined by tube formation assay using conditioned medium of fucoidan-treated cells. Figure 2a shows tubular structure formed by endothelial cells in each group. As quantified by counting branch points from tubes formed between discrete endothelial cells in Figure 2b, fucoidan reduced T98G cell-induced and THP1 cell-induced tube formation by 43% ± 6% and 52% ± 6%, respectively.

Marchantin C increases sFlt-1 level in the supernatant of T98G cells

ELISA was performed to detect the influence of marchantin C on sFlt-1 level in the supernatant of T98G or THP1 cells. As shown in Figure 3 (first two groups), marchantin C increased the sFlt-1 level in the supernatant of T98G cells to about two fold that of its control group, whereas the sFlt-1 level in the supernatant of THP1 cells was not significantly influenced by marchantin C.

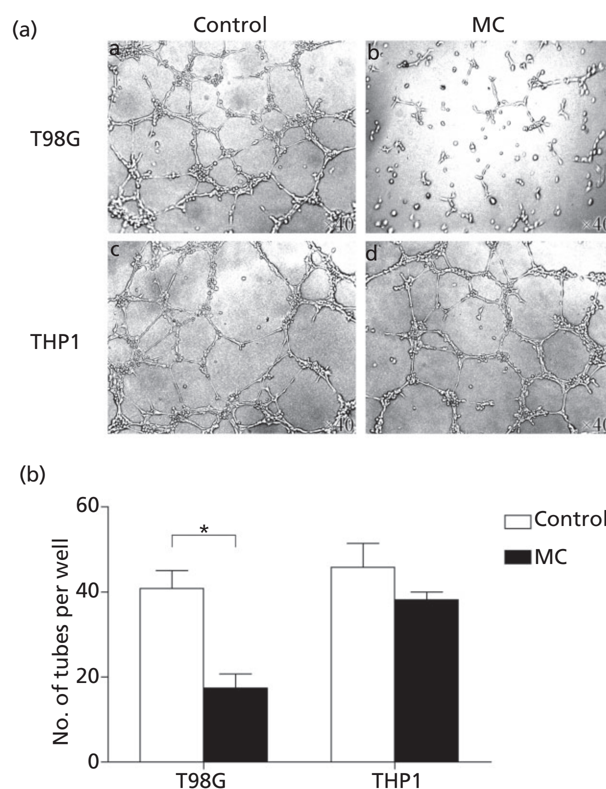

Figure 1 The effect of marchantin C on T98G and THP1 cell-induced angiogenesis. T98G glioma cells or THP1 monocytes were treated with marchantin C (5 μM for T98G, 8 μM for THP1) for 24 h, the conditioned media were used for tube formation assay of human umbilical vein endothelial cells. (a: a, b) T98G cell-induced tube formation was inhibited by marchantin C (MC). (a: c, d) THP1 cells induced tube formation was not significantly influenced by marchantin C (MC). (b) Quantification of tube formation was done by counting branch points from tubes formed between discrete endothelial cells in three random fields per well, *$P < 0.05$.

Fucoidan increases sFlt-1 level in the supernatants of both T98G and THP1 cells

The effects of fucoidan on sFlt-1 level in supernatants were determined in the same way as for marchantin C. As shown in Figure 3 (last two groups), fucoidan increased sFlt-1 level in the supernatant of T98G cells to about ten fold that of its control group and increased sFlt-1 level in the supernatant of THP1 cells to about seven fold that of its control group.

Inhibition of angiogenesis was recovered by sFlt-1 antibody

For the three groups in which angiogenesis was inhibited by drugs (marchantin C-T98G, fucoidan-T98G and

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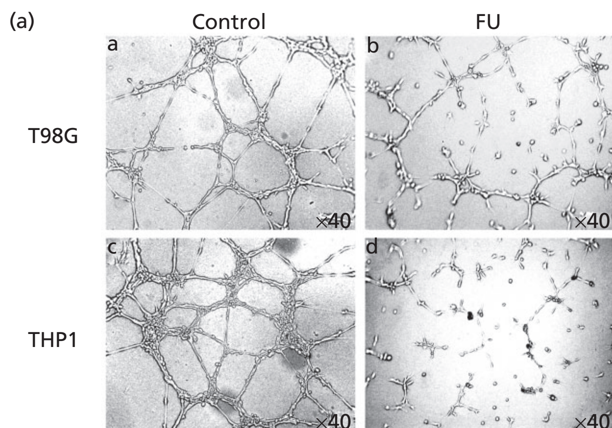


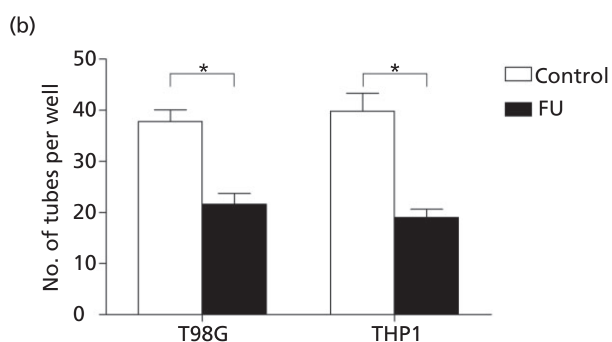


Figure 2 The effect of fucoidan on T98G and THP1 cell-induced angiogenesis. T98G glioma cells or THP1 monocytes were treated with fucoidan (100 μg/ml) for 24 h and the conditioned media were used for tube formation assay of human umbilical vein endothelial cells. (a: a, b) T98G cell-induced tube formation was inhibited by fucoidan (FU). (a: c, d) THP1 cell-induced tube formation was also inhibited by fucoidan (FU). (b) Quantification of tube formation was done by counting branch points from tubes formed between discrete endothelial cells in three random fields per well, $^{*}P < 0.05$.

fucoidan-THP1), conditioned media were pretreated with a specific neutralizing antibody of sFlt-1 before use in the tube formation assay. Figure 4a shows tubular structure formed by endothelial cells in each group. The level of tube formation was quantified by counting branch points from tubes formed between discrete endothelial cells. sFlt-1 antibody recovered the inhibited tube formation in the marchantin C-T98G group, fucoidan-T98G group and fucoidan-THP1 group to 76% ± 5%, 95% ± 7% and 70% ± 8% of the control group, respectively, (Figure 4b).

Discussion

As most anti-tumour chemical drugs are developed from natural active components, extracting anti-tumour chemicals from natural plants followed by structural modification and reconstruction has been paid more and more attention in anti-tumour pharmaceutical research. For example, taxol,

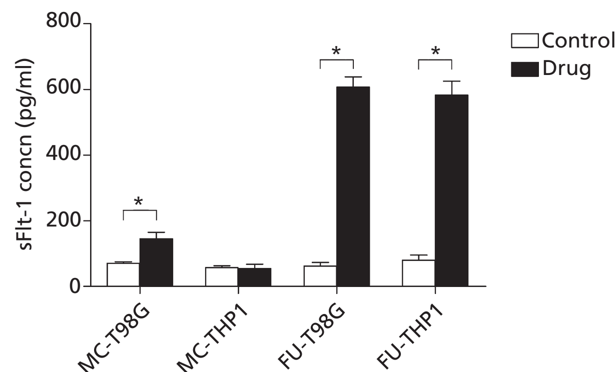
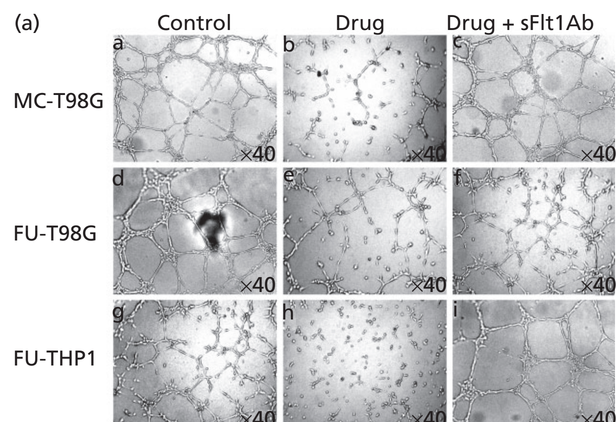


Figure 3 The effect of marchantin C and fucoidan on sFlt-1 level in the supernatants of T98G and THP1 cells. T98G cells or THP1 cells were treated with marchantin C (5 μM for T98G, 8 μM for THP1) or fucoidan (100 μg/ml) for 24 h and the conditioned media were used for ELISA according to the manufacturer's instruction. sFlt-1 level were significantly elevated in marchantin C-T98G (MC-T98G), fucoidan-T98G (FU-T98G) and fucoidan-THP1 (FU-THP1) groups. No significant change was observed in marchantin C-THP1 (MC-THP1) group. Each value represents means ± SD from three independent experiments, $^{*}P < 0.05$.



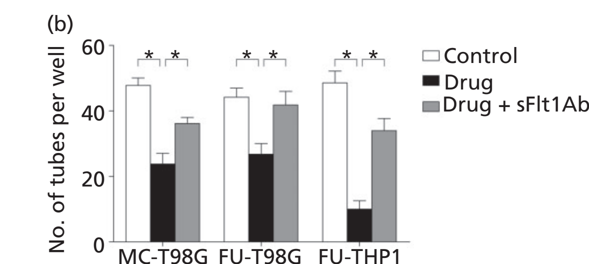


Figure 4 Depletion of sFlt-1 resulted in recovery of angiogenesis inhibited by marchantin C or fucoidan. Conditioned media were pretreated with a neutralizing antibody against sFlt-1 (2 μg/ml) for half an hour before use in the tube formation assay. (a) The inhibited tube formation in the three groups (marchantin C-T98G (MC-T98G), fucoidan-T98G (FU-T98G), fucoidan-THP1 (FU-THP1)) was recovered by pretreatment with sFlt-1 antibody. (b) Quantification of tube formation was done by counting branch points from tubes formed between discrete endothelial cells in three random fields per well, $^{*}P < 0.05$.

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which is isolated from the plant *Taxus brevifolia*, has been used universally in anti-tumour chemotherapy. Marchantin C and fucoidan are both natural components of plants with a variety of biological actions. However, their effects on glioma have not been extensively studied, especially in the field of tumour-induced angiogenesis. In this study, we witnessed the different patterns of these two drugs in inhibiting glioma-induced angiogenesis and further studied the underlying molecular mechanism in two main types of cells from the glioma microenvironment, glioma cells and infiltrating monocytes.

First, by endothelial cell tube formation assay *in vitro*, we found that the two drugs both inhibited glioma-induced angiogenesis, with marchantin C focusing on T98G cells and fucoidan influencing both T98G and THP1 cells. Then, as VEGF plays a pivotal role in the process of angiogenesis and both glioma cells and monocytes were reported to have the ability to secrete sFlt-1, an endogenous sequestrator of VEGF, we further examined the level of sFlt-1 in the supernatants of drug-treated glioma cells and monocytes by ELISA. In all the four groups (marchantin C-T98G, marchantin C-THP1, fucoidan-T98G, fucoidan-THP1), three groups (marchantin C-T98G, fucoidan-T98G, fucoidan-THP1), in which induced angiogenesis was inhibited, showed elevation of sFlt-1 level. Whereas, in the marchantin C-THP1 group in which induced angiogenesis was not influenced, sFlt-1 level was not significantly changed either. Finally, to determine whether the inhibition of angiogenesis was attributed to elevated sFlt-1 in the supernatant of T98G or THP1 cells, sFlt-1 in the supernatant was immunodepleted by a neutralizing antibody before performing the tube formation assay. As we had anticipated, depletion of sFlt-1 restored the previously inhibited tube formation to a certain degree in all three groups. However, we found that in glioma cells, the up-regulated sFlt-1 level by the two drugs does not confirm to their tube formation level. We hypothesize that marchantin C might also induce other anti-angiogenic factors (e.g. endostatin, thrombospondin or TIMP-1) from glioma cells. But the inhibited angiogenesis could at least partially be attributed to up-regulated sFlt-1. Taking the previously mentioned anti-tumour effects of the two drugs into consideration, we suggest that combination of marchantin C and fucoidan may effectively reduce angiogenesis in glioma tissue and meanwhile directly inhibit proliferation and invasion of glioma cells and even induce their apoptosis. Moreover, anti-angiogenic therapy may result in a transient normalization of malformed tumour vasculature, which would enhance the efficacy of concomitant radiotherapy or chemotherapy. All these actions will contribute to the treatment of malignant gliomas. In our subsequent study, we will investigate the combined effect of marchantin C and fucoidan both *in vitro* and *in vivo*, including anti-angiogenic effect and anti-tumour effect. Besides, other potential anti-angiogenic factors induced from glioma cells by marchantin C and the fundamental mechanism of the anti-angiogenic effect will be further studied.

Conclusions

In this study, for the first time, we demonstrated that marchantin C and fucoidan, which are extracts from natural plants, can inhibit glioma-induced angiogenesis, which is at least partially attributed to the increased sFlt-1 secretion from glioma cells or monocytes. Marchantin C mainly affects T98G cells while fucoidan affects both T98G and THP1 cells. As a potent endogenous VEGF sequestrator released by VEGF-producing cells, such as glioma cells and monocytes, sFlt-1 could be an optimal target of anti-angiogenic therapy for its effectiveness in inhibiting VEGF signal transduction without the reverse effects in other anti-angiogenic approaches such as bevacizumab, sunitinib or sFlt-1 gene delivery. So combination of marchantin C and fucoidan should result in up-regulation of overall sFlt-1 level in the glioma microenvironment and stronger inhibition of glioma-induced angiogenesis; yet more research is needed for the combined effects and underlying mechanisms.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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