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Vitamin C Down-Regulates VEGF Production in B16F10 Murine Melanoma Cells via the Suppression of p42/44 MAPK Activation

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ABSTRACT

It is known that vitamin C induces apoptosis in several kinds of tumor cells, but its effect on the regulation of the angiogenic process of tumors is not completely studied. Vascular endothelial growth factor (VEGF) is the most well-known angiogenic factor, and it has a potent function as a stimulator of endothelial survival, migration, as well as vascular permeability. Therefore, we have investigated whether vitamin C can regulate the angiogenic process through the modulation of VEGF production from B16F10 melanoma cells. VEGF mRNA expression and VEGF production at protein levels were suppressed by vitamin C. In addition, we found that vitamin C suppressed the expression of cyclooxygenase (COX)-2 and that decreased VEGF production by vitamin C was also restored by the administration of prostaglandin E2 which is a product of COX-2. These results suggest that vitamin C suppresses VEGF expression via the regulation of COX-2 expression. Mitogen-activated protein kinases are generally known as key mediators in the signaling pathway for VEGF production. In the presence of vitamin C, the activation of p42/44 MAPK was completely inhibited. Taken together, our data suggest that vitamin C can down-regulate VEGF production via the modulation of COX-2 expression and that p42/44 MAPK acts as an important signaling mediator in this process. J. Cell. Biochem. 112: 894–901, 2011.

KEY WORDS: VITAMIN C; VEGF; COX-2; P42/44MAPK

Vitamin C is a well-known anti-oxidant and scavenges reactive oxygen species and reactive nitrogen species to protect cells from oxidative damages [Johnson et al., 2003; Coulter et al., 2006; Muthuvel et al., 2006]. The effects of vitamin C on the immune system have also been extensively studied, and the increase of interferon production and phagocytic activity has been documented [Siegel and Morton, 1984; Wintergerst et al., 2006]. In addition, it has been reported that vitamin C acts as a pro-oxidant in the presence of metal iron and shows a potent anti-tumor activity [Martin et al., 2006]. Recently, there have been several reports that vitamin C induces the apoptosis in murine melanoma cells via the down-regulation of transferrin receptor expression and that cytochrome *c* is an important mediator of vitamin C-induced apoptosis [Kang et al., 2003, 2005]. However, the effects of vitamin C

Abbreviations used: VEGF, vascular endothelial growth factor; COX, cyclooxygenase; MAPK, mitogen-activated protein kinase; HIF, hypoxia-inducible factor; PGE2, prostaglandin E2.Ha Na Kim and Hyemin Kim contributed equally to this work.

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on the regulation of the angiogenic process of tumors are not completely studied.

Vascular endothelial growth factor (VEGF) is widely recognized as a potent angiogenic factor. It is a 36–46 kDa heparin-binding homodimeric protein and has the following five family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. They are well-known products which are up-regulated by hypoxia and HIF-1 [Jiang et al., 1997; Mazure et al., 1997; Ravi et al., 2000; Zhong et al., 2000; Zundel et al., 2000; Laughner et al., 2001; Fukuda et al., 2002]. In addition, its expression is induced by several inflammatory cytokines and growth factors, including IL-1, IL-6, and transforming growth factor (TGF)-β [Nakahara et al., 2003; Konishi et al., 2005]. Moreover, the up-regulation of VEGF production can be induced by prostaglandin (PG) E2, which is derived from arachidonic acid by cyclooxygenase (COX)-2 [Pai et al., 2001; Miura et al., 2004; Ding et al., 2005]. Recently, Knowles et al. [2003] have reported that vitamin C could regulate tumor growth and angiogenesis via the suppression of hypoxia-inducible factor (HIF)-1 expression. In addition, we have thesis reporting inhibition of COX-2 expression by the treatment of vitamin C in cancer cell [Lee et al., 2008].

There are several reports regarding intracellular signaling molecules targeted by vitamin C. p38MAPK is the most well-known target of vitamin C. The activation of NF-κB and IL-18 production are inhibited by vitamin C through p38MAPK activation [Andrews, 2000; Cho et al., 2005]. It is reported that the activation of p38MAPK, ERK (p42/44MAPK), and JNK is critical for the angiogenic process in human monocytes by M-CSF signals [Curry et al., 2008]. In addition, VEGF production in microvascular endothelium is dependent on JNK- and ERK-dependent pathway [Milkiewicz et al., 2007]. On the contrary, the anti-angiogenic effect of resveraterol, which has potent anti-cancer and anti-angiogenic properties, is achieved by the inhibition of PI3K/AKT and MEK/ERK pathways. In addition, FOXO transcription factor may play an important role in this process [Srivastava et al., 2009]. Even though it seems that the activation status ERK is different in normal cells and tumor cells, the role and status of EKR on VEGF production in tumor cells are still not clarified.

Therefore, we aim to investigate the down-regulation of VEGF production by vitamin C its related mechanisms via the modulation of COX-2 expression and p42/44MAPK phosphorylation in this study.

MATERIALS AND METHODS

CELL AND CULTURE CONDITION

B16F10 murine melanoma cells were used in this study and maintained in continuous log phase growth and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Hypoxic cell culture condition was induced by the addition of $CoCl_2$ (100 μmol/L) to some cultures under normoxic condition.

ANTIBODIES AND CHEMICALS

Antibody to cyclooxygenase (COX)-2 was purchased from Santa Cruz Biotechnology (Palo Alto, CA). Vitamin C, recombinant PGE2, NS-398 (COX-2 specific inhibitor), and PD98059 (p42/44 MAPK inhibitor) were purchased from Sigma (St. Louis, MO).

RT-PCR

Total RNA was isolated and cDNA was made, after B16F10 murine melanoma cells (1×10^6) were exposed to 1 mM vitamin C for 1, 3, and 6 h. cDNA was amplified with β-actin primers (sense: 5′-AAGAGCTATGAGCT GCC TGA-3′, antisense: 5′-CAGG AGGAG-CAATGATCTTG-3′; product = 220 bp), VEGF primers (sense: 5′-AACAAATGCTTTCTCCGCTC-3′, 5′-TGAACTT TCTGC TCTCTTGG-3′; product = 618 bp). PCR products were electrophoresed and the density of each band was analyzed.

VEGF ELISA

The concentration of VEGF in the culture supernatant was measured by ELISA. Murine VEGF ELISA kits were purchased from R&D Systems (Minneapolis, MN) and ELISA was performed according to the manufacturer's instructions. Briefly, B16F10 murine melanoma cells were cultured in the presence or absence with vitamin C and then culture supernatants were collected and added to anti-VEGF Ab coated well. After 1 h incubation at 37°C, the wells were washed four times with PBS–Tween 20 (pH 7.4), alkaline phosphatase conjugated anti-murine VEGF Ab was added and then incubated for another 1 h. After the wells were washed four times with PBS–Tween 20, substrate solution was added and incubated for another 1 h. The relative absorbance was measured at 450 nm and then VEGF concentration was calculated with VEGF standard curve.

COX-2 siRNA TRANSFECTION

The siRNA targeted COX-2 was purchased from Santa Cruz Biotechnology. Cells in exponential phase of growth were plated in six-well plates at 5×10^5 cells/well, grown for 24 h then transfected with 20 nM of siRNA using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNA were chosen based on dose–response studies. After examination of transfection efficiency under fluorescent microscopy, silencing was examined by immunoblotting at 24–48 h after transfection. Control cells were transfected with control siRNA with oligofectamine and serum-reduced medium (mock).Inhibitor Studies

After B16F10 murine melanoma cells (1×10^6 cells/ml) were incubated in the presence of 20 μM of NS-398 and 10 μM of PD98059, and then culture supernatant was collected and changes in VEGF production were examined by ELISA.

IMMUNOBLOTTING

B16F10 melanoma cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 (v/v) in 20 mM Tris–HCl, pH 8.3, 150 mM NaCl. The lysis buffer contained 1 mM PMSF, 1 mM Na_4VO_3, 10 mM NaF, and 1 mM $Na_4P_2O_7$. Briefly, the cells were washed twice with PBS and resuspended in 200 μl of lysis buffer. After 5 min on ice, the cells were spun for 5 min at 14,000 rpm and 4°C in a microcentrifuge. Supernatants were transferred to fresh tubes and the concentration of cytosolic proteins by the BCA method. Thirty micrograms of each sample was then

added to SDS-loading buffer (0.5 M Tris–HCl (pH 6.8), 1 M 2-ME, 10% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and boiled for 10 min. The boiled samples were then loaded into 12% polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk proteins. Blocked membranes were then incubated with a mouse developed anti-murine COX-2 Ab (1:100; Santa Cruz Biotechnology) for 1 h at room temperature, washed three times (5 min each) with PBS containing 0.1% Tween 20, and incubated with a HRP-conjugated anti-mouse IgG secondary Ab (1:1000; Sigma). The expression of COX-2 was detected using the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ).

STATISTICAL ANALYSIS

Data were expressed as mean ± SD of each group in independent experiments. For comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. A value of $P < 0.05$ was considered statistically significant. Statistical tests were carried out using GraphPad InStat (GraphPad Software).

RESULTS

VITAMIN C DOWN-REGULATES VEGF PRODUCTION FROM B16F10 MURINE MELANOMA CELLS

As we already confirmed that there was no lethal effect on B16F10 melanoma cells by 1 mM of vitamin C, We first examined whether the expression of VEGF mRNA is changed by 1 mM of vitamin C treatment by RT-PCR. VEGF mRNA expression was decreased at 1 h after vitamin C treatment. However, it was restored at 6 h after vitamin C treatment (Fig. 1A). Next we examined the changes on VEGF production at protein level by ELISA. When the cells were exposed to 1 mM of vitamin C, VEGF production was definitely suppressed in a time-dependent manner (Fig. 1B).

In general, VEGF production is prominent phenomenon in hypoxia. Even though we showed the changes of VEGF production at mRNA and protein levels in Figure 1, it was done under normoxia. Therefore, we did the same experiment under hypoxia. To make hypoxic condition, 100 μM of $CoCl_2$ was added to culture medium for 12 h and then the VEGF production was measured. When it compared with the VEGF production in normoxia, it is relatively higher in hypoxia. In addition, VEGF production was definitely suppressed by the treatment of vitamin C (Fig. 1C).

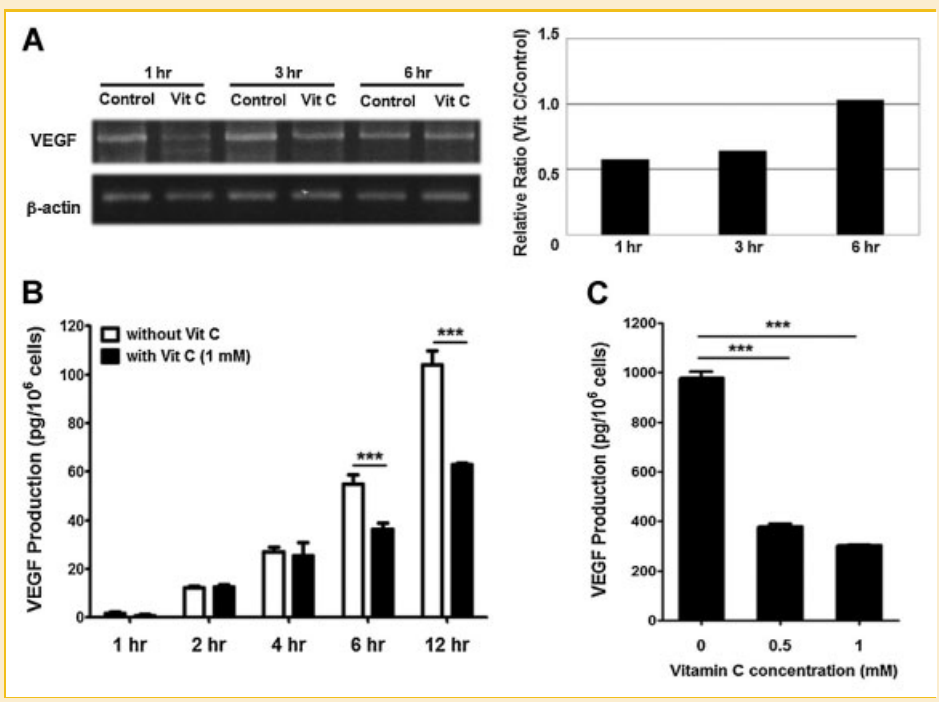

Fig. 1. The down-regulation of VEGF expression in B16F10 melanoma by vitamin C. A: After cells were incubated with 1 mM vitamin C for 1, 3, and 6 h, RNA was extracted using Trizol, and cDNA was prepared. RT-PCR was performed as described in the Materials and Methods Section. Negatives of direct positive images were measured using an analytical software (Scion Image Program, Frederick, MA), and a relative quantity of ethidium-stained bands, which represents integrated area under the curve of densitometric tracing, was reported as the ratio of VEGF to β-actin. Results are representative of the three independent experiments. B: Cells were incubated in the presence or absence of 1 mM vitamin C for 1, 2, 4, 6, and 12 h, and then culture supernatants were collected. VEGF concentration was measured by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. ***P-value was <0.001, when it compared with control. C: Cells were incubated in the presence 0.5 and 1 mM of vitamin C under hypoxia induced by the treatment of 100 μM of $CoCl_2$ for 18 h and then VEGF concentration was measured by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. ***P-value was <0.001, when it compared with control.

VITAMIN C DOWN-REGULATES VEGF PRODUCTION THROUGH THE SUPPRESSION OF CYCLOOXYGENASE (COX)-2 EXPRESSION

There are several reports regarding the regulation of VEGF production by COX-2 and its product, PGE2. In addition, Han et al. reported that the repression of constitutive COX-2 expression in human myeloid leukemia, HL-60. However, there are no reports about the regulatory effect of vitamin C on the VEGF production through the regulation of COX-2 expression. For this reason, we investigated whether COX-2 is involved in the decrease of VEGF production by vitamin C. First, we measured the amount of VEGF when the cells were cultured in the presence of vitamin C and COX-2 specific inhibitor, NS-398, respectably. As we expected, VEGF production was decreased by NS-398, similar with VEGF production in the presence of vitamin C (Fig. 2A). In addition, the change on COX-2 expression by vitamin C was examined and we found that COX-2 expression was decreased by vitamin C in a time-dependent manner (Fig. 2B).

PGE2 RESTORES DECREASED VEGF PRODUCTION BY VITAMIN C

Since we have already showed that COX-2 expression and PGE2 production is down-regulated by vitamin C, we investigated whether decreased VEGF production by vitamin C is recovered by the treatment of exogenous PGE2. As shown in Figure 3A, when the cells were culture in the presence of 50 μM of PGE2, VEGF production was relatively higher than normal control. It suggests that VEGF production is regulated by COX-2 and its product, PGE2. However, when the cells were cultured in the presence of vitamin C and PGE2 simultaneously, PGE2 induced increase of VEGF production is suppressed and vitamin C induced decrease of VEGF production is recovered. The involvement of COX-2 and PGE2 on the production of VEGF was investigated on COX-2 siRNA transfectant. As we expected, VEGF production was decreased by COX-2 siRNA transfection and it was similar with the treatment of vitamin C and NS-398. Decreased VEGF production was remarkably restored by the treatment of exogenous PGE2 (Fig. 3C).

VITAMIN C DOWN-REGULATES VEGF PRODUCTION THROUGH THE SUPPRESSION OF p42/44 MAPK

To clarify the intracellular signaling pathway involved in the regulation of VEGF production by vitamin C, we investigated the change on p42/P44 MAPK by the treatment of vitamin C, since it is already reported that p42/P44 MAPK play an important role in the intracellular signal pathway for VEGF production among several kinds of kinases. When the cells were cultured in the presence of 10 μM of PD98059, a p42/44 MAPK specific inhibitor, we found decreased VEGF production, similar with the treatment of vitamin C (Fig. 4A). In addition, the changes on the phosphorylation of p42/44 MAPK upon vitamin C treatment were investigated by immunoblotting. As shown in Figure 4B, the phosphorylation of p42/44 MAPK was decreased by vitamin C treatment in a time-dependent manner.

DISCUSSION

It is well-known that vitamin C plays an important role as an anti-oxidant and an essential nutrient for human beings [Coulter et al., 2006]. It also has an anti-tumor activity from growth inhibition and induction of apoptosis. However, there are no reports regarding the role of vitamin C on the regulation of the angiogenic process and metastasis of tumors. Therefore, we examined the inhibitory effect of vitamin C on angiogenic process through the regulation of VEGF production.

As we expected, the expression of VEGF mRNA transcripts was effectively down-regulated at 1 h after treatment of vitamin C and its protein production was down-regulated at 6 h after treatment and lasting to 12 h. However, VEGF mRNA expression is re-increased at 3 h after treatment of vitamin C. It might be caused by the unique characteristic of vitamin C. It is generally known that vitamin C is very rapidly oxidized to semidehydroascorbate (SDA) and dehydroascorbate (DHA) in culture supernatant and plasma

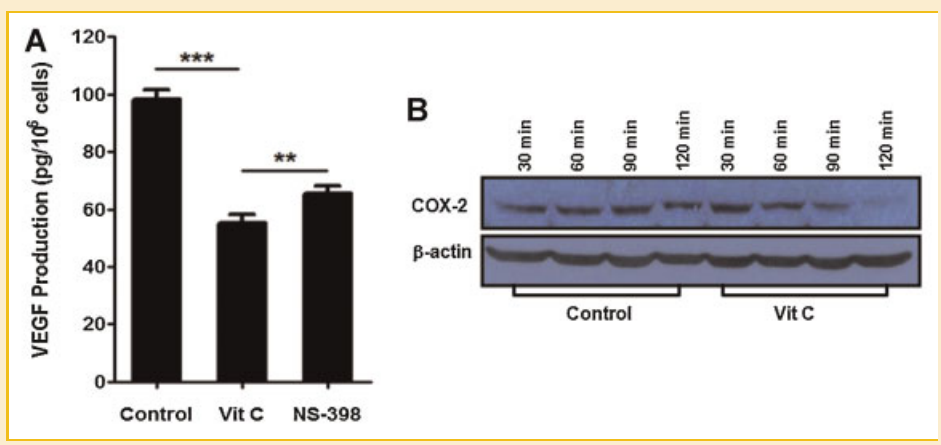

Fig. 2. The regulatory effect of vitamin C on VEGF production in B16F10 melanoma cells through the inhibition of COX-2 activity. A: Cells were incubated with 1 mM vitamin C or 20 μM NS-398 for 12 h, and then the change of VEGF production was measured by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. $^{***}P$-value was <0.001, $^{**}P$-value was <0.01, when it compared with control. B: Cells were treated with 1 mM vitamin C for 30, 60, 90, and 120 min, and the cells were lysed using Triton X-100 lysis buffer. The change of COX-2 expression was measured by immunoblotting as described in the Materials and Methods Section. Results are representative of three independent experiments.

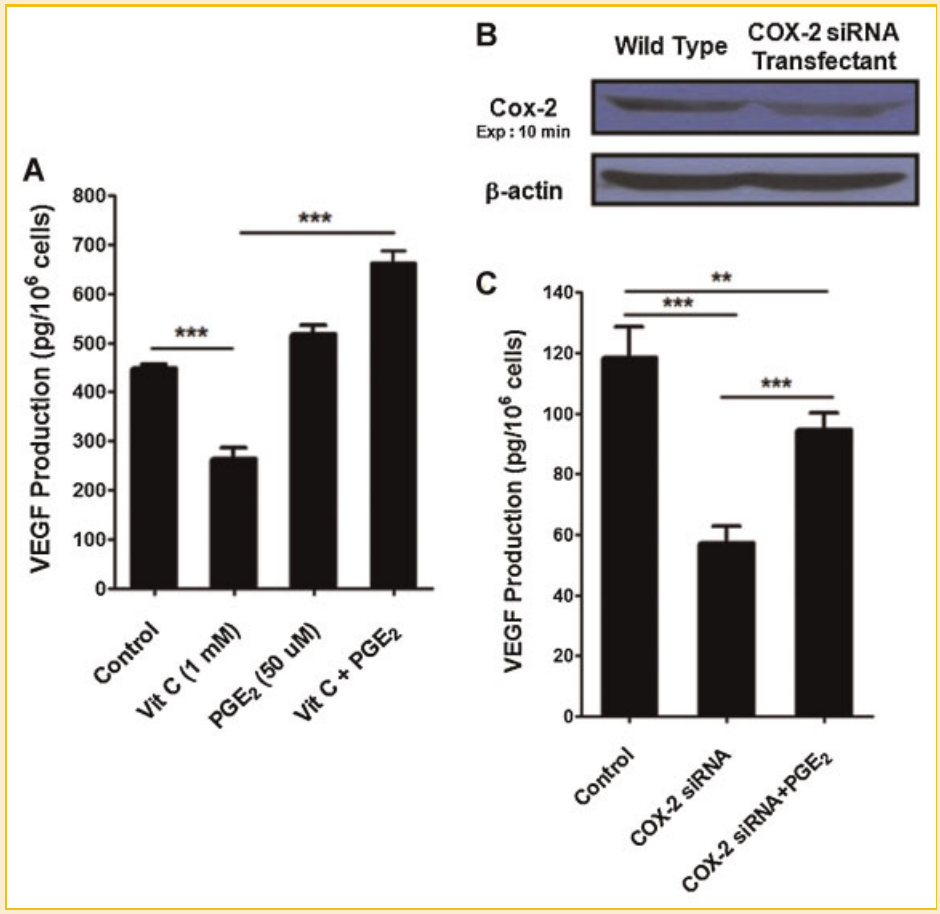

Fig. 3. The restoration of VEGF production in B16F10 melanoma cells by the treatment of PGE2. A: Cells were cultured in the presence or absence of vitamin C and PGE2 for 18 h, and then the change of VEGF production was examined by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. ***P-value was <0.001, when it compared with control. B: COX-2 siRNA was transfected into B16F10 melanoma cells as described in the Materials and Methods Section and then the down-regulation of COX-2 expression was confirmed by immunoblotting as described in the Materials and Methods Section. C: COX-2 siRNA transfected B16F10 melanoma cells were cultured in the presence or absence of vitamin C and PGE2 for 12 h, and then the change of VEGF production was examined by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. ***P-value was <0.001, when it compared with control.

after finishing its anti-oxidant action [Linster and Van Schaftingen, 2007; Lee, 2009; Mandl et al., 2009]. In the case of DHA, it can be irreversibly degraded into diketo-L-gulonic acid and has no more anti-oxidant activity [Mandl et al., 2009]. Even though DHA can be recycled to vitamin C by DHA reductase after uptake into the cells, it is rapidly lost within several minutes, if it is not to be taken up by the cells. However, intracellular uptake process for DHA is quite different with vitamin C. While vitamin C has two kinds of specific channel proteins only for the transport of vitamin C, sodium dependent vitamin C transporter (SVCT)-1 and -2, DHA shares glucose transporters (GLUTs) with glucose [Linster and Van Schaftingen, 2007]. However, GLUTs have higher affinity to glucose than DHA, most DHA oxidized from vitamin C is degraded in culture supernatant without uptake by cells. Therefore, it is supposed that oxidized forms of vitamin C including DHA gradually increases in culture medium during the incubation and it had seldom taken by the cells and then lost. It was reflected in the lost of suppressive effect of vitamin C on VEGF mRNA expression as time goes on. According to the general concept regarding the process from transcription to translation, it takes 4–18 h for mRNA to be changed into protein, Therefore, inhibition of VEGF production at 6 h, even at 12 h after treatment of vitamin C, is affected by the suppression of VEGF mRNA expression at 1 h after treatment of vitamin C. Taken together, the regulation of VEGF production from tumor can be effectively achieved by continuous exposure to vitamin C.

The concentration of vitamin C used in this experiment is 1 mM. Since it is generally known that vitamin C concentration in serum is 70–80 μM, the effect of vitamin C in vitro is very controversial. However, it depends on the route of administration. That is, when we take vitamin C via oral route, it only reaches 70–80 μM in serum, but it reaches up to 15–17 mM when 100 g of vitamin C is taken via intravenous (IV) injection [Padayatty et al., 2004]. In general, 50–100 g of vitamin C is used for the treatment of cancer via intravenous route injection [Riordan et al., 1995, 2004]. Even though we do not present here, we already confirmed that serum concentration of vitamin C in cancer patients can be reached up to

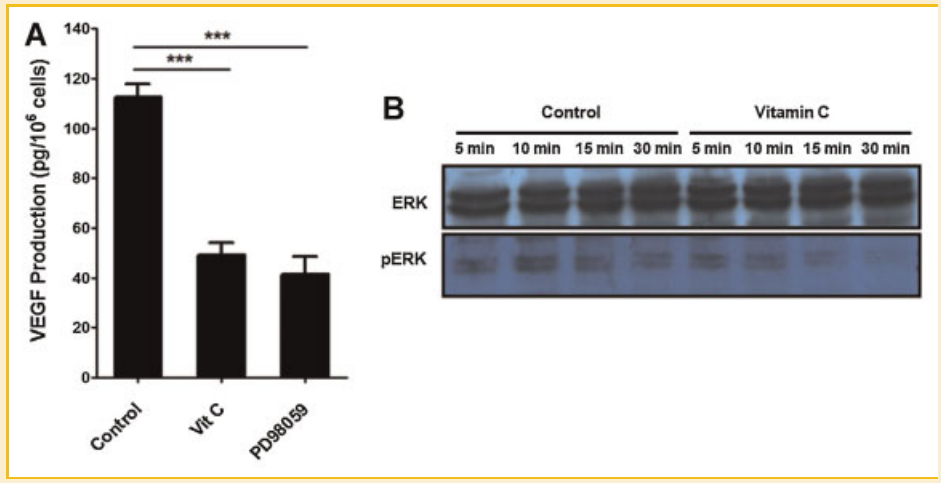

Fig. 4. The decrease of VEGF production in B16F10 melanoma cells through the inhibition of p42/44 MAPK activation. A: Cells were incubated with 1 mM vitamin C or 10 μM PD98059 for 12 h, and then the change of VEGF production was measured by ELISA as described in the Materials and Methods Section. Values are the mean ± SD. Results are representative of three independent experiments, each performed in triplicate. $^{**}P$-value was <0.001, when it compared with control. B: Cells were incubated with 1 mM vitamin C for 5, 10, 15, and 30 min, and then B16F10 melanoma cells were lysed using Triton X-100 lysis buffer. The p42/44 MAPK and phosphorylated p42/44 MAPK expressions measured by immunoblotting as described in the Materials and Methods Section. Results are representative of more than three independent experiments.

1.5 mM by IV injection of 10 g of vitamin C. Therefore, there are two advantages of vitamin C administration into cancer patients. One is the increase of anti-cancer immune responses, since it is already reported that vitamin C enhances natural killer (NK) cell activity, and the other is suppression of angiogenic process via the modulation of VEGF production.

Angiogenic process is not only necessary for the metastasis of tumors, but also strongly needed for the wound healing process [Zetter, 1990; Meadows et al., 1991; Faria et al., 1997; Abe et al., 2001; Norrby, 2006]. In addition, it is well known that vitamin C promotes wound healing process by facilitating the synthesis of collagen types I and II [Gross, 2000; Munday et al., 2005; Sato et al., 2006]. Moreover, there is a report that angiogenic process in collagen matrices is enhanced by covalent incorporation of VEGF [Koch et al., 2006]. Therefore, the effect of vitamin C on the production of VEGF from normal cells during wound healing process should be evaluated, even though it suppresses VEGF production from tumor cells. We hypothesize that it is caused by two sides of vitamin C. That is, vitamin C acts as a pro-oxidant on tumor cells, but it acts as an anti-oxidant normal cells. In our previous reports, vitamin C induces apoptosis on tumor cells via the mitochondrial pathway and suppresses IL-18 production from tumor cells. In both of case, intracellular ROS dramatically increased by the treatment of vitamin C [Kang et al., 2003; Cho et al., 2005]. However, vitamin C showed anti-oxidant properties in UVB-irradiated human skin epidermal keratinocytes, HaCaT for the prevention of skin inflammation through the increase of IL-8 and MCP-1 [Kang et al., 2007]. It means that vitamin C might facilitate wound healing process through the increase of VEGF production rather than suppression of its production. Although it is still not cleared what kinds of factors are related with the pro-oxidant properties of vitamin C, metal iron concentration is thought be as a key factor [Martin et al., 2006]. Therefore, it also provides the scientific basis for further research regarding the involvement of Fe^{2+} on the regulation of VEGF production by vitamin C.

HIF-1 is well-known as regulatory factors for VEGF production [Jiang et al., 1997; Mazure et al., 1997; Ravi et al., 2000; Zhong et al., 2000; Zundel et al., 2000; Laughner et al., 2001; Fukuda et al., 2002]. It is generally known that HIF-1 expression is prominently increased under hypoxic condition and facilitates angiogenic process through increase of several kinds of angiogenic factors such as VEGF and IL-18. There is report regarding the regulatory effect of vitamin C on HIF-1 expression by Knowles et al. Based on their report, HIF-1 expression is effectively down-regulated by vitamin C. Even though they did not present the down-regulation of VEGF production in their report, it suggests that vitamin C could regulate VEGF production as well, since increasing HIF-1 expression is correlated with VEGF production. Therefore, the result presented in manuscript is coincided with their report, since we evaluated the effect of vitamin C on the regulation of VEGF production under hypoxic condition as well. HIF-1 is a heterodimer which is composed of HIF-1α subunit and HIF-1β/ARNT subunit. HIF-1α subunits are post-translationally modified by a series of oxygen-dependent enzymatic hydroxylations at specific amino acid residues [Bruick and McKnight, 2001; Epstein et al., 2001; Hewitson et al., 2002; Lando et al., 2002]. In this process, prolyl-4-hydroxylase, 2-OG-dependent dioxygenases, and Fe^{2+} are important factors, and vitamin C acts as an important co-factor. Therefore, the involvement of these factors in the regulation of VEGF production by vitamin C is further investigated.

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