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Cinnamon extract suppresses tumor progression by modulating angiogenesis and the effector function of CD8$^+$ T cells

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ABSTRACT

Cinnamon is one of the most widely used herbal medicines with diverse bioactive effects. However, little evidence has been reported about the potential anti-tumor effects of cinnamon. *In vitro* and *in vivo* system, cinnamon treatment strongly inhibited the expression of pro-angiogenic factors and master regulators of tumor progression not only in melanoma cell lines but also in experimental melanoma model. In addition, cinnamon treatment increased the anti-tumor activities of CD8$^+$ T cells by increasing the levels of cytolytic molecules and their cytotoxic activity. In conclusion, cinnamon extract has the potential to be an alternative medicine for tumor treatment.

1. Introduction

Cancers are the most life-threatening health problems in the world [1]. Although many different types of anti-tumor agents are available, severe side effects and toxicity limit their applications [2]. Recently, complementary and alternative medicine (CAM) is becoming a popular treatment for various cancers. Among the CAMs, herbal medicine is one of the methods used in cancer therapy [3].

Oriental herbal medicine including traditional and folk-healing methods has been used for the treatment of malignancies for thousands of years. Currently, numerous scientific studies support herbal medicine as a potent anti-cancer drug [4]. However, the development of herbal medicine as an anti-cancer agent needs substantial research in order for it to meet strict criteria such as those on standardization, quality control, safety, toxicity, and clinical trials [5]. More importantly, elucidation of action mechanism of herbal medicines will turn CAM into 'evidence-based medicine' [6,7].

Cinnamomum cassia bark is the outer skin of an evergreen tall tree belonging to the family Lauraceae, which contains large amounts of bioactive molecules including essential oils (cinnamic aldehyde and cinnamyl aldehyde), tannin, mucus, and carbohydrates. Many studies have shown the diverse biological functions of cinnamon including anti-inflammatory [8], anti-oxidant [9,10], anti-microbial [9,11], and anti-diabetic effects [12–14]. An anti-tumor effect of cinnamon was previously suggested *in vitro* [15,16] without *in vivo* evidence or a working mechanism. It is currently unclear whether cinnamon has anti-tumor activities *in vivo* and, if it has, elucidation of its mechanism of action will be important for its use as a CAM.

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In this study, we tested the anti-cancer efficacy of cinnamon extract and elucidated its underlying mechanisms both *in vitro* and *in vivo* using an experimental melanoma model. Treatment with cinnamon extract decreased the expression of pro-angiogenic factors (EGF, VEGF, and TGF-β) and master regulators of tumor progression (Cox-2 and HIF-1α) both in melanoma cell lines and in a melanoma mouse model. Moreover, administration of cinnamon extract significantly inhibited tumor progression by inhibiting angiogenesis while increasing the cytolytic activity of $CD8^+$ T cells.

2. Materials and methods

2.1. Animals

C57BL/6 mice (6–8 weeks, male) were purchased from SLC (Japan) and maintained under specific pathogen-free conditions in an animal facility at the Gwangju Institute of Science and Technology (GIST). All of the animal experiments were approved by the GIST Animal Care and Use Committee.

2.2. Preparation of cinnamon extracts (CE)

Dried *Cinnamomum cassia* bark (Hwajin Distribution Co., Seoul, Korea) was pulverized and extracted in hot water for 3 h in a hot water extractor. The extract was filtered and the supernatant was concentrated with a rotary evaporator. The extract was then freeze-dried resulting in a powder extract. The powder extract was suspended in sterilized distilled water at the appropriate concentrations. HPLC assay was performed and relative amounts of trans-cinnamic acid and cinnamic aldehyde were used as standard makers for the quality control of CE composition in each experiment (Supplementary Fig. 1).

2.3. Cell lines

B16F10 and Clone M3 mouse melanoma cells were obtained from the Korean Cell Line Bank (Seoul National University, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, USA), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were cultured with different doses of cinnamon extract for 24 h and harvested for further analysis.

2.4. RNA Isolation, cDNA synthesis, quantitative RT-PCR, and standard RT-PCR

Total RNA was prepared using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol. For reverse transcription, cDNA was generated using 1 μg of total RNA, oligo (dT) primer (Promega) and Improm-II Reverse Transcriptase (Promega) in a total volume of 20 ul. One microliter of cDNA was amplified using the following RT-PCR primer sets: L32 (245 bp; 5′-GAGGACCA AGAAGTTCATCAG-3′ and 5′-GCACAGTAAGATTTGTTGCAC-3′); HPRT (126 bp; 5′-TTATGGACAGGACTGAAAGAC-3′ and 5′- GCTTTAATGTAATCCAGCAGGT-3′); β-actin (154 bp; 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′- CCAGTTGGTAACAA TGCCATGT-3′); EGF (175 bp; 5′-TTCTCACAAGGAAAGAGC ATCTC-3′ and 5′-GTCCTGTCCCGTTAAGGAAAAC-3′); FGF (62 bp; 5′-ACCCACACGTCAAACTACAAC-3′ and 5′-CACTCC CTTGATAGACACAACTC-3′); TGF-β (133 bp; 5′-CTCCCGTG GCTTCTAGTGC-3′ and 5′-GCCTTAGTTTGGACAGGATCTG-3′); VEGF-α (105 bp; 5′-GCACATAGAGAGAATGAGCTTCC-3′ and 5′-CTCCGCTCTGAACAAGGCT-3′); Cox-2 (74 bp; 5′-TGAGCAACTATTCCAAACCAGC-3′ and 5′-GCACGTAGTCTTC-GATCACTATC-3′), and HIF-1α (187 bp; 5′-ACCTTCATCG-GAAACTCCAAAG-3′ and 5′-ACTGTTAGGCTCAGGTGAACT-3′).

2.5. Immunoblotting

Proteins were resolved by 10% SDS–PAGE gels, transferred onto a PVDF membrane (Bio-RAD) and subjected to Western blot analysis using anti-Cox-2 (Cayman), anti-HIF-1α (Novus), and peroxidase-conjugated secondary antibodies (DAKO). Proteins were visualized with a chemiluminescence kit (Amersham Bioscience). Tubulin beta (anti-tubulin; Santa Cruz) was used as a loading control.

2.6. Melanoma induction and anti-tumor assay

Mouse melanoma B16F10 (1×10^6 cells/0.1 ml) cells were injected subcutaneously (sc) into the flanks of C57BL/6 mice (6 week old male). One week after the injection, the mice were divided into three groups (Control, intra-tumoral (IT) and oral administration (OA) groups) and treated with either 10 mg/dose (400 μg/g mouse weight) of cinnamon extract in 100 μl of PBS or same volume of PBS alone as a sham control by different routes (intra-tumoral injection or oral administration) for 30 days. During the treatment period, the tumor size was measured with Vernier calipers every 2 days, and tumor volumes were calculated using the standard formula: $\text{width}^2 \times \text{length} \times 0.52$. Mice were sacrificed for further analysis after 30 days of treatment.

2.7. H&E staining and Immunohistochemistry

Paraffin sections (3 mm) of tumor tissues and lingual lymph nodes from PBS, OA, and IT groups were stained with H&E following the previous report with minor modification [17]. For the detection of CD31 expression in these tissues, immunohistochemistry was performed with rabbit-anti-mouse CD31 (Abcam) and anti-rabbit Alexa555 (Molecular Probes). After staining procedure, tissue sections were analyzed under Fluoview microscope (Olympus).

2.8. Isolation and activation of $CD8^+$ T cells

$CD8^+$ T cells were purified from tumor draining lymph nodes (axillary, superficial, brachial, and lingual lymph node) using a $CD8^+$ T cell isolation kit (Miltenyi, 130-090-895). $CD8^+$ T cells (5×10^6/ml) were stimulated with 10 μg/ml plate-bound anti-CD3ε, 10 μg/ml soluble anti-CD28 and recombinant human IL-2 (rhIL-2, 100 U/ml) in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, penicillin–streptomycin, nonessential amino acids, sodium pyruvate, vitamins, HEPES, and 2-mercaptoethanol for 5 days. Recombinant human IL-2 was provided by the National Cancer Institute, Preclinical Repository. Anti-CD3 (145.2C11) and anti-CD28 (37.51) were purchased from Pharmigen.

2.9. JAM test

The cytotoxicity of $CD8^+$ T cells was assessed using the JAM test with minor modifications [18,19]. B16F10 and $CD8^+$ T cells were used as target cells and effector cells, respectively. Target cells (B16F10; mouse melanoma) were labeled with 5 μCi/ml of [H^3]-thymidine (NEN) for 12 h at 37 °C, harvested, washed three times with PBS, and seeded in 96-well U-bottom plates at a density of 1×10^4 cells/well. Activated $CD8^+$ T cells were washed and added in various ratios to the target cells. After 16 h co-culture, radioactivity was measured on a microplate beta counter (Beckman). Data are expressed as the percentage cytotoxicity as calculated by the following formula: [(cpm spontaneous – cpm experimental)/cpm spontaneous] × 100.

2.10. Statistical analysis

A two-tailed Student's *t*-test was employed where $P < 0.05$ was considered to be statistically significant ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$).

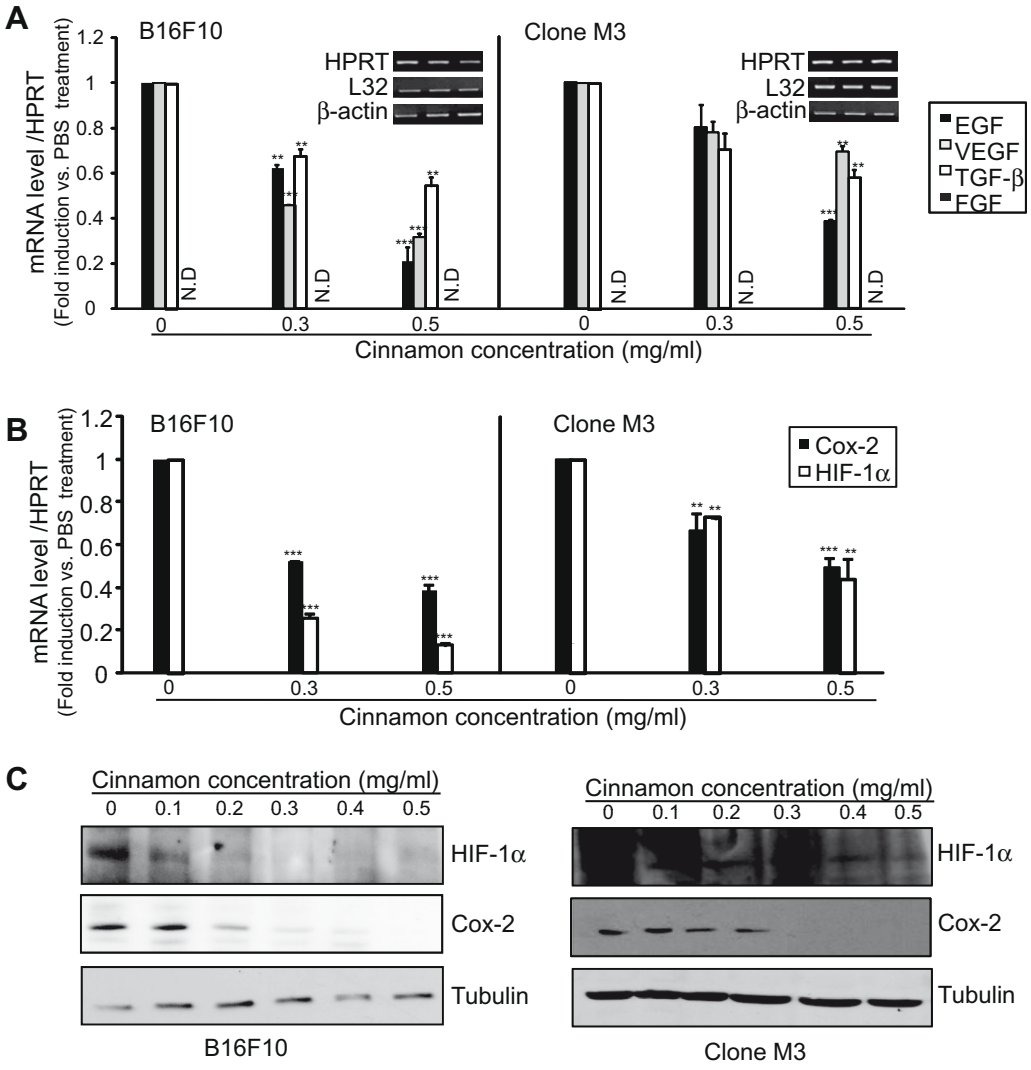

Fig. 1. Cinnamon treatment reduces the levels of pro-angiogenic factors, Cox-2 and HIF-1α. Mouse melanoma cells (B16F10; left and Clone M3; right) were cultured with different doses of cinnamon extract. (A) After 24 h of cinnamon treatment, the expression levels of pro-angiogenic molecules (EGF, VEGF-α, TGF-β, and FGF) were measured by quantitative RT-PCR. The levels of mRNA (B) and protein and (C) of Cox-2 and HIF-1α were measured by quantitative RT-PCR and immunoblotting. The mRNA expression levels in each group were normalized with HPRT (house-keeping gene) and then fold induction of each target gene was compared to PBS groups (expression level of PBS group: 1). Data are representative of three independent experiments. Not detectable (ND). Two (**) or three asterisks (***) indicates $p < 0.005$ or $p < 0.001$, respectively. Data are representative of three individual experiments.

3. Results

3.1. Cinnamon extract inhibits the expression of pro-angiogenic growth factors

To ensure the quality and purity of each preparation of cinnamon extract, HPLC analysis was performed by measuring the content of known active compounds such as *trans-cinnamic acid* and *cinnamic aldehyde* (*Supplementary* Fig. 1). Each preparation of cinnamon extracts showed similar levels of *trans-cinnamic acid* and *cinnamic aldehyde* (*Supplementary* Fig. 1 and Supplementary Table 1).

We initially performed a cytotoxicity test to define the optimal concentration at which cinnamon did not induce cell damage. Cinnamon treatment up to 0.5 mg/ml did not induce cell death within 24 h (data not shown). Next, to test the anti-tumor effects of cinnamon extract *in vitro*, we treated mouse melanoma cell lines (B16F10 and Clone M3) with cinnamon extract at different doses and analyzed the expression levels of angiogenic growth factors that play important roles in tumor progression (Fig. 1A). Cells treated with either 0, 0.3, or 0.5 mg/ml of cinnamon were cultured for 24 h. The expression or protein levels of various growth factors including EFG, FGF, VEGF-α, and TGF-β were tested by quantitative RT-PCR and ELISA, respectively. The mRNA expression levels in each group were normalized with HPRT (house-keeping gene) and then fold induction of each target gene was compared to PBS groups (expression level of PBS group: 1). Cinnamon treatment in B16F10 and Clone M3 cells inhibited the levels of the growth factors both in mRNA (Fig. 1A) and in protein (*Supplementary* Fig. 2). These results suggest that cinnamon extract could inhibit the levels of growth factors that are critical for tumor progression in melanoma cells. No change was observed in the expression level of the housekeeping genes L32, β-actin and HPRT between cinnamon-treated and control (PBS-treated) cells (Fig. 1A). Cox-2 and HIF-1α play important roles in the progression of various type of cancers [20,21] by stimulating angiogenesis, invasion, and apoptosis [20–23]; therefore, we tested whether cinnamon could block the activity of these molecules. To test this, melanoma cells were treated with cinnamon for 24 h, then the levels of Cox-2 and HIF-1α were analyzed by PCR (Fig. 1B) and immunoblotting (Fig. 1C) in B16F10 and Clone M3 cells. Treatment with cinnamon extract decreased Cox-2 and HIF-1α expression in a dose-dependent manner. For example, treatment of melanoma cells with 0.5 mg/ml cinnamon extract abolished the expression of Cox-2 (Fig. 1C) while the β-tubulin levels remained constant in all of the samples tested.

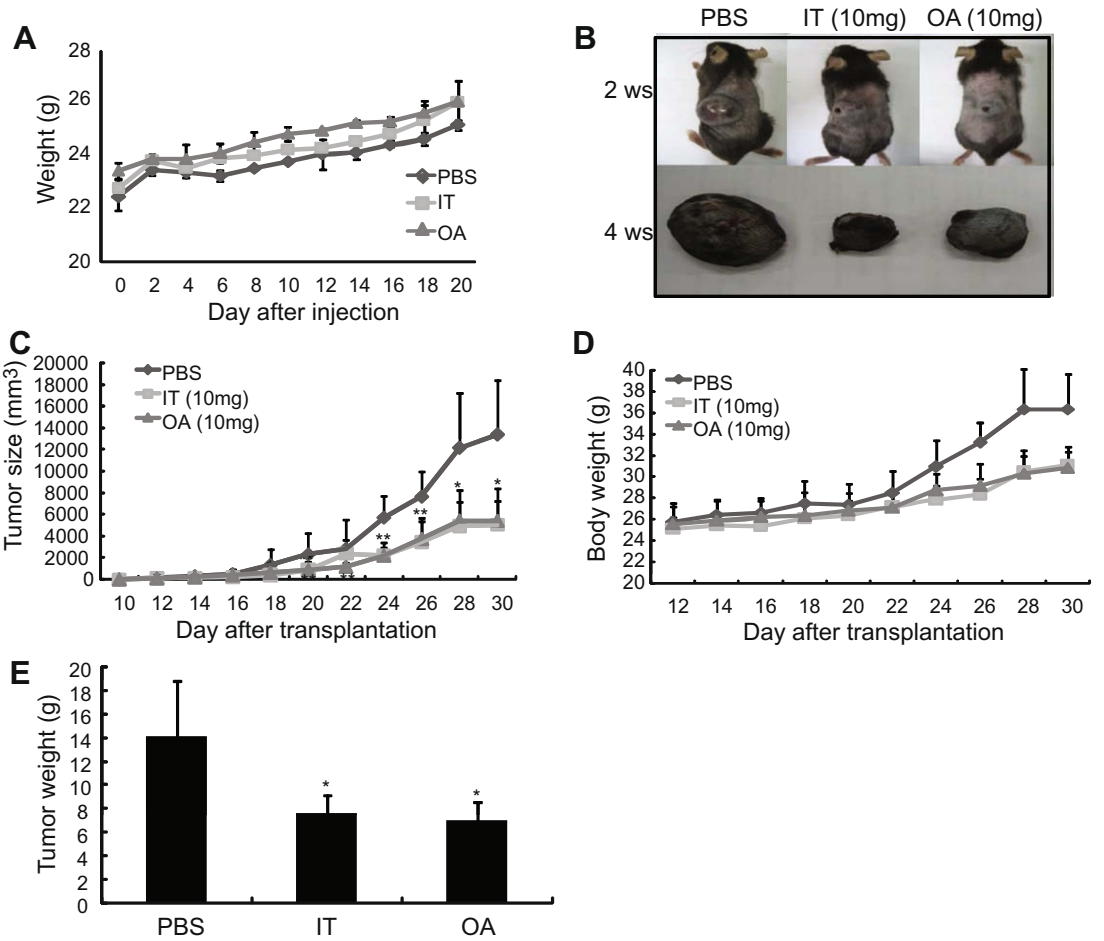

Fig. 2. Administration of cinnamon extract inhibits tumor growth *in vivo*. (A) No cytotoxicity of cinnamon extract in normal mice. PBS (oral administration) or cinnamon extract (10 mg/dose (400 μg/g mouse weight) by oral administration (OA) or by intra-tumoral injection (IT), indicated, respectively, was administered every 2 days for 20 days and then changes in the body weight of each group were measured. To test the anti-cancer effect of cinnamon *in vivo*, B16F10 cells (1×10^6) were transplanted by intra-dermal injection. Ten days after transplantation, PBS (oral administration) or cinnamon (10 mg) were administered daily by oral administration (OA) or intra-tumoral injection (IT) for 20 days. (B) Photographs of representative tumors from each group at weeks two and four. During the treatment period, (C) tumor size, (D) body weight were measured daily, and (E) After 4 weeks of treatment, each group of mice was sacrificed and the tumors were removed to compare the weight variation among the treatment groups. Error bars indicated SD. Data are representative of three independent experiments. One (∗), or Two (∗∗) asterisks indicate $p < 0.05$ or $p < 0.005$, respectively.

3.2. Cinnamon treatment inhibits melanoma progression in vivo

Before testing the anti-cancer activity of cinnamon extract in an experimental melanoma model, we first tested the *in vivo* toxicity of cinnamon extract. We fed or injected into normal healthy mice with either PBS [control group (Cont); treated by oral route], or 10 mg/dose (400 μg/g mouse weight) of cinnamon by oral administration (OA) or intra-tumoral injection (IT) and change of body weight was monitored for 20 days after treatment. No significant change in body weight was observed between the treatment groups (Fig. 2A). Lower concentrations (less than 10 mg/dose) of cinnamon did not affect changes in body weight (data not shown). In addition, no cytotoxicity was observed in response to cinnamon administration (10 mg/dose) when we examined the intestines and skin tissue by histological analysis (data not shown). These data indicated that doses up to 10 mg of cinnamon were safe for the treatment of mice by oral-administration or subcutaneous injection. Next, to test whether cinnamon extract exhibited any anti-cancer effects, experimental melanomas were established by the subcutaneous injection of B16F10 into C57BL/6 mice. Treatment was initiated ten days after tumor plantation by the administration of cinnamon extract orally (OA) or by intra-tumor injection (IT) at 2 day intervals for 30 days after transplantation. Tumor size and body weight were measured throughout the treatment period. Treatment with cinnamon extract by IT or OA significantly ($p < 0.05$–0.01) inhibited tumor growth compared with the control group after three weeks (Fig. 2B and C). Changes in body weight during the treatment period were measured to monitor the *in vivo* tumor growth. The control group exhibited a faster weight gain than the cinnamon-treated groups after 22 days of treatment due to vigorous tumor growth (Fig. 2D). The tumor weight was measured after the final treatment (Fig. 2E). IT injection or OA of cinnamon extract suppressed tumor growth compared with the control PBS group (Fig. 2E). These data suggest that cinnamon extract displays anti-tumor activity when it is administered orally or by intra-tumor injection (Fig. 2).

3.3. Cinnamon treatment inhibits angiogenesis by down-regulating pro-angiogenic factors

In order to elucidate the underlying mechanism of anti-tumor effect *in vivo*, we initially tested the anti-angiogenic activity of cinnamon based on the finding that cinnamon extract reduced the levels of pro-angiogenic factors (EGF, VEGF-α, TGF-β, and FGF) *in vitro* (Fig. 1). To test the anti-angiogenic effect of cinnamon *in vivo*, melanoma mice were sacrificed after being treated with cinnamon (IT or OA) or PBS for 30 days and the newly synthesized blood vessels around the tumor forming region were analyzed (Fig. 3A). Vasodilatation and vascularization around the tumor sites were evident in the PBS-treated control group (PBS, Fig. 3A); however, these were significantly reduced in response to OA and IT cinnamon treatment (Fig. 3A). To investigate tumor metastasis, the size and weight of lymph nodes (LN) (axillary, brachial LN, Fig. 3B) and spleen (Fig. 3C) around tumor sites were measured. Cinnamon treatment significantly reduced the size and weight of draining lymph nodes and spleens compared with the control group (Fig. 3A–C). To check effects of cinnamon in vasodilatation and vascularization in detail, first, we compared histological aspects of tumor tissue, lingual lymph node, and kidney among the three groups. Cinnamon treatment (OA or IT route) significantly decreased vessel formations and the number of abnormal shape of cells both in tumor tissues and lymph node compared with PBS groups (Fig. 3D). No significant difference was observed in kidney (data not shown). To further confirmation of these results, we performed immuno-histochemical analysis with anti-CD31 antibody to check differential expression of CD31 protein, a marker for vascular cell [24] (Fig. 3E). Cinnamon treated group (OA or IT route) showed significantly reduced the level of CD31 positive cells than PBS groups (Fig. 3E). In addition, cinnamon treatment significantly ($p < 0.005$) down-regulated the expression levels of pro-angiogenic factors (VEGF-α, FGF, EGF, and TGF-β) in the isolated tumors measured by quantitative RT-PCR (Fig. 3F).

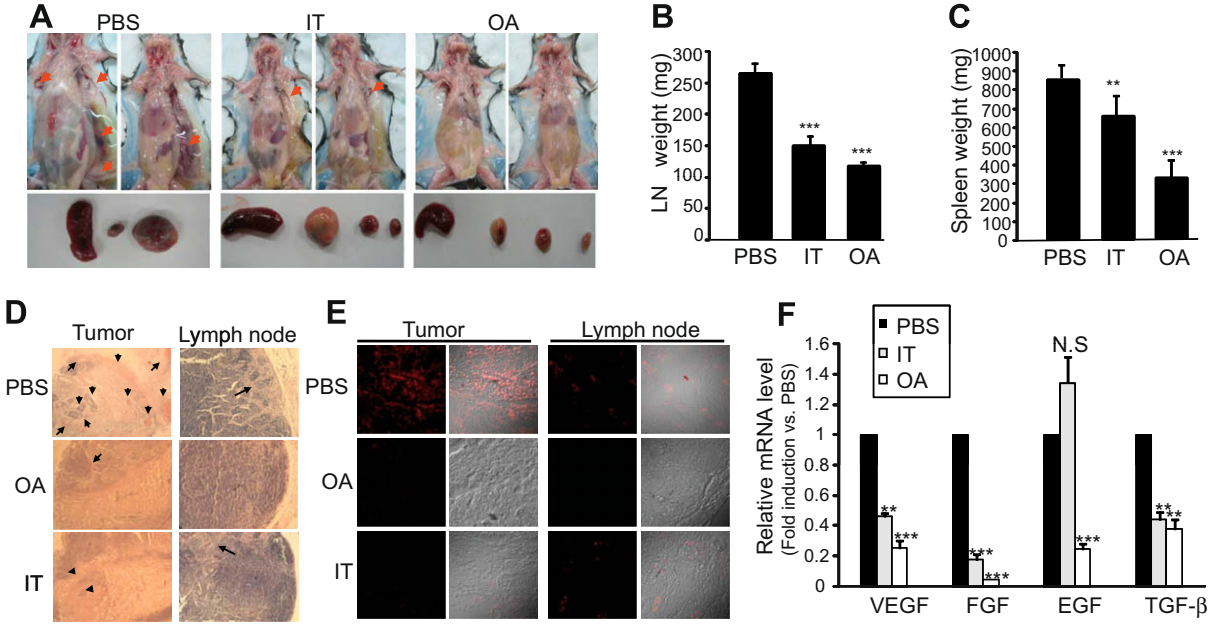

Fig. 3. Cinnamon treatment inhibits vasodilatation and neo-vascularization by reducing pro-angiogenic molecules. (A) After 20 days treatment, mice were sacrificed from each treatment group and angiogenic progression was measured by observing the level of newly synthesized blood vessels in the tumor site (upper pictures) and the metastatic level in tumor draining spleen and lymph nodes (below pictures). Changes in tumor draining lymph node (LN) (B) and in spleen (C) of melanoma mice were compared among the treatment groups. (D) Histological analysis of tumor tissue and lingual lymph node by H&E staining. (E) Immuno-histochemical analysis of CD31 protein levels as a marker for vascular cell and (F) The expression levels of pro-angiogenic molecules (VEGF-α, EGF, FGF, and TGF-β) were measured by quantitative RT-PCR. The mRNA expression levels in each group were normalized with HPRT (housekeeping gene) and then fold induction of each target gene was compared to PBS groups (expression level of PBS group: 1). Error bars indicated SD. Not significant (NS; $p > 0.05$). Two (**) or three asterisks (***) indicate $p < 0.005$ or $p < 0.001$, respectively. Data are representative of three independent experiments.

3.4. Cinnamon treatment down-regulates the levels of HIF-1α and Cox-2 in tumor tissue

HIF-1α and Cox-2 play pivotal roles in tumor progression and are important targets for tumor therapy [23,25]. Cinnamon treatment down-regulated the levels of HIF-1α and Cox-2 in mouse melanoma cell lines (Fig. 1). We therefore investigated whether the anti-tumor effect of cinnamon treatment *in vivo* was also related to the down-regulation of HIF-1α and Cox-2 in tumor tissues. The levels of HIF-1α and Cox-2 in the tumor tissues were determined by quantitative RT-PCR (Fig. 4A) and immunoblot analysis (Fig. 4B). Cinnamon treatment significantly reduced the expression of HIF-1α and Cox-2 transcript levels compared with the control group (Fig. 4A). Interestingly, oral administration (OA) of cinnamon extract was more effective at down-regulating the levels of HIF-1α and Cox-2 compared with intra-tumor (IT) injections at the level of both mRNA (Fig. 4A) and protein (Fig. 4B). Cinnamon treatment down-regulated the level of Cox-2 mRNA by 40% (IT group) and 90% (OA group), respectively (Fig. 4A). These results suggest that the potent anti-tumor effect of cinnamon extract is associated with a down-regulation of HIF-1α and Cox-2, which mediates tumor suppression *in vivo*.

3.5. Cinnamon treatment potentiates CD8$^+$ T cell activity

Treatment with cinnamon extract suppressed tumor progression by inhibiting the degree of angiogenesis (Fig. 3) and the levels of HIF-1α and Cox-2 (Fig. 4). Since CD8$^+$ T cells predominantly eliminate intracellular pathogens and tumor tissue [26], we hypothesized that the anti-tumor activity of cinnamon extract may also modulate the cytotoxic activity of CD8$^+$ T cells. To test this, CD8$^+$ T cells isolated from tumor draining lymph nodes (axillary, superficial, brachial, and lingual lymph node) were stimulated with anti-CD3/CD28/IL-2. And then, the expression levels of cytolytic molecules were determined by quantitative RT-PCR (Fig. 5A). CD8$^+$ T cells from the IT and OA groups highly expressed cytolytic molecules (IFN-γ and TNF-α), granzymes (granzyme B and granzyme C) and perforin molecules compared with CD8$^+$ T cells from the control group (Fig. 5A). Since the expression level of cytolytic molecules is directly linked with the killing activity of CD8$^+$ T cells [27], we tested the hypothesis that CD8$^+$ T cells from the cinnamon-treated groups exhibited an increased CTL response than those from the control group. We performed the JAM test to test this hypothesis. CD8$^+$ effector T cells isolated from different treatment groups were co-cultured with [3H]-thymidine labeled B16F10 cells (target cells) and their killing activities were compared (Fig. 5B). CD8$^+$ T cells from the IT and OA groups showed significantly increased killing activity than those in the control group in a dose-dependent manner (Fig. 5B). Upon increasing the effector cell ratio up to 1–20, the increase in killing activity of CD8$^+$ T cells from the IT and OA groups was significantly higher ($p < 0.001$) than those in the control group (Fig. 5B). These results indicate that in addition to an anti-angiogenic activity, cinnamon extract potentiates the cytolytic activity of CD8$^+$ T cells in tumor draining lymph nodes.

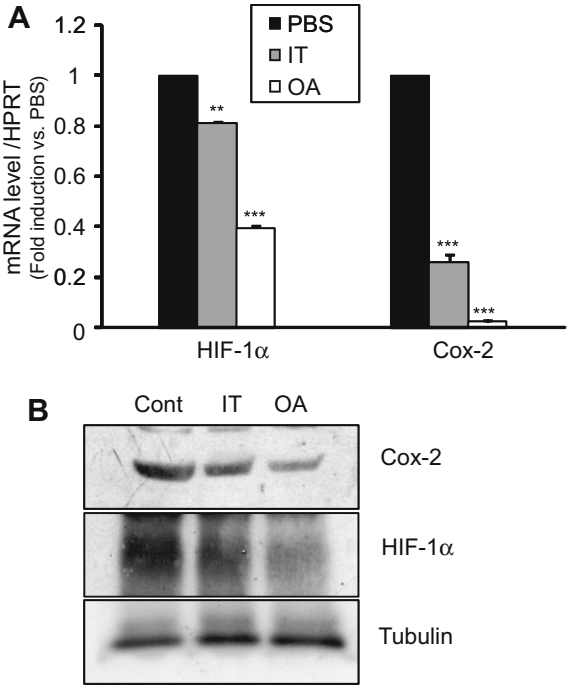

Fig. 4. Cinnamon treatment down-regulates the levels of Cox-2 and HIF-1α in tumor tissue. Total RNA and protein were isolated from tumor tissues from each treatment group and the levels of Cox-2 and HIF-1α in mRNA (A) and protein (B) were analyzed by quantitative RT-PCR and immunoblotting, respectively. The mRNA expression levels in each group were normalized with HPRT (house-keeping gene) and then fold induction of each target gene was compared to PBS groups (expression level of PBS group: 1). Error bars indicated SD. Two (**) or three asterisks (***) indicate $p < 0.005$ or $p < 0.001$, respectively. Data are representative of three independent experiments.

4. Discussion

The serious side effects of conventional chemotherapies limit their therapeutic application in diverse cancers. Recently complementary and alternative medicines (CAM) such as traditional herbal medicines have become an alternative therapeutic modality in treating cancer patients [6]. However, incomplete studies on their therapeutic mechanism limit CAM for cancer treatment.

In this study, we have demonstrated the potent anti-tumor efficacy of cinnamon extract and have elucidated its underlying mechanism using a mouse melanoma model system. Cinnamon extract significantly reduced tumor progression by down-regulating the tumor-associated growth factors such as EGF, VEGF-α, TGF-β, Cox-2, HIF-1, and neovascularization, while increasing the cytolytic activity of CD8$^+$ T cells.

Cinnamon extract was previously shown to inhibit the growth of hematologic tumor cell growth [15]; however, the role of cinnamon extract in *in vivo* tumor progression remained to be determined. We found that treatment of melanoma cell lines with cinnamon extract abrogated the expression of Cox-2 and HIF-1α (Fig. 1B and C). Administration (oral ingestion or intra-tumoral injection) of cinnamon extract significantly inhibited *in vivo* tumor growth, metastasis, and neo-vascularization progression of melanomas. To elucidate the underlying mechanism of the anti-tumor effect of cinnamon, we tested the possibility that cinnamon may modulate angiogenesis and the effector function of CD8$^+$ T cells that mediate cytotoxicity against tumor cells.

Tumor cells recruit new blood vessels by excessive production of pro-angiogenic factors that play a pivotal role in tumor progression and tumor survival. These include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), Interleukin 8 (IL-8), placenta-like growth factor (PLGF), transforming growth factor beta (TGF-β), platelet-derived endothelial growth factor (PDGF), pleiotrophin, and other factors [28,29]. Indeed, inhibition of tumor angiogenesis is thought to be a good target for cancer treatments. Cinnamon treatment significantly reduced the levels of pro-angiogenic factors (EGF, FGF, VEGF, and TGF-β) (Figs. 1A and 3F) and neo-vascularization

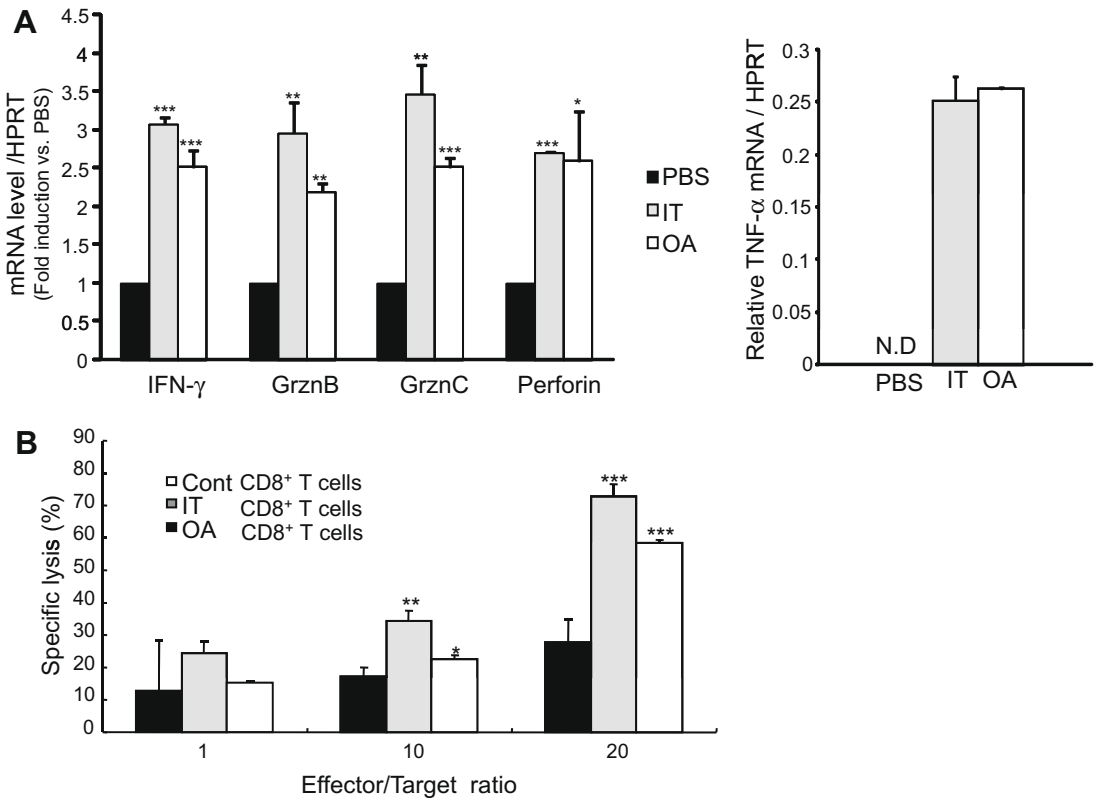

Fig. 5. Cinnamon treatment enhances the cytolytic activity of $CD8^+$ T cells. Cinnamon treatment was started after one week of tumor transplantation. After four weeks of cinnamon extract treatment, $CD8^+$ T cells in tumor draining lymph nodes (axillary, superficial, brachial, and lingual lymph node) were isolated from each group. (A) The expression levels of cytolytic molecules (TNF-α, IFN-γ, granzyme B, granzyme C, and perforin) were measured by quantitative RT-PCR. The mRNA expression levels in each group were normalized with HPRT (house-keeping gene) and then fold induction of each target gene was compared to PBS groups (expression level of PBS group: 1) and (B) the cytotoxicity of $CD8^+$ T cells in each group was compared using the JAM test. $CD8^+$ T cells were co-cultured with [3H]-thymidine labeled B16F10 cells for 12 h and the radioactivity in each sample was measured. Error bars indicated SD. Data are representative of three independent experiments. Not detectable (ND). One (∗), two (∗∗) or three asterisks (∗∗∗) indicate $p < 0.05$, $p < 0.005$ or $p < 0.001$, respectively.

in tumor tissues and tumor draining lymph nodes (Fig. 3A–C). Examination of morphological changes and the size of lymphoid organs in melanoma mice also supported the potent anti-angiogenic effect of cinnamon (Fig. 3A–C). The size of the spleen and tumor draining lymph nodes was significantly decreased in the cinnamon-treated groups, especially in the OA group (Fig. 3A–C). Conclusively, these data strongly suggests that oral administration of cinnamon strongly inhibits not only angiogenesis but also metastasis of tumor tissues.

Cinnamon treatment significantly reduced the levels of Cox-2 and HIF-1α in melanoma cell lines and in the melanoma mouse model (Figs. 1 and 4). Cox-2 and HIF-1α are well known as master regulators in cancer progression [20,21,23]. They share common roles in tumor progression to aggravate angiogenesis and metastasis. HIF-1 is a transcription factor that responds to changes in tissue oxygenation and is a key regulator of tumor angiogenesis and metastasis [21,23]. Hypoxic conditions upregulate angiogenic factors and promote tumor angiogenesis [30,31]. Cyclooxygenase-2 (Cox-2) is an enzyme that catalyzes arachidonic acid to prostaglandins. Cox-2 is predominantly expressed in synoviocytes, fibroblasts, osteoblasts, activated endothelial cells and tumor cells [20,32]. Cox-2 expression is induced by pro-inflammatory and mitogenic stimuli such as growth factors (EGF, FGF, and VEGF) and cytokines (TNF-α and IL1-β). Enhanced expression of Cox-2 is linked with tumor progression by inducing immune suppression as well as angiogenic and metastatic progression [20,25,33]. Hence, the decrease in Cox-2 and HIF-1α expression in the tumor tissues in response to cinnamon treatment may mediate the potent anti-tumor activity of cinnamon.

The tumor microenvironment induces active immune tolerance and escapes immune surveillance, in which tumor cells can easily foster proliferation, survival and migration without being attacked by the immune system [34]. In order to achieve an effective anti-tumor immune response, appropriately activated immune cells should maintain their anti-tumor activity to overcome the immune suppressive tumor microenvironment [35]. Although $CD8^+$ T cells have a central role in the elimination of tumors, the tumor microenvironment can actively suppress $CD8^+$ T cells that mediate the anti-tumor response *in vivo*. $CD8^+$ T cells at tumor sites or tumor draining lymph nodes frequently exhibit functional defects such as defective antigen specific cytolytic activity [36], lack of perforin expression [37], ineffective granule

exocytosis [38], poor adhesion [39], defective cytokine production and abnormal proliferation [40,41]. Enhanced $CD8^+$ T cell activity is therefore critical to eradicate tumor cells, especially in tumor regions. In this study, administration of cinnamon extract significantly inhibited tumor growth compared with the control (PBS-treated) group (Figs. 2 and 3). Interestingly, administration of cinnamon extract by intra-tumor injection or oral administration resulted in similar tumor-suppressing potencies. Oral administration (OA) induced a more potent down-regulation in the levels of pro-angiogenic factors (Fig. 3F), HIF-1α and COX-2 (Fig. 4) compared with intra-tumor injection (IT). However, effector function of cytotoxic $CD8^+$ T cells was more significantly increased by an intra-tumor injection (IT) (Fig. 5B). Administration of cinnamon extract enhanced the cytolytic activity of $CD8^+$ T cells by increasing the expression of cytolytic molecules such as cytokines (IFN-γ and TNF-α), granzymes (Grzn B and Grzn C), and perforin. In addition, cinnamon extract significantly increased the specific lysis rate in the JAM test (Fig. 5B). In this study, we mainly suggested that cinnamon extract could suppress tumor progression by modulating angiogenesis and the effector function of $CD8^+$ T cells. Recently we also questioned, in addition to the anti-cancer mechanisms described in this study, whether any additional mechanisms are also involved in anti-melanoma effect of cinnamon extract such as induction of active cell death or growth inhibition. Indeed, we found that long term treatment of cinnamon extract (more than 48 h) could induce active cell death and growth inhibition in several cancer lines such as melanoma, breast cancer, colorectal cancer, and hepatoma cell lines (manuscript in preparation).

Regardless of delivery routes (either OA or IT), cinnamon extract showed potent anti-melanoma effect although little differences was observed in regulation of tumor-associated specific growth factor (Figs. 3 and 4). The amount of delivered anti-tumoral compounds would be quite higher in IT system than in OA system. How come orally delivered extract showed anti-melanoma effect similar to intratumorally delivered? We assumed that active compounds of cinnamon extract may need further metabolic pathway to modify from inactive to active form like pro-drugs in gastrointestinal tract, as suggested in other study [42]. Further investigation is necessary to elucidate the action mechanisms related with different anti-tumor efficacies depending on the routes of administration of cinnamon extract.

In conclusion, our data suggest that cinnamon extract strongly diminishes tumor growth, angiogenesis and vascularization by inhibiting the levels of pro-angiogenic factors and Cox-2 and HIF-1α in tumors tissues. In addition, cinnamon extract enhances the cytotoxic activity of $CD8^+$ T cells in the tumor microenvironment, which collectively leads to an active immune response to tumors. Our results constitute the first evidence of the potent anti-tumor activity of cinnamon together with a detailed mechanism of action. Collectively, our data suggest the potential use of cinnamon extract as an anti-melanoma agent by targeting angiogenesis and the cytolytic effector function of $CD8^+$ T cells.

Conflict of interest

Authors do not have conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.01.015.

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