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Vanillin Enhances TRAIL-Induced Apoptosis in Cancer Cells through Inhibition of NF-κB Activation

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Abstract. *Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent which selectively kills cancer cells with little effect on normal cells. However, TRAIL resistance is widely found in cancer cells. We have previously reported antimetatstatic and antiangiogenic effects of vanillin, a flavoring agent from vanilla. Here we have evaluated the sensitizing effect of vanillin on a TRAIL-resistant human cervical cancer cell line, HeLa. Materials and Methods: Cell viability after treatments was determined by the WST-1 cell counting kit. Apoptosis was demonstrated by detection of caspase-3 activation and cleavage of poly (ADP-ribose) polymerase using immunoblot analysis. Effect of treatments on TRAIL signaling pathway and nuclear factor κB (FN-κB) activation was studied using immunoblot analysis and luciferase reporter assay. Results: Pretreatment of HeLa cells with vanillin enhanced TRAIL-induced cell death through the apoptosis pathway. Vanillin pretreatment inhibited TRAIL-induced phosphorylation of p65 and transcriptional activity of NF-κB. Conclusion: Vanillin sensitizes HeLa cells to TRAIL-induced apoptosis by inhibiting NF-κB activation.*

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent because of its selective induction of apoptosis in various types of cancer cells, independently of growth rate and p53 status, without toxic effect on normal cells (1). Presently, the use of recombinant soluble TRAIL or agonistic anti-TRAIL receptor monoclonal antibody is being investigated in clinical trials for cancer therapy, either its own or in combination with other anticancer agents (2). However, inherent TRAIL resistance has been observed in many cancer cell lines and primary cells obtained from tumors. In addition to pro-apoptotic signaling, TRAIL also activates anti-apoptotic signaling pathways including nuclear factor-κB (NF-κB), phosphoinositide 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases (MAPKs), which have been shown to be involved in TRAIL resistance (3). Currently, there is much interest in screening for natural products that can sensitize cancer cells to TRAIL-induced apoptosis for their use in combination with TRAIL (4).

Vanillin, a widely used flavoring agent from vanilla, has been shown to exhibit several chemopreventive properties, including antioxidant (5), antimutagenesis (6), and anticarcinogenesis *in vivo* (7, 8). Previously, we reported the antimetastatic effect of vanillin in a mouse model (9). Vanillin was also able to suppress the metastatic potential of human lung cancer cells and reduced *in vivo* angiogenesis (10).

In the present work, we have studied the ability of vanillin to sensitize HeLa human cervical cancer cells to TRAIL, and then investigated its effect on anti-apoptotic TRAIL signaling pathways, including NF-κB, PI3K/Akt, and MAPKs.

Materials and Methods

Chemicals. Vanillin (Sigma-Aldrich Japan K.K., Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO) and kept as a stock solution at –20˚C. The final concentration of DMSO was kept below 0.2% throughout the study. Recombinant human TRAIL and recombinant human tumor necrosis factor α (TNFα) were purchased from

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Peprotech (London, UK). Primary antibodies specific to caspase-3, poly (ADP-ribose) polymerase (PARP), and phosphorylated form of Akt (Ser^{473}), extracellular signal-regulated kinase (ERK) (Thr^{202}/Tyr^{204}), p38 (Thr^{180}/Tyr^{182}), c-Jun NH2-terminal kinase (JNK) (Thr^{183}/Tyr^{185}) and p65 (Ser^{536}) were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies specific to actin, Akt, ERK, p38, JNK, and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. HeLa human cervical cancer cells (ATCC, Rockville, MD, USA) stably transfected with NF-κB-luciferase reporter plasmid were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 ug/ml streptomycin. The culture was maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assay. Viability of cells after treatment was determined by WST-1 Cell Counting Kit (Wako Pure Chemical Industries, Osaka, Japan) as previously described (9). Cells were seeded into a 96-well plate (6×10^3/100 μl/well). After 24 hours of incubation, vanillin-containing medium (100 μl) was added to the wells, and cells incubated for 30 min. Aliquots of TRAIL in medium (5 μl) were added to each well, and cells further incubated for 3 hours or 24 hours. WST-1 solution (10 μl) was added to each well at 2 hours before the end of the experiment. The absorbance at 450 nm was measured using a microplate reader. Cell viability was determined from the absorbance of soluble formazan dye generated by living cells.

Immunoblot analysis. Immunoblot analysis was performed as previously described (11). Cells were seeded and grown overnight in a 6-well plate (1×10^6/2 ml/well). After treatment, the cells were scraped and lysed in whole-cell lysis buffer (25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cell lysates were subjected to electrophoresis in 7.5% or 10% SDS-PAGE, and electrophoretically transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA, USA). The membranes were treated with BlockAce (Dainippon Pharmaceutical, Co. Ltd., Osaka, Japan) for at least 2 hours, and probed with the indicated primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). Bands were visualized using ECL reagents (Amersham Bioscience, Piscataway, NJ, USA).

Luciferase reporter assay. NF-κB transcriptional activity in the HeLa cells stably transfected with NF-κB-luciferase reporter was determined by luciferase assay. Cells were seeded in a 96-well plate (3×10^4/100 μl/well) and left overnight. The cells were pretreated with vanillin (2 mM) for 30 min, and further incubated with TRAIL (200 ng/ml) or TNFα (20 ng/ml) for 3 hours. Cells were then lysed with 20 μl passive lysis buffer (Promega, Medison, WI, USA), and 5 μl of cell lysate were mixed with 50 μl of luciferase substrate solution (PicaGene, Toyo Ink, Tokyo, Japan). Luminescence was determined using a luminometer (Atto, Tokyo, Japan).

Statistical analysis. Data are expressed as mean±S.D. and analyzed by Student's *t*-test to determine the significance of differences between groups. A *p*-value lower than 0.05 was considered to be significant.

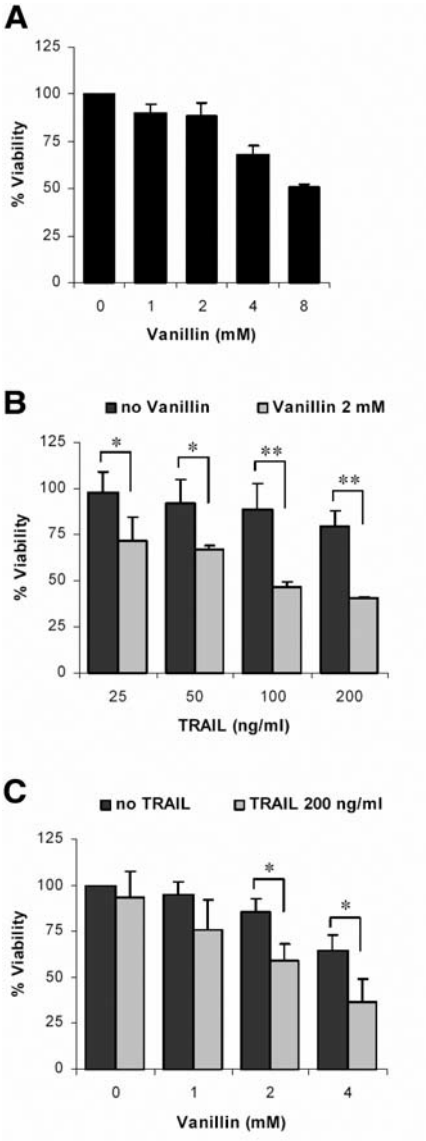

Figure 1. *Effect of vanillin on TRAIL-induced cell death in HeLa cells. Viability of HeLa cells after treatment was determined by WST-1 assay. A: Cells were treated with vanillin alone for 24 hours. B: Cells were pretreated with vanillin (2 mM) or vehicle (0.2% DMSO) for 30 min, and further incubated with different concentrations of TRAIL for 24 hours. C: Cells were pretreated with different concentrations of vanillin for 30 min, and further incubated in the presence or absence of TRAIL (200 ng/ml) for 24 hours. Data are expressed as mean±S.D. from at least three independent experiments, and significant differences between groups are shown by *p<0.05 and **p<0.01.*

Results

Vanillin enhances TRAIL-induced apoptosis. Initially, cytotoxicity of vanillin in HeLa cells was determined after 24 hours of treatment with 1-8 mM vanillin. The concentration range of 1-2 mM vanillin was observed to be

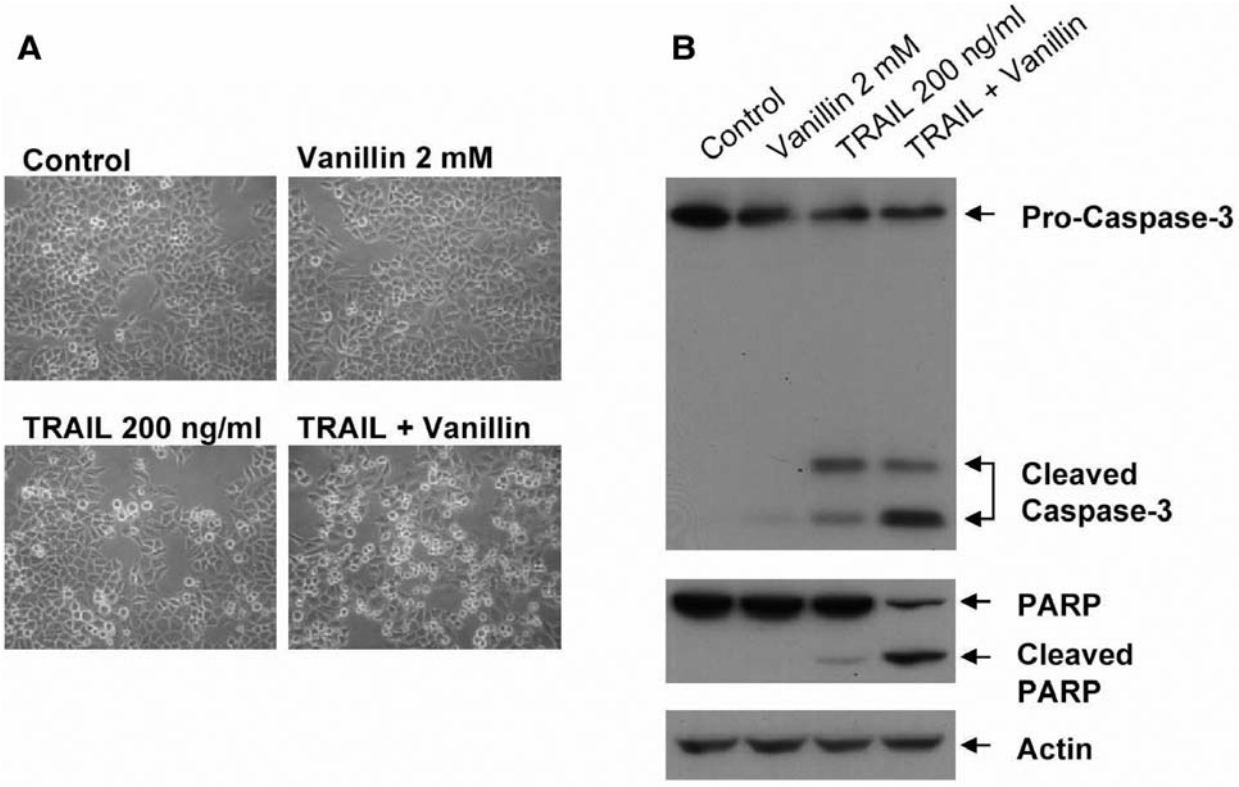

Figure 2. *Vanillin enhances TRAIL-induced apoptosis. HeLa cells were pretreated with vanillin (2 mM) for 30 min, followed by incubation in the presence or absence of TRAIL (200 ng/ml) for 12 hours. A: Photographs were taken at original magnification of ×50. B: Whole cell lysates were analyzed by immunoblot for apoptotic proteins, caspase-3 and PARP, using actin as loading control.*

non-toxic, with cell viability being greater than 89% (Figure 1A). Therefore, the highest non-cytotoxic concentration of vanillin (2 mM) was selected for further studies. HeLa cells are normally resistant to TRAIL, and treatment with 25-200 ng/ml TRAIL for 24 hours resulted in only 2-20% cell death (Figure 1B). However, when cells were pretreated with 2 mM vanillin for 30 min before 25-200 ng/ml TRAIL treatment, TRAIL-induced cell death was significantly enhanced, with 28-60% cell death being observed (Figure 1B). The enhancement of TRAIL-induced cell death by vanillin occurred in a dose-dependent manner, with significant enhancement being observed with 2-4 mM vanillin when combined with 200 ng/ml TRAIL (Figure 1C).

Massive cell death was observed in HeLa cells at 12 hours after treatment with a combination of 2 mM vanillin and 200 ng/ml TRAIL (Figure 2A). Cleavage of pro-caspase-3 and poly(ADP-ribose) polymerase (PARP) are hallmarks of cells undergoing apoptosis. The apoptosis-inducing effect of TRAIL was enhanced by vanillin pretreatment, as clearly shown by increased cleavage of pro-caspase-3 and PARP (Figure 2B).

Effect of vanillin on TRAIL signaling pathway. We further investigated effect of vanillin on anti-apoptotic TRAIL signaling including NF-κB, PI3K/Akt and MAPKs. After 12 hours of treatment, TRAIL (200 ng/ml) induced activation of NF-κB and all MAPKs, as revealed by increased phosphorylation of NF-κB p65, ERK, JNK and p38, whereas Akt was already constitutively activated and was not affected by TRAIL (Figure 3). TRAIL-induced NF-κB activation was markedly suppressed by vanillin, while other pathways were not affected (Figure 3).

Vanillin inhibits TRAIL-induced NF-κB activation. We further confirmed the inhibitory effect of vanillin on TRAIL-induced NF-κB activation in HeLa cells by using luciferase reporter assay. TNFα was used as a potent inducer of NF-κB activation. Treatment with 20 ng/ml TNFα or 200 ng/ml TRAIL for 3 hours induced activation and increased transcriptional activity of NF-κB by 5.9- and 1.8-fold, respectively, compared to control (Figure 4A and 4B). Vanillin pretreatment suppressed the TNFα-induced NF-κB transcriptional activity by approximately 50%, and completely

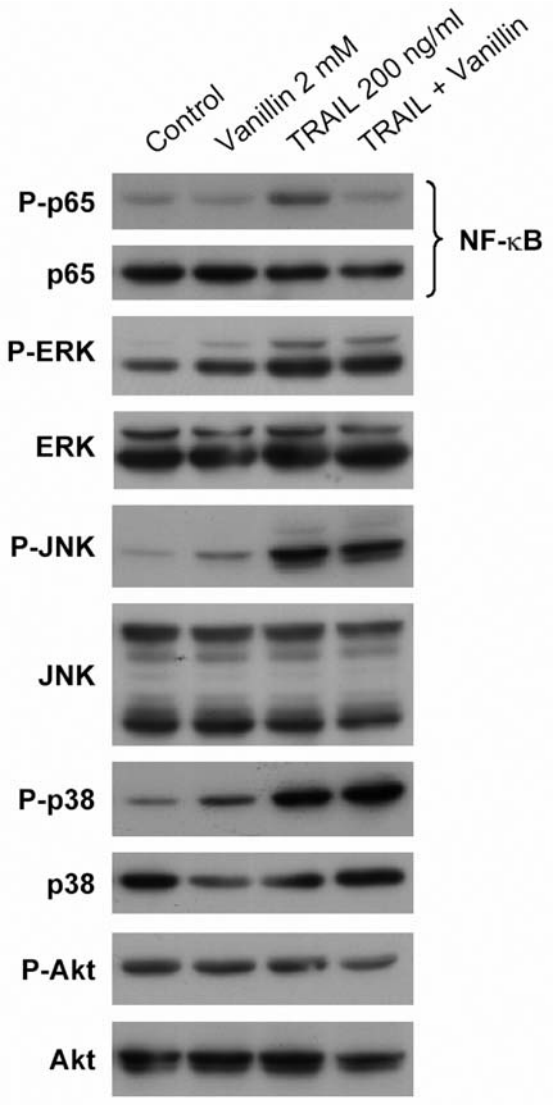

Figure 3. *Effect of vanillin on downstream signaling of TRAIL. HeLa cells were pretreated with vanillin (2 mM) for 30 min, followed by incubation in the presence or absence of TRAIL (200 ng/ml) for 12 hours. Whole cell lysates were then analyzed by immunoblot for levels of total and phosphorylated forms of Akt, ERK, JNK, p38 and NF-κB p65.*

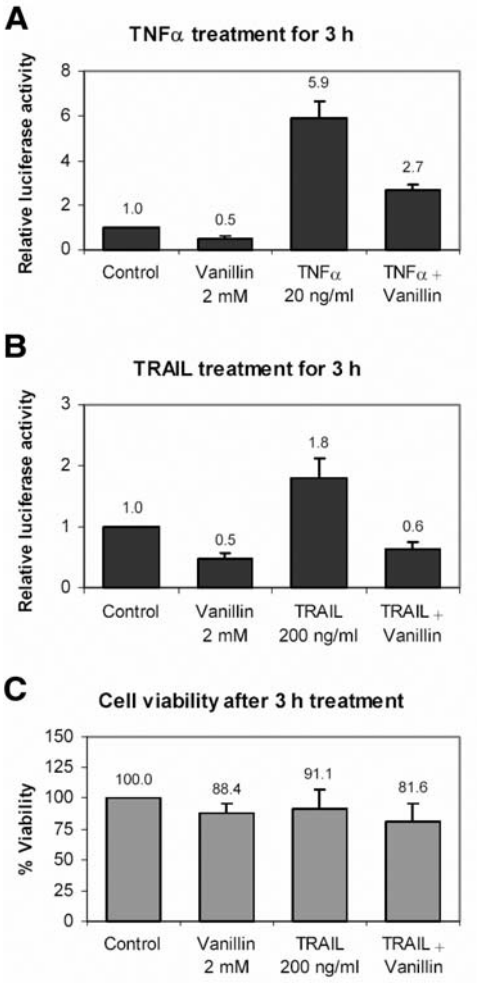

Figure 4. *Effect of vanillin on TRAIL-induced NF-κB transcriptional activity. HeLa cells stably transfected with NF-κB-luciferase reporter plasmid were pretreated with vanillin (2 mM) for 30 min, and further incubated with TNFα (20 ng/ml)(A) or TRAIL (200 ng/ml) (B) for 3 hours. NF-κB-mediated luciferase gene expression was then determined by luciferase activity assay. C: Cell viability after 3 hours of treatment was determined by WST-1 assay. Data are expressed as mean±S.D. from three independent experiments.*

abolished the TRAIL-induced NF-κB transcriptional activity (Figure 4A and 4B). The inhibition by vanillin during 3 hours of treatment was not due to the loss of cell viability, as revealed by the more than 80% cell viability remaining at the end of treatment (Figure 4C).

Discussion

Anti-apoptotic function of NF-κB activation in TRAIL signaling is mediated by the up-regulation of several anti-apoptotic genes such as cellular FLICE-inhibitory protein (*cFLIP*), B-cell lymphoma-extra large (*Bcl-XL*) and X-linked inhibitor of apoptosis protein (*XIAP*) (3). It has been shown in HeLa and other cancer cell lines that inhibition of NF-κB can sensitize cancer cells to TRAIL-induced apoptosis (12, 13). Our results provide evidence for the first time that vanillin is capable of sensitizing human cancer cells to TRAIL-induced apoptosis. The mechanism of vanillin for TRAIL sensitization in HeLa cells is, at least in part, due to inhibition of TRAIL-induced NF-κB activation.

Our results demonstrated that vanillin suppressed TRAIL-induced NF-κB activation by inhibiting phosphorylation of p65 and transcriptional activity of NF-κB. In addition to

TRAIL, vanillin also inhibited NF-κB activation induced by inflammatory cytokine TNFα. Recent reports showed that vanillin inhibited NF-κB activation induced by other inflammatory stimuli including lipopolysaccharide and trinitrobenzene sulfonic acid (TNBS) (14, 15), as well as a potent carcinogen, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (16). The concentration range of vanillin used to inhibit TPA-induced NF-κB activation in HepG2 human hepatocellular carcinoma cells was 2.5-5 mM (16), which is consistent with the concentrations used to inhibit TRAIL- or TNFα-induced NF-κB activation in HeLa cells.

In HeLa cells, TRAIL stimulated phosphorylation of NF-κB p65 and all MAPKs, but not Akt (Figure 3). We previously demonstrated that vanillin (1-4 mM) suppressed cell migration of A549 human lung cancer cells through inhibiting the PI3K/Akt pathway (10). However, in HeLa cells, where Akt is constitutively activated and not affected by TRAIL stimulation, the vanillin concentrations required to inhibit Akt phosphorylation in unstimulated cells after 24 hours of treatment was 5 mM or more, whereas 2.5 mM vanillin did not have an effect (unpublished data). Thus the TRAIL-sensitization effect of vanillin in HeLa cells in this study is likely to be a result of inhibiting NF-κB activation.

NF-κB activation is a key step in the inflammatory response. The *in vivo* anti-inflammatory effect of vanillin has been demonstrated in a mouse model. The preventive effects of vanillin on TNBS-induced colitis (a chronic inflammatory disease) in BALB/c mice was observed in the mice daily given vanillin at an oral dose of 36.3 mg/kg/day for 3 days before TNBS treatment (15). The therapeutic effect of oral administered vanillin was also observed in the TNBS-treated mice daily given vanillin at the same dose for 7 days after TNBS treatment (15). Interestingly, by using transgenic mice carrying luciferase gene driven by NF-κB-responsive element for monitoring the *in vivo* NF-κB activity, bioluminescence imaging revealed decreased NF-κB-mediated bioluminescent signal in the colons of TNBS-treated mice after 7 consecutive days off treatment of vanillin (15). The results from the animal model indicate that oral administration of vanillin at 36.3 mg/kg/day for a period reduced the NF-κB-mediated response *in vivo*.

Pharmacokinetic study of vanillin over 24 hours, after a single oral administration of vanillin in rat at 100 mg/kg, showed two peaks at 0.25 and 4 hours after administration, suggesting its possible reabsorption *via* enterohepatic recirculation (17). The maximal plasma concentration of vanillin that was observed in the second peak (at 4 hours) was 290 ng/ml (~2 μM), which is far lower than the millimetre concentration ranges used to inhibit *in vitro* NF-κB activation in our study and other reports. However, the *in vivo* inhibitory effect of vanillin on NF-κB-mediated response was evidenced by repeated oral dose of 36.3 mg/kg/day for 3-7 days (15). Therefore, it is possible that vanillin, which is a lipophilic compound, may be stored in fat tissues and released over time (17), or accumulated at the sites of action.

In conclusion, vanillin is regarded as a safe compound for use in food and drugs by the Food and Drug Administration (FDA), as the reported oral LD50 in rats was 1.58-2.8 g/kg (18). Since vanillin also exhibits antimetastatic and antiangiogenic effects (9, 10, 16), it has potential as a lead compound for the development of less toxic anticancer agents possessing multiple therapeutic effects, in prevention of metastasis and angiogenesis, as well as in sensitizing cancer cells to TRAIL-induced apoptosis.

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