Extract of *Cordyceps militaris* inhibits angiogenesis and suppresses tumor growth of human malignant melanoma cells

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Abstract. Angiogenesis is essential for tumor development and metastasis. Among several angiogenic factors, vascular endothelial growth factor receptor (VEGF) is important for tumor-derived angiogenesis and commonly overexpressed in solid tumors. Thus, many antitumor strategies targeting VEGF have been developed to inhibit cancer angiogenesis, offering insights into the successful treatment of solid cancers. However, there are a number of issues such as harmful effects on normal vascularity in clinical trials. Taking this into consideration, we employed *Cordyceps militaris* as an antitumor approach due to its biological safety *in vivo*. The herbal medicinal mushroom *Cordyceps militaris* has been reported to show potential anticancer properties including anti-angiogenic capacity; however, its concrete properties have yet to be fully demonstrated. In this study, we aimed to elucidate the biological role of *Cordyceps militaris* extract in tumor cells, especially in regulating angiogenesis and tumor growth of a human malignant melanoma cell line. We demonstrated that *Cordyceps militaris* extract remarkably suppressed tumor growth via induction of apoptotic cell death in culture that links to the abrogation of VEGF production in melanoma cells. This was followed by mitigation of Akt1 and GSK-3β activation, while p38α phosphorylation levels were increased. Extract treatment in mouse model xenografted with human melanoma cells resulted in a dramatic antitumor effect with downregulation of VEGF expression. The results suggest that suppression of tumor growth by *Cordyceps militaris* extract is, at least, mediated by its anti-angiogenicity and apoptosis induction capacities. *Cordyceps militaris* extract may be a potent antitumor herbal drug for solid tumors.

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Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; PDGF, platelet derived growth factor; PlGF, placental growth factor; TIMP, tissue inhibitor of metalloproteinases; bFGF, basic fibroblast growth factor; GSK-3β, glycogen synthase kinase-3β; MM, malignant melanoma; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; PKC-β, protein kinase Cβ; TGF, transforming growth factor

Key words: *Cordyceps militaris*, melanoma, angiogenesis, vascular endothelial growth factor receptor, Akt and GSK-3β

Introduction

Angiogenesis, the sprouting of new capillaries from pre-existing vessels, is essential for physiological processes including embryonic development and tissue remodeling but is also involved in pathological conditions such as tumor development (1). Over 90% of solid tumors are highly angiogenic (2). Tumor angiogenesis results from an imbalance between pro-angiogenic factors such as VEGF and endogenous anti-angiogenic factors such as angiostatin and endostatin (3). Tumor cells vary in their angiogenic potential associated with the amounts of bFGF and VEGF secreted (4,5). In addition, it appears tumors that produce multiple angiogenic factors show increased rates of primary tumor expansion, survival and also metastasis (6-8). An angiogenic switch is considered prerequisite for cancer progression (9). Many triggers that are commonly encountered in precancerous or cancer cells, including low pO2, low pH, hyper/hypoglycemia, hyperthermia, mechanical stress, immune/inflammatory responses and genetic mutations, can turn on this angiogenic switch (2,10,11).

The central molecule in physiological and pathological angiogenesis is VEGF (11,12). VEGFs are a heparin-binding family of glycoproteins, which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (placental growth factor) (13). VEGF has an important role in inducing and maintaining angiogenesis in tumors (14). Among the VEGF family members, VEGF-A is the major player in angiogenesis that activates two

tyrosine kinase receptors, VEGFR1 and VEGFR2, of which VEGFR2 has the most important role in VEGF-induced angiogenesis (15,16). VEGF-A is overexpressed in many types of solid tumors and correlates with vascularity, grade and prognosis (17).

Malignant melanoma (MM) is the most lethal skin cancer. It is highly angiogenic, highly metastatic and resistant to treatment. *In vivo* murine studies have shown that aggressive melanoma cell lines express higher levels of VEGF than non-aggressive cell lines (2). Furthermore, significant increases in secretion and stromal deposition of VEGF are observed during the switch from radial to vertical growth of MM, suggesting a significant role of VEGF in cancer invasiveness (18). Therefore, downregulation of VEGF is a potential approach to combat MM progression.

Due to the essential role of VEGF in angiogenesis during cancer progression, many drugs have been developed and widely used to inhibit VEGF, such as anti-VEGF neutralizing antibody and small molecules that block the tyrosine kinase activity of VEGFRs. However, these approaches only benefit a small fraction of cancer patients as cancer cells can become refractory to VEGF inhibitors (19,20). Therefore, alternative approaches that provide a better outcome but are also economically justified are needed. One such approach is the utilization of herbal drugs, such as *Cordyceps militaris (C. militaris)*, a medicinal mushroom that is known to contain cordycepin, adenosine, exo-polysaccharides, and sterols (21-24). Among these metabolic active ingredients, cordycepin is known to have anticancer properties such as inhibition of purine metabolism, RNA/DNA synthesis, and the mTOR pathway (22). Therefore, many cordycepin analogues and synthetic ingredients have been developed for anticancer drugs. However, due to the limited availability in its natural environment and increasing demands, the price of *C. militaris* has greatly increased (25,26).

Despite its many anticancer properties, it has not been determined whether *C. militaris* extract has an anti-angiogenic property in human MM cancer cells. In the present study, we evaluated the role of *C. militaris* extract for inhibition of angiogenesis and tumor growth of a human MM cancer cell line. We found that *C. militaris* extract prepared by a newly developed method remarkably suppressed the growth of tumor cells through mitigation of VEGF production in the human MM cancer cell line *in vitro* and *in vivo*.

Materials and methods

Cell lines and chemicals. The human malignant melanoma cell line MeWo (HTB-65) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) in 2002, and after expanded culture, $1x10^6$ cells/tube were kept in liquid nitrogen. MeWo cells from stocks were cultured in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and used within five passages. *C. militaris* extract was kindly supplied by CAITAC Corp. (Okayama, Japan). Cordycepin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-VEGF polyclonal Ab (no. ab46154) and rat anti-mouse CD31 monoclonal Ab (no. DIA-310) were purchased from Abcam (Cambridge, UK) and Dianova (Hamburg, Germany), respectively.

Plasmid constructs. To construct a mammalian expression vector, the CMV promoter-intron (CMVi) from the phCMV-FSRTM vector (Genlantis, San Diego, CA, USA) was inserted into the promoter-less pIDT-SMART vector (Integrated Device Technology, San Jose, CA, USA) and this was named pIDT-CMViR (27). The vector could efficiently express cargo cDNAs. In addition to GFP cDNA, human cDNAs encoding the full lengths of p38α, GSK3β, PI3K (p85), and Akt1 were then each inserted into the pIDT-CMVi vector. Transient transfection of the plasmids to MeWo cells was performed using Xfect transfection reagent (Clontech Takara, Mountain View, CA, USA).

Antibody array. Screening for angiogenesis-related factors produced by MeWo cells was carried out by using the Human angiogenesis Antibody Array (R&D Systems, Minneapolis, MN, USA). MeWo cells were treated with *C. militaris* extract or cordycepin and cultured for 48 h in serum-free RPMI medium. The medium incubated for the last 24 h was used for the assay. Extracts prepared from MeWo cells cultured with or without *C. militaris* extract for 48 h in serum-free RPMI medium were also analyzed using a proteome profiler array (Human Phospho-MAPK Array; R&D Systems).

Evaluation of cell growth and apoptosis. For growth assay, MeWo cells were cultured in RPMI medium with 10% fetal bovine serum for 24 h prior to treatment with 1%, 5% or 10% *C. militaris* extract or with 20, 100 or 200 μM cordycepin. Cells were counted in triplicates at day 1 before treatment and subsequently on days 2, 3, 5 and 7 after seeding. Re-treatment was performed on days 3 and 5 for the remaining cultures after counting. Cells were detached with 0.20% trypsin-EDTA solution, stained with 0.3% trypan blue in PBS, and counted with a haemocytometer. For apoptosis assay, cells were incubated with 1, 5 or 10% *C. militaris* extract or with 20 and 50 μM cordycepin for 48 h before identification of apoptotic cells showing nuclear shrinkage or fragmentation by staining with Hoechst 33342 (Invitrogen) for 30 min.

RNA extraction and real-time quantitative reverse transcription (qRT)-PCR. Total RNA was extracted using TriPure Isolation Reagent (Roche, Tokyo, Japan). Reverse-transcription was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR was performed using FastStart SYBR Green Master (Roche, Basel, Switzerland) with specific primers for VEGF-A (tta aacgaacgtacttgcagatg, gagagatctggttcccgaaa), VEGF-B (ctg gccaccagaggaaagt, ccatgagctccacagtcaag), and GAPDH (agc cacatcgctcagacac, gcccaatacgaccaaatcc) on a LightCycler 480 system II (Roche). The levels of amplicons were expressed relative to GAPDH as an internal control using the ΔΔCt method.

In vivo tumor growth assay. Two millions MeWo cells were subcutaneously implanted in each of 6 week-old female Balb/c nu/nu mice, and then 400 μl *C. militaris* extract was injected subcutaneously to surround the tumor (not directly injected into the tumor) every two days after the size of the tumor had reached ~100 mm^3. The results were compared with results for mice that were injected with saline alone as an untreated

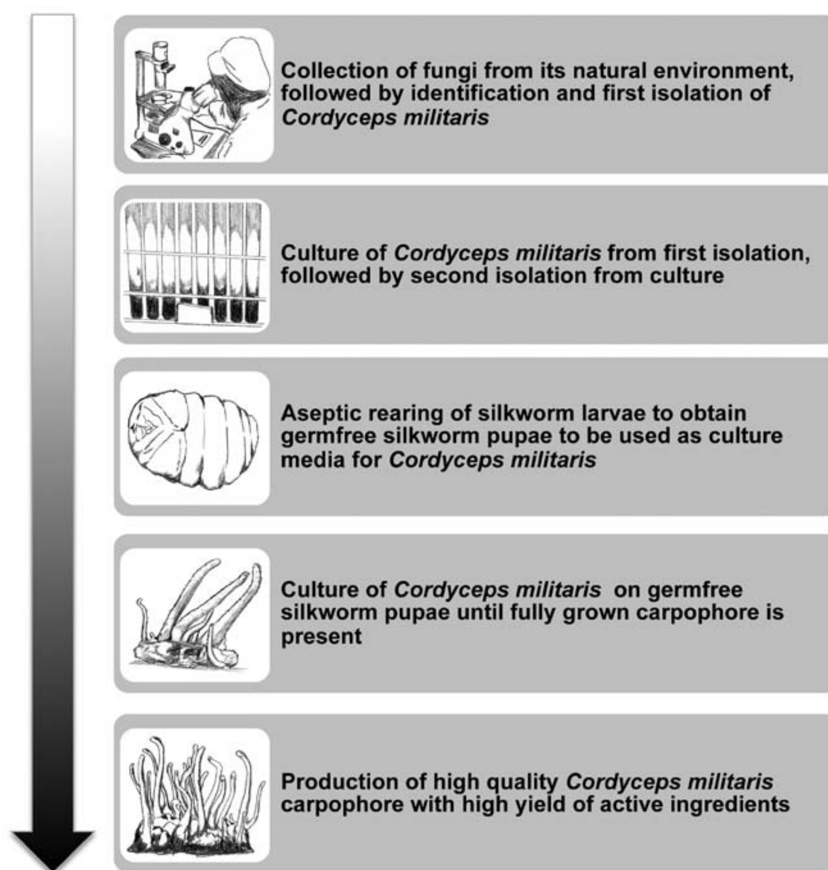

Figure 1. Production of high-quality *C. militaris* extract. *C. militaris* collected from wild mushrooms goes through a 2-step isolation process. After culturing the first isolation product, the culture is resampled for a second isolation of *C. militaris*. The highly purified product of the second isolation is then infected onto germfree silkworm pupae reared through a highly controlled aseptic method. Aseptic rearing of silkworms is highly renewable, assuring production of pupae all year round and also producing other beneficial materials such as germfree silk powder. Active ingredients of *C. militaris* can only be harvested after complete growth of the fungal carpophore.

control group following the same protocol. For comparison, the volume and weight of the subcutaneous tumors were measured in each group. Tumor volumes in each group were calculated every two days as (length x width^2)x0.5 mm^3.

Statistical analysis. Data are expressed as means ± SD. We employed simple pair-wise comparison with Student's t-test (two-tailed distribution with two-sample equal variance), and $P<0.05$ was considered significant.

Results

Extract of C. militaris has an ability similar to that of synthetic cordycepin for controlling cell growth and inducing apoptosis. A number of culture techniques for this mushroom have been established, for example, storage/stock culture, pre-culture, popular/indigenous culture (spawn culture, husked rice culture and saw dust culture) and special/laboratory culture (shaking culture, submerged culture, surface liquid culture and continuous/repeated batch culture), which vary in places and environment and with aim of the culture (28). We developed a method for isolation and culture of *C. militaris* in order to produce high-quality extract with a high yield of active ingredients under sterilized conditions but economically, and we used it for the purposes of this study (Fig. 1). We evaluated the ability of increasing percentages of *C. militaris* extract in controlling cell growth and apoptosis compared to that of increasing concentrations of synthetic cordycepin. The efficiency of the *C. militaris* extract for controlling MeWo cell growth rate and apoptosis was similar to that of synthetic cordycepin (Fig. 2A-C). For the following experiments, we used 5% extract of *C. militaris* and 20 μM cordycepin to determine the underlying mechanism.

Extract of C. militaris reduces production of VEGF and transcription of VEGF-A and VEGF-B. In order to uncover the role of *C. militaris* extract and cordycepin in angiogenesis, we used a human angiogenesis antibody array. Of note, both 5% extract and 20 μM cordycepin significantly decreased production of angiogenin, VEGF and PDGF-AA. Unexpectedly, 5% *C. militaris* extract had a stronger effect on production of VEGF and TIMP-1 compared to cordycepin (Fig. 3A). A higher dose of cordycepin (100 μM) reduced the production of VEGF by less than that in the case of 20 μM cordycepin, suggesting that a lower dose of cordycepin is required for inhibiting the production of VEGF (Fig. 3A, lowest panel). We also used qRT-PCR analysis to examine whether protein level is associated with mRNA level by 5% *C. militaris* extract treatment. Interestingly, not only the mRNA levels of VEGF-A but also that of VEGF-B were significantly reduced

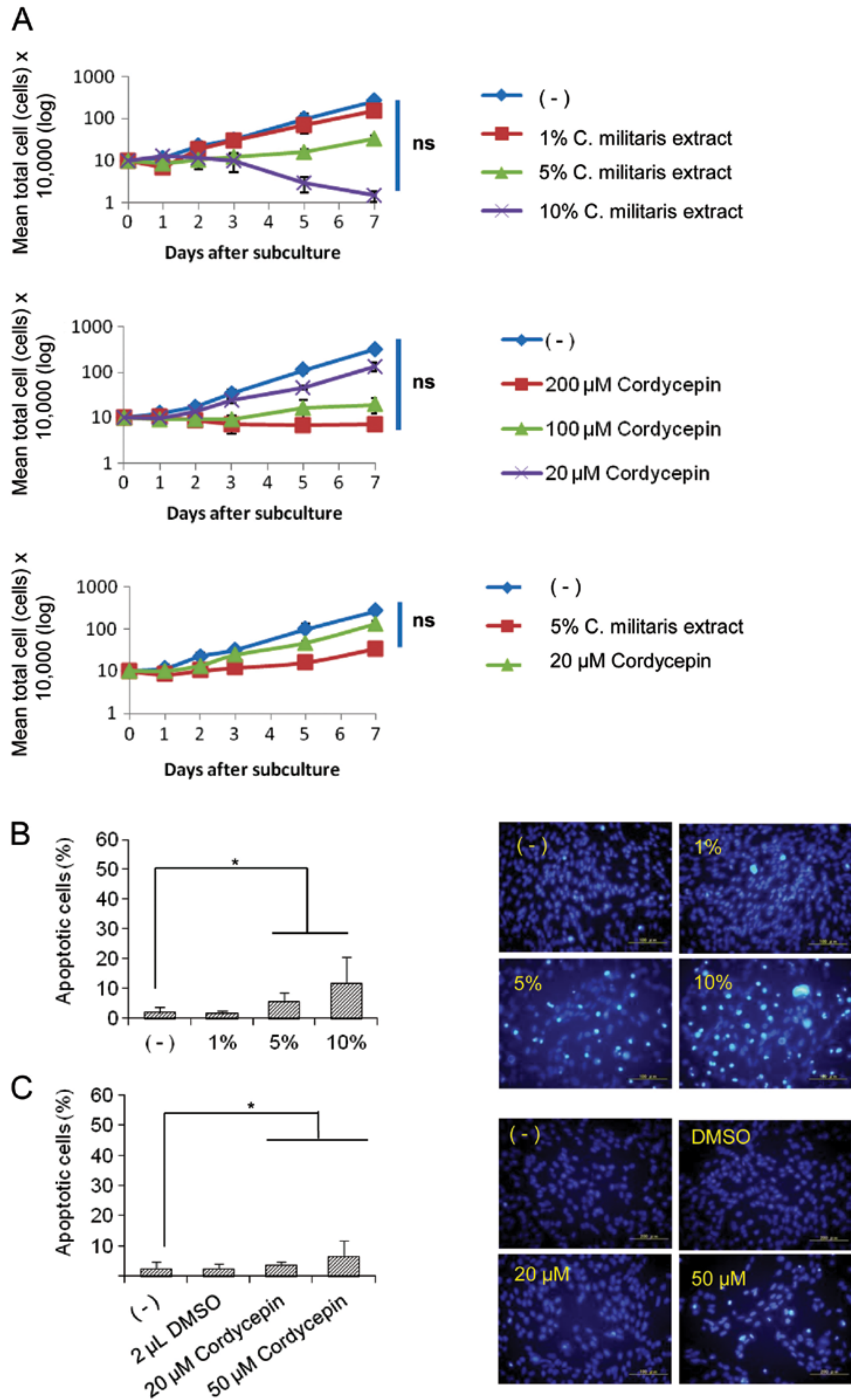

Figure 2. Ability of *C. militaris* to control cell growth and cell apoptosis is comparable to that of synthetic cordycepin. (A) Control of cell growth after treatment with *C. militaris* extract or cordycepin. MeWo melanoma cells were cultured in groups of triplicates for 7 days. The groups were then treated with increasing concentrations of *C. militaris* extract or cordycepin on the remaining cultures on days 1, 3 and 5. Cell counts using a hemocytometer were performed on days 1, 2, 3, 5 and 7 after seeding. Results suggested that the effect of 5% *C. militaris* extract was comparable to that of 20 μM cordycepin; ns, not significant. Induction of apoptosis by *C. militaris* extract (B) or cordycepin (C). MeWo cells were incubated with increasing concentrations of *C. militaris* extract or cordycepin for 48 h. Apoptotic cells were identified after staining with Hoechst 33342. Representative image (right) and quantified data (left) are shown in (B and C), respectively. Statistically significant differences compared to the control were found in treatment groups of 5 and 10% *C. militaris* extract and 20 and 50 μM of cordycepin.

by treatment with *C. militaris* extract (Fig. 3B). Treatment with 20 μM cordycepin only reduced the mRNA level of VEGF-A (Fig. 3C). The results indicate that *C. militaris* extract has a broader effect than that of cordycepin in controlling angiogenesis, suggesting that other ingredients in the extract reduce the mRNA level of VEGF-B.

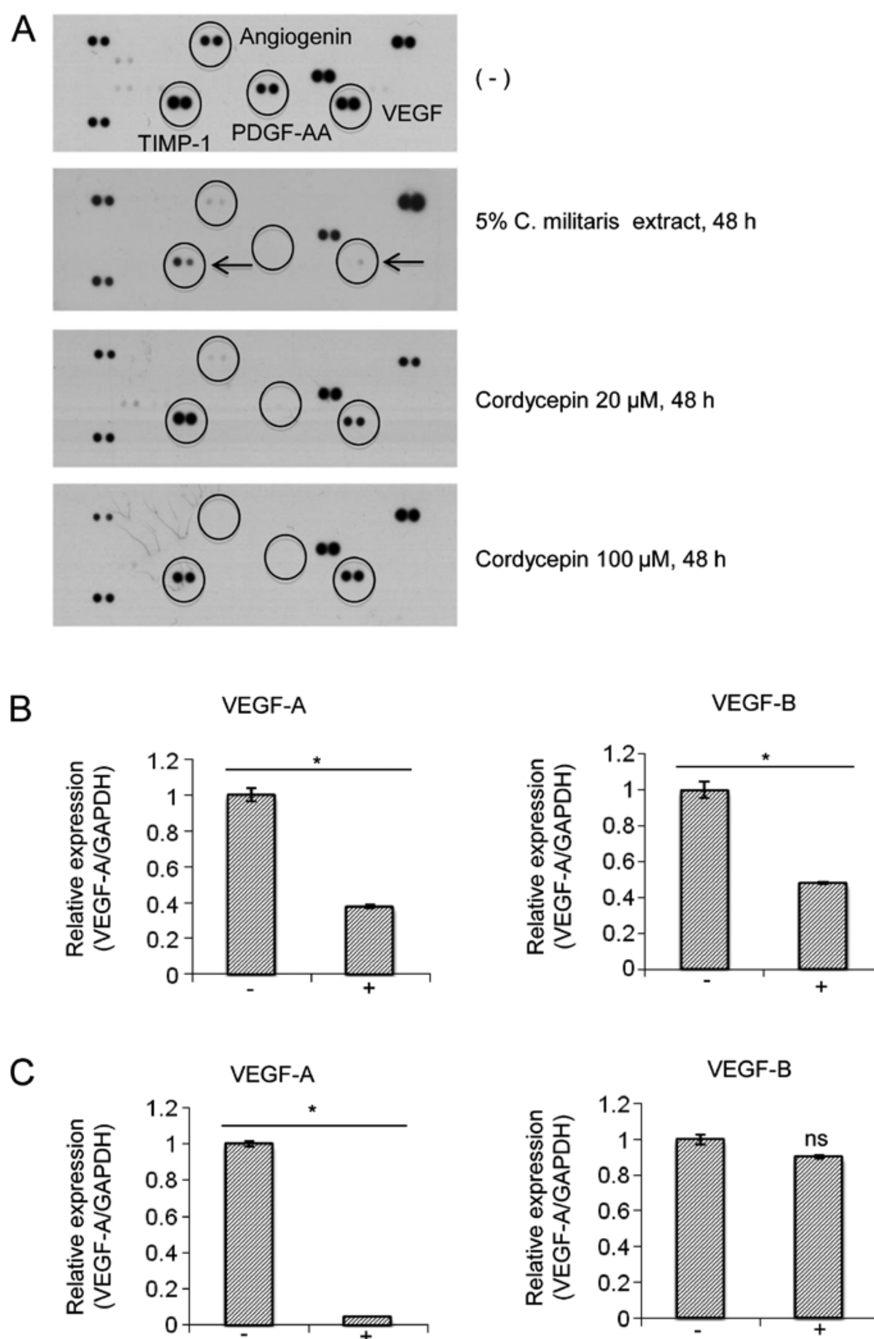

Figure 3. Reduced production of VEGF and transcription of both VEGF-A and VEGF-B by *C. militaris* extract. (A) Dot blot-human angiogenesis array of MeWo culture medium after *C. militaris* extract or cordycepin treatment. Culture media of MeWo cells incubated with control, 5% *C. militaris* extract, 20 μM cordycepin, or 100 μM cordycepin for 48 h were used to detect the production of various angiogenesis-related proteins. All treatment groups showed lower production of angiogenin, TIMP-1, PDGF-AA, and VEGF compared to the control group. Production of TIMP-1 and VEGF was especially reduced in the 5% *C. militaris* treatment group. (B and C) qPCR of VEGF-A and VEGF-B. Transcription levels of VEGF-A and VEGF-B were quantified from MeWo cells after incubation with either 5% *C. militaris* extract (B) or 20 μM cordycepin (C) for 48 h. Transcription levels of VEGF-A and VEGF-B were significantly decreased after treatment with 5% *C. militaris* extract, while only VEGF-A was decreased after treatment with 20 μM cordycepin. *P<0.05 significantly different from the control group. ns, not significant.

Extract of C. militaris reduces the production of VEGF through downregulation of Akt1 and GSK 3α/3β. In order to elucidate the molecular mechanism underlying the properties of *C. militaris* extract, we used a human phospho-MAPK array. The extract of *C. militaris* reduced Akt1 and GSK 3α/3β levels but increased p38α level (Fig. 4A). Furthermore, we investigated the roles of PI3K, Akt1, GSK-3β and p38α, in regulating mRNA levels of VEGF-A and VEGF-B by qRT-PCR in human MM cells (MeWo) transiently overexpressing the aforementioned protein kinases. The messenger RNA level of VEGF-A was upregulated by cells overexpressing p38α, PI3K and Akt1. The mRNA level of VEGF-B was upregulated by cells overexpressing p38α, GSK-3β, PI3K and Akt1. GSK-3β showed specificity for regulating the mRNA of VEGF-B, suggesting that the additional effect of *C. militaris* extract treatment in decreasing VEGF-B was at least in part due to

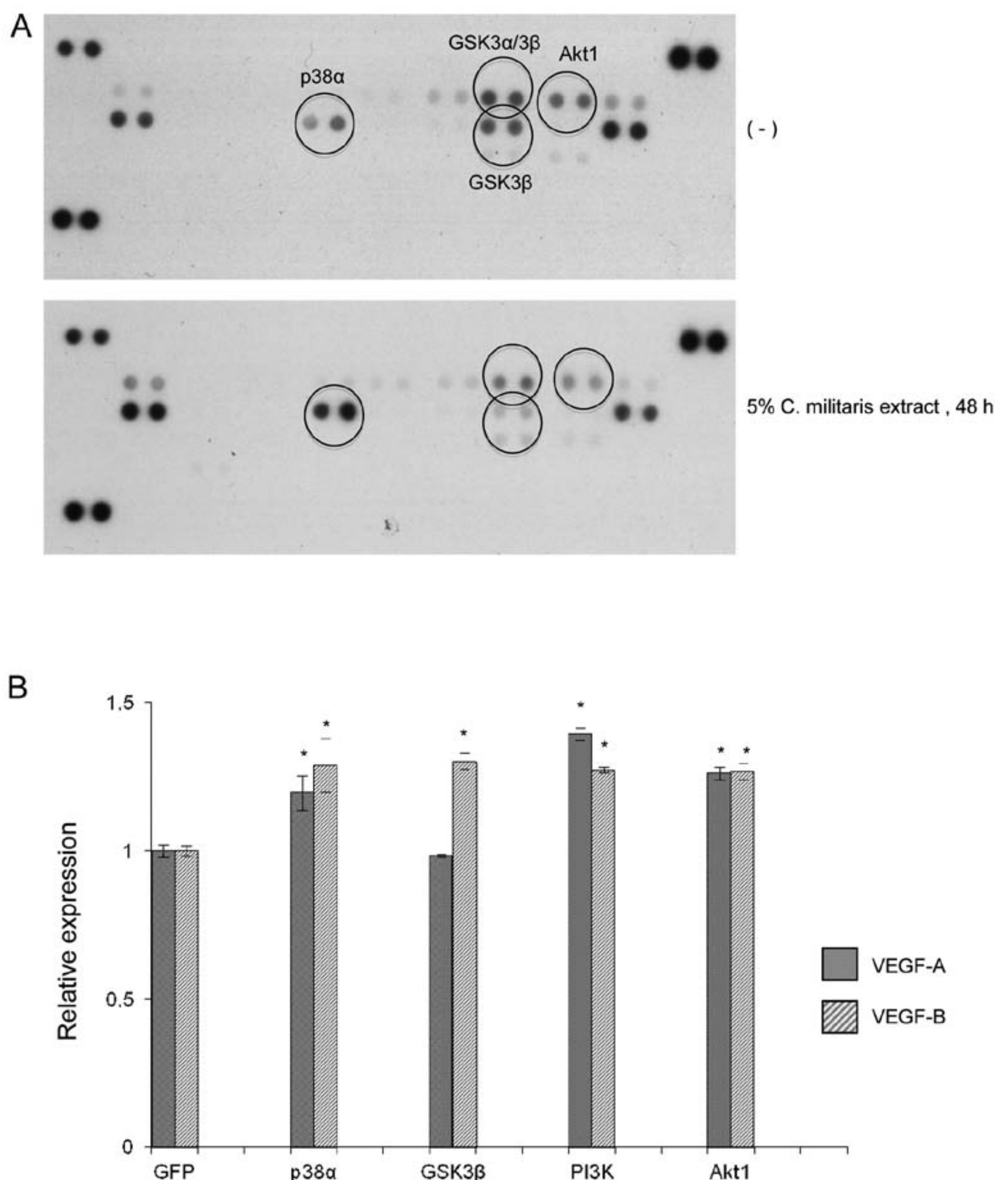

Figure 4. Reduced production of VEGF by *C. militaris* extract was related to the downregulation of GSK 3α/3β and Akt1. (A) Phospho-MAPK array of MeWo cell lysate after treatment with control or 5% *C. militaris* extract. Cell lysate from MeWo cells incubated with control or 5% *C. militaris* extract for 48 h showed phosphorylation of several protein kinases, particularly p38α, GSK3α/3β, and Akt1. Addition of 5% *C. militaris* extract resulted in an increase in phosphorylation of p38α but decrease of GSK3α/3β and Akt1. (B) qRT-PCR of VEGF-A and VEGF-B mRNA levels in MeWo cells overexpressing p38α, GSK3β, PI3K, and Akt1. MeWo cells overexpressing p38α, GSK3β, PI3K or Akt1 showed a significant increase of VEGF-B mRNA levels, while VEGF-A mRNA was only found to significantly increase in cells overexpressing p38α, PI3K, and Akt1, indicating the possibility that 5% *C. militaris* extract lowered VEGF-B phosphorylation levels through GSK3β. $^{*}P<0.05$ significantly different from the control group.

its ability to decrease phosphorylation of GSK-3β. Moreover, Akt1 upregulated mRNA of VEGF-A and VEGF-B to similar levels (Fig. 4B), indicating a possible role of *C. militaris* extract in the Akt/ GSK-3β pathway.

In vivo growth retardation of subcutaneously implanted melanocytic tumors by C. militaris extract. A mouse model of a xenografted melanocytic tumor was generated by subcutaneous injection of a human MM cell line (MeWo) in the flank region of Balb/c nude mice. The tumor cells gradually grew at the implanted site, forming a visual tumor mass after 3-4 weeks. When the tumors had grown to ~100 mm^3 in each mouse, the mice were divided into two groups, which were treated with or not treated with subcutaneous administration of *C. militaris* extract (vehicle: saline only) once every two days for 37 days consecutively. At day 37 after the initial treatment, tumor size in the *C. militaris* extract-treated group was significantly smaller than that in the untreated group (Fig. 5A). Histological examination of the excised melanocytic tumors from mice treated with *C. militaris* extract showed massive necrotic/degenerative lesions inside the tumors, which were observed less in tumors of the untreated mice group (Fig. 5B, upper panel). In immunohistochemical analysis with anti-VEGF antibody, expression of VEGF on tumor cells was diffusely and abundantly observed in untreated tumors, whereas tumor cells lacking VEGF expression or with weak expression were detected in tumors from *C. militaris* extract-treated mice (Fig. 5B, left lower panel). Moreover, anti-mouse CD31 staining revealed

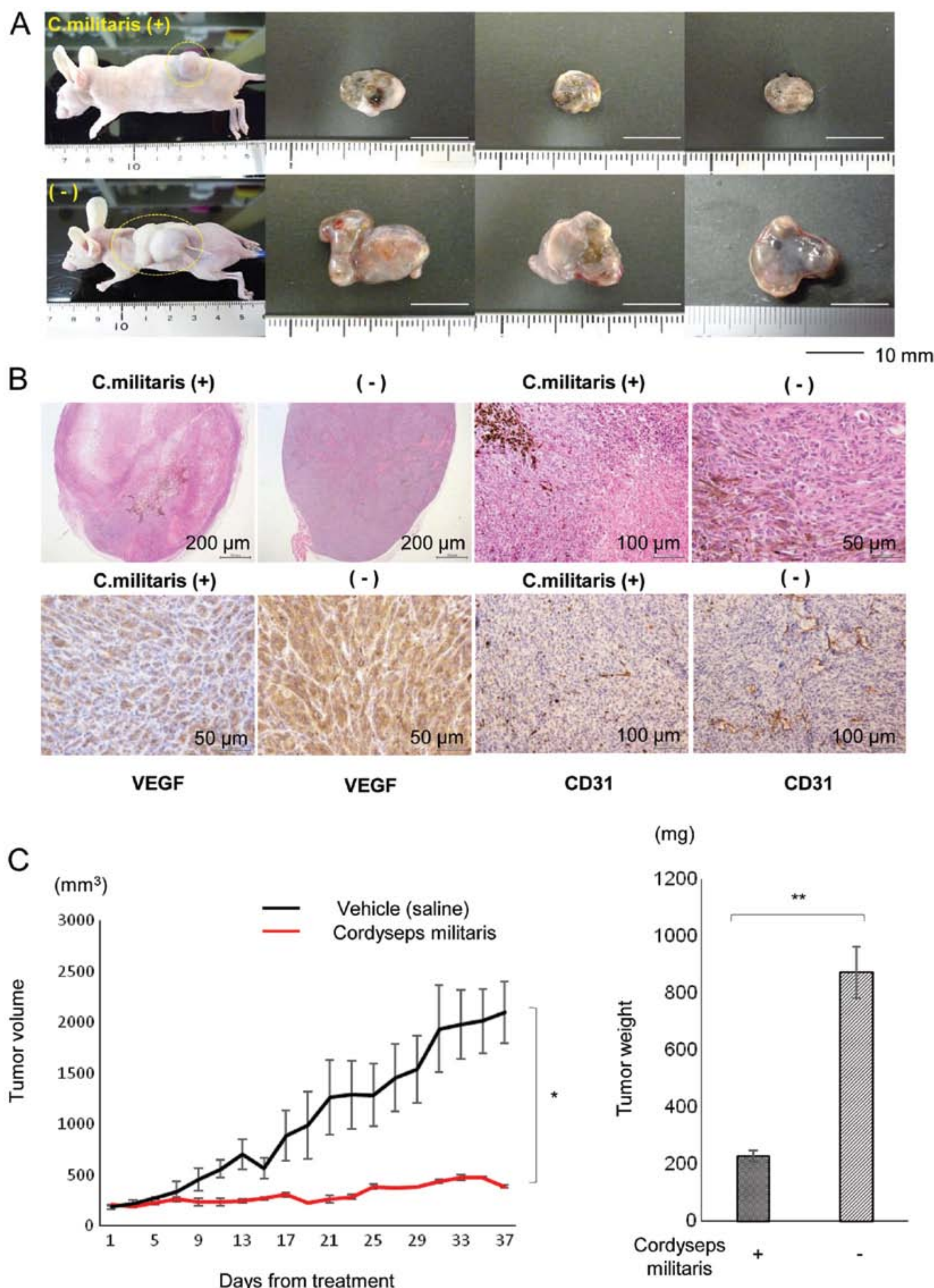

Figure 5. Treatment with *C. militaris* extract reduced growth of subcutaneously xenografted melanomas. (A) Gross appearance of xenografted tumors. Tumors with subcutaneous melanoma tissues were excised from mice after 37 days with or without *C. militaris* extract treatment. Compared to the control group, *C. militaris*-treated mice showed small tumors. (B) Histology (H&E staining) and immunohistochemistry of excised tumors of mice treated with *C. militaris* or saline (control). Expression of VEGF on tumor cells and morphology of vascular vessels in tumor tissues stained by CD31 mAb are shown. Both VEGF and CD31 expression were reduced in *C. militaris*-treated tumors. (C) Growth of subcutaneously xenografted melanomas in mice after treatment with *C. militaris* or saline. Mice bearing subcutaneous MeWo xenografts were treated with saline or the supernatant of *C. militaris* after tumor volumes had reached ~100 mm^3 (day 1). Tumor-bearing mice in each group were subjected to subcutaneous administration of the supernatant or saline once every two days for 37 days consecutively. Tumor volumes were measured daily from day 1 to day 37. Statistical significance of tumor volumes and that of tumor masses were evaluated by Student's t-tests. Saline (n=3, mean volume of 2101 mm^3 on day 37) vs. *C. militaris* (n=3, mean volume 384 mm^3 on day 37); $^{*}P=0.069 >0.05$. Final masses of tumors excised on day 37 in each group are also shown in the graph (right). Saline (n=3, mean weight of 874 mg) vs. *C. militaris* (n=3, mean weight of 230 mg); $^{**}P=0.031 <0.05$. Means and SE (standard error) of triplicates are shown.

undeveloped small blood vessels with thin walls in many of the *C. militaris* extract-treated tumors, whereas the tumors in the untreated mice had developed well-forked vessels with prominent lumens (Fig. 5B, right lower panel). These results were in line with results of the Directed In Vivo Angiogenesis Assay (DIVAA) (Fig. 6A) and chorioallantoic membrane assay

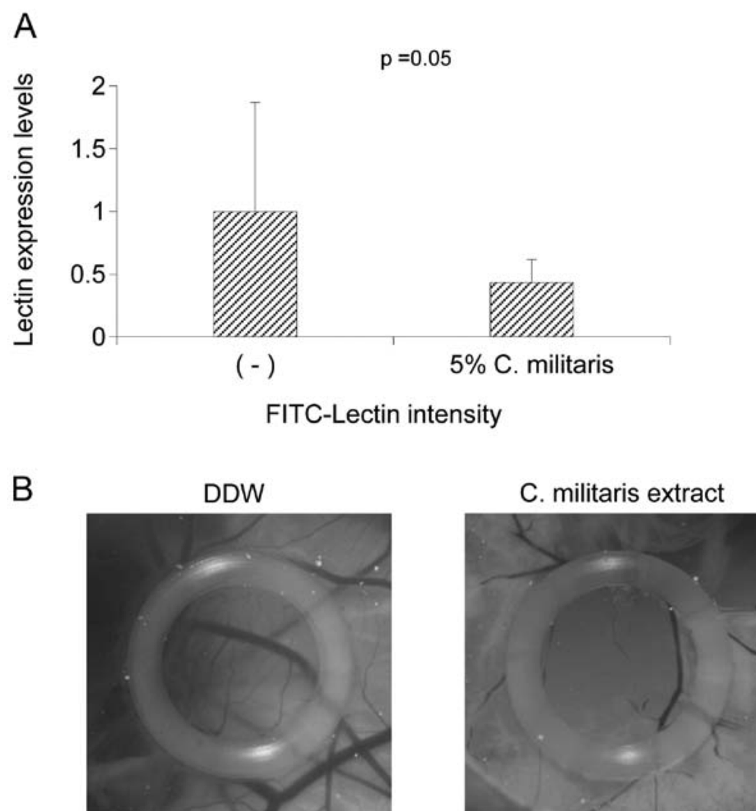

Figure 6. Treatment with *C. militaris* extract reduced angiogenesis. (A) Decrease of FITC-lectin intensity after treatment with 5% *C. militaris* extract. Using the Cultrex Directed In Vivo Angiogenesis Assay (DIVAA) (Trevigen, Gaithersburg, MD, USA), angioreactors containing a mixture of BME and DDW or BME and 5% *C. militaris* extract were implanted subcutaneously into the flanks of mice for 16 days. After extraction, FITC-lectin intensity was measured using excitation 485 nm, emission 510 nm. (B) Chorioallantoic membrane (CAM) assay showed that neovascularization was inhibited by *C. militaris* extract. Eight days post-fertilized chicken egg shells were cut to place silicon rings on the CAM. The inner spaces of the silicon rings were filled with DDW or 50 μl of *C. militaris* extract. Egg shells were covered using transparent tape, and eggs were incubated for 48 h. Results were observed by decrease or increase of vascular branch formation from main capillary vessels.

(CAM) (Fig. 6B) that revealed decreased angiogenic activity by treatment with *C. militaris* extract. Consequently, the tumors derived from *C. militaris* extract-treated mice were less than one-fifth in volume and approximately one-fourth in weight of those of untreated mice, indicating an antitumor effect of *C. militaris* extract for MM-derived tumors *in vivo* (Fig. 5C).

Discussion

In this study, we found that *C. militaris* extract, which was isolated and cultured by a newly developed method under sterilized conditions, has an anti-angiogenic property by decreasing the production of VEGF from a human MM cell line (MeWo). We showed that treatment with 5% *C. militaris* extract reduced the mRNA levels of VEGF-A and VEGF-B and production of VEGF protein in MeWo cells. Furthermore, we demonstrated that tumor growth in the mouse model was significantly decreased by *C. militaris* extract treatment with reduced vascular density observed. For many tumors, vascular density is a prognostic indicator of metastatic potential, with highly vasculated primary tumors having a higher incidence of metastasis than that of poorly vasculated tumors (4).

Several important signaling pathways, including mitogen-activating protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositol 3-kinase (PI3K)/Akt, have been found to be induced by angiogenic growth factors in endothelial cells (29). Activation of these pathways can promote migration, proliferation, differentiation and survival of endothelial cells (30-32). Activation of the PI3K/Akt pathway in tumor cells can also increase VEGF production, by both hypoxia-inducible factor-1 (HIF-1)-dependent and independent mechanisms (33,34). The PI3K/Akt pathway also modulates expression of other angiogenic factors such as nitric oxide and angiopoietins (35).

In order to elucidate the molecular mechanism by which *C. militaris* extract affects the production of VEGF in MeWo cells, we detected reduction of protein kinase GSK 3α/3β and Akt1 but increase of p38α by *C. militaris* extract treatment. GSK3β is known to be involved in a variety of cellular processes including glycogen metabolism, insulin signaling, cell proliferation, apoptosis, neuronal function and embryonic development (36-38). When GSK3β is phosphorylated by Akt, the kinase activity is lost, i.e., GSK3β is negatively regulated by Akt. In accordance with our results, Tekle *et al* reported that treatment of non-small cell lung cancer (NSCLC) with a PKC-β inhibitor increased apoptosis and reduced Akt phosphorylation followed by activation of GSK3β, and mitigation of VEGF production (39). Graff *et al* (40), Hanauske *et al* (41), and Lee *et al* (42) also reported inhibition of tumor growth by a PKC-β inhibitor through marked reduction of VEGF production in breast, thyroid, head/neck, non-small cell lung cancer,

pancreatic cancer and melanoma cancer cell lines and also in patient-derived tumor explants. This suggests a possible involvement of PKC-β in VEGF production through a regulation of Akt-GSK3β pathway. Further investigation is needed to clarify the mechanism.

In this study, we also found a decrease in Akt1 level induced by treatment with *C. militaris* extract. Akt plays an important role in both physiological and pathological angiogenesis through effects in both endothelial cells and cells producing angiogenic signals, such as tumor cells (43). Akt1 is the predominant isoform ubiquitously expressed in a variety of tissues and endothelial cells (44). Akt1 is vital for the regulation of vascular permeability, angiogenic responses and subsequent vascular maturation (45,46). Akt1 is necessary for inside-out integrin signaling. Impaired integrin function often results in decreased extracellular matrix assembly and deposition; this, in turn, might affect the integrity of the endothelial monolayer and its permeability, an essential part of the angiogenic response (47,48). Therefore, we suggest that angiogenesis in the tumor-bearing mice was perturbed by treatment with *C. militaris* extract, which further lead to a decrease in the supply of nutrition or growth factors required by tumor cells to proliferate.

A recent study exploring the role of MAPK and PI3K signaling in angiogenesis of squamous cell carcinoma of the head and neck revealed that EGFR activation induces VEGF-A expression that requires both PI3K and MAPK (49). p38 MAP kinases (p38) are activated by extracellular or intracellular stresses (50,51). and lipopolysaccharide (LPS) (52), and thus their activation generally promotes growth inhibition and/or induces apoptosis (50,53). Our study showed an increase in p38α upon treatment with *C. militaris* extract, suggesting additional inhibition of tumor growth through apoptotic induction, which supports its anti-angiogenic properties.

Despite accumulating evidence indicating an important role of VEGF signaling in angiogenesis, we realize that there are many other angiogenic pathways with observable effects on vessel growth *in vivo* that have not been completely characterized. These include chemokines, integrins (54), several transcriptional regulators, Wnt ligands and their frizzled receptors (55), other members of the FGF, PDGF and TGF-β superfamilies, and the VEGF homolog PlGF that transmit angiogenic signals through VEGFR1 (56). Identifying their role in vessel branching or other types of vessel growth will lead to the generation of a better comprehensive model that can serve as a source for future drug development. In this study, we focused on the role of *C. militaris* extract in affecting VEGF production of a human MM cell line in angiogenesis and tumor growth. Further studies are required to enhance the understanding of a complex angiogenic pathway, especially other angiogenic pathways independent of VEGF.

In conclusion, the extract of *C. militaris* that we isolated and cultured by a newly developed method is a very promising anticancer agent. It has properties targeting several cellular signaling pathways that are involved in induction of apoptosis and inhibition of the angiogenesis of tumor cells. These properties were mediated by reducing GSK3β and Akt1 phosphorylation, which led to decreased VEGF production and increased phosphorylation of p38α that induced apoptosis. These results make the effect of *C. militaris* a good candidate for different combination regimens, including combinations such as pre-treatment or co-treatment with other novel targeting agents and cytotoxic drugs commonly used in a clinical setting.

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