Effect of Cell Differentiation for Neuroblastoma by Vitamin K Analogs

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Background: Lack of receptor tyrosine kinase (TrkA), a high-affinity nerve growth factor (NGF) receptor, is closely associated with the malignant progression of neuroblastoma (NB) and its prognosis. Vitamin K3 (VK3) analogs inhibit the activity of protein tyrosine phosphatases (PTPases), which causes hydrolysis of the phosphate groups bound to the tyrosine residues on tyrosine kinase, resulting in sustained tyrosine phosphorylation.

Methods: In order to reverse this abnormal NGF/TrkA signal transduction in NB cells, we synthesized new VK3 analogs and examined their activity against NB cells.

Results: VK3 analogs increased or maintained the expression level of c-fos mRNA in the NB cells, which express the downstream genes of NGF/TrkA signal transduction. Moreover, the expression level of GAP-43 mRNA, which is a marker of neurite outgrowth and neuronal differentiation, was increased and morphological differentiation was also observed. VK3 analogs (especially COOH analog) continued to express c-fos and GAP-43 mRNAs and induced differentiation of NB cells after stimulation of NGF by strong inhibition of PTPase without affecting TrkA autophosphorylation.

Conclusions: Vitamin K3 analogs may have potential as clinical therapeutic agents for NB.

Key words: vitamin K3 – neuroblastoma – TrkA – PTPase – differentiation

INTRODUCTION

Neuroblastoma (NB) is the most common solid malignant tumor in the pediatric age group. Advanced NB is characterized by abnormal nerve growth factor (NGF)/high-affinity NGF receptor (TrkA) signal transduction, and this abnormal signaling is known to be significantly associated with the patient prognosis (1,2). In the early stages of NB (stage I, II and IVS), trkA mRNA is expressed at high levels, which is associated with a good prognosis. In contrast, the prognosis of NB patients showing low trkA mRNA expression levels is known to be very poor. Moreover, NB cells lacking the intracellular signal transduction system of NGF/TrkA, despite showing high levels of trkA expression, have been shown to be proliferative (3). By binding to TrkA, NGF normally autophosphorylates some tyrosine residues on the intracellular domain of TrkA, and through this phosphorylation signal information is transmitted inside the cell. The signal conveyed inside the cell is transmitted further to nuclear factors such as Ras, v-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1), mitogen-activated protein kinase kinase and mitogen-activated protein kinase (MAPK). The signal transduction is transmitted to a group of early genes (e.g. c-fos) and a group of late genes (e.g. GAP-43) present downstream of the NGF/TrkA signal transduction system (4), which influences various biological reactions such as cell differentiation and apoptosis (5,6).

Protein tyrosine phosphatases (PTPases) are enzymes that hydrolyze the phosphate groups from autophosphorylated tyrosine residues. Vitamin K3 (VK3; menadione) analogs with a thioether group have been shown to have the ability to inhibit this PTPase activity, resulting in sustained phosphorylation (7–9). Thus, we conducted this study to examine whether VK3 analogs might inhibit PTPase activity without affecting TrkA autophosphorylation, and because we had some good results to lead to differentiated cells from...
NB cells, we report that these agents may be useful for the treatment of NB patients.

**PATIENTS AND METHODS**

**REAGENTS**

The reagents used in this experiment were vitamin K1 (VK1) (Wako, Osaka, Japan) and VK3 (menadione) (Wako). Thioether analogs of VK3 were synthesized by using known methods (10) (Fig. 1).

**CULTURES**

A human NB cell line (IMR-32) was obtained from the RIKEN cell bank and used at 5.0 × 10^6 cells/dish (35 × 10 mm), in this experiment. This cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 7.5% (W/V) sodium bicarbonate under 5% CO2 at 37°C.

**EXTRACTION OF TOTAL RNA**

Total RNA was extracted with RNAiso (Takara Bio, Shiga, Japan) in accordance with the manufacturer’s instructions.

**CORRECTED RNA QUANTIFICATION AND REVERSE TRANSCRIPTASE REACTION**

Total RNA was extracted and corrected using the Agilent RNA6000 Nano LabChip® (Agilent Technologies, CA, USA) and quantified with the Agilent 2100 Bioanalyzer® (Agilent Technologies). The quantified and corrected total RNA (0.8 μg) was subjected to reverse transcriptase (RT)–PCR using the RNA PCR Kit® (AMV) ver. 3.0 (Takara), to synthesize cDNA.

**QUANTITATIVE PCR**

cDNA synthesized by the RT reaction was applied to real-time PCR [stage 1: 95°C × 10 s (1 cycle), stage 2: 95°C × 5 s, 62°C × 20 s (45 cycle) and stage 3: melt curve] by SYBR Premix EX Taq® (Takara). The primer sequences were as follows: c-fos (sense: 5’-CCC TCA GTG GAA CCT GTC AA-3’, antisense: 5’-GAT GAT GCT GGG AAC AGG AA-3’, its gene product is 83 bp); GAP-43 (sense: 5’-TTG GTG TTG TTA TGGCAA G-3’, antisense: 5’-GAG GAA AGT GGA CTC CCA CAG-3’, its gene product is 120 bp). Synthesis of the primers was entrusted to TaKaRa (Takara). We used human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) as the internal marker.

**DETERMINATION OF THE MOST EFFECTIVE CONCENTRATION**

VK1, VK3 and VK3 analogs were added to obtain a final concentration of 1.0 × 10^-14–1.0 × 10^-6 M with dimethyl sulfide and allowed to react for 2 h. Only NGF 7s (Roche Diagnostics, Mannheim, Germany) was added at 100 ng/μl, and the reaction was allowed to continue for 0.5 h. The c-fos mRNA expression levels at each concentration and in the presence of only NGF were compared with the level in the control sample contained neither VK3 analogs nor NGF (Fig. 2).

**DETERMINATION OF THE MOST EFFECTIVE REACTION TIME**

IMR-32 cells (5.0 × 10^6 cells/60 mm dish) were added with VK1, VK3 and VK3 analogs at the maximum effective concentration (shown in underlines in each graph in Fig. 2) and treated for each 0.75, 1.5, 4, 8, 18 or 24 h. NGF was also treated for each 0, 0.5, 0.75, 2, 4, 8 or 12 h. We determined the most effective reaction time that yielded the highest level of c-fos mRNA expression. Controls contained neither VK3 analogs nor NGF (Fig. 3).
Figure 2. Expression of c-fos mRNA for each concentration of VK1, VK3 and VK3 analogs. IMR-32 cells (5.0 × 10⁶ cells/60 mm dish) were treated with some concentrations (1 × 10⁻¹⁴–1 × 10⁻⁶ M) of VK1, VK3 and VK3 analogs for 2 h and analyzed the amount of c-fos mRNA at each concentration (n = 3). By comparing with it and control (containing neither VK3 analogs nor NGF) accounted P value (*P < 0.05, **P < 0.01 and ***P < 0.001). NGF, nerve growth factor.
Determination of the Duration Time with VK3 Analogs and NGF

After IMR-32 cells (5.0 × 10^6 cells/60 mm dish) were incubated with VK3 analogs (OH, OCH3, MOM, COOH, and diol) at each maximum effective concentration and NGF, the mixture was allowed to react for 0, 0.5, 1, 2, 4, 8 or 15 h and the level of c-fos mRNA expression for each time duration was analyzed. Controls contained neither VK3 analogs nor NGF (Fig. 4).
Morphological Differentiation and Neurite Outgrowth

Both of VK3 analogs (OH, OCH3, MOM, COOH and diol) at the maximum effective concentration with the maximum level of c-fos mRNA expression and NGF were added at the same time and incubated for 48 h at 37°C in 5% CO2. After 48 h, the IMR-32 cells were observed at random in selected fields under a microscope (×20, Fig. 5A). The proportion of cells in which the length of neurite outgrowth was 2 or more times when compared with that of the cell body relative to the total number of cells calculated (Fig. 5B); this served as the indicator of neurite outgrowth (11).

GAP-43 mRNA Expression

Each of the VK3 analogs (OH, COOH and diol) and NGF were added to the IMR-32 cells for the same time durations and incubated for 0, 1, 2, 4, 6, 15 or 26 h. The expression level of GAP-43 mRNA as an indicator of neurite differentiation was quantified (Fig. 6).

Inhibition of T-Cell Protein Tyrosine Phosphatase Activity

T-cell protein tyrosine phosphatase (TC-PTPase) activity was evaluated using the Cyclrex® TC-PTPase Fluorometric Assay Kit (Cyclex, Nagano, Japan). First, 10× PTP assay buffer and 10× fluoro-phospho-substrate were added to the microtiter plate wells. Then, the VK3 analogs were added and mixed well. The reactions were initiated by adding recombinant TC-PTPase and mixing thoroughly at room temperature for 15 min. Development buffer and development reagent were then added to each well of the microtiter plate and mixed thoroughly. Finally, stop solution was added.

Figure 4. Expression of c-fos mRNA for duration time by using VK3 analogs and NGF. The duration time was examined by both of each high maximum effective concentration of VK3 analogs (shown in underline in each graph in Fig. 2) and NGF. IMR-32 cells were incubated for 0, 0.5, 0.75, 2, 4, 6 and 15 h (n = 3). We analyzed amount of c-fos mRNA for each sample. By comparing with it of each VK3 analogs for 0.5 h and control for 0.5 h accounted P value (**P < 0.01, ***P < 0.001).
to each well of the microtiter plate and mixed thoroughly. A fluorescence plate reader (emission 510–530 nm, excitation 482–502 nm) was used for the detection. The inhibition rate of sodium orthovandate was used as the control.

**TRKA Kinase Activity**

TrkA kinase activity was evaluated using the Cyclrex® TrkA Kinase Assay Screening Kit (Cyclrex). Microplate wells were coated with recombinant ‘Tyrosine kinase

![Figure 5. Morphological differentiation of IMR-32 cells at 48 h in the presence of each VK3 analog and NGF. Morphological differentiation of IMR-32 cells at 48 h in the presence of VK3 analogs and NGF [×20, A(d–h)]. Control did not contain any VK3 analog or NGF. Dimethyl sulfoxide (DMSO) was only DMSO, and only NGF was it without VK3 analogs [×20, A(a–c)]. Neurite outgrowth was quantitated by comparing the neurite length and the somal diameter. The neurite index was calculated as the ratio of the number of cells with neurites (longer than 2× somal diameter) to those without neurites (n = 3, B). By comparing with the neurite index of each VK3 analog with NGF and DMSO accounted P value (*p < 0.05, **p < 0.01).
substrate-1’. The kinase assay buffer containing VK3 analogs in duplicate was prepared. Then, 0.1 U/ml of TrkA-positive control was added to the wells of the assay plate on ice. Kinase reaction buffer was added to each well and incubated at 30°C for 30 min. All the wells were washed and horseradish peroxidase-conjugated detection anti-phosphotyrosine monoclonal antibody (PY-39) was added to each well, followed by incubation at room temperature for 60 min. All the wells were washed followed by the addition of substrate to each well and incubation at room temperature for 15 min. Finally, stop solution was added to each well. The absorbance of each well was measured using a spectrophotometric plate reader at the wavelengths of 450/595 nm. TrkA-positive control® was used as the control.

STATISTICAL ANALYSIS
All the statistical analyses were performed by using GraphPad Prism 4 (Graphpad, CA, USA). Data were expressed as standard error of mean. Mean values of continuous variables were compared using analysis of variance and did Bonferroni’s comparison was performed after logarithmic transformation of the data. The significance level was set at ≤0.05 (*P < 0.05, **P < 0.01 and ***P < 0.001).

RESULTS AND DISCUSSION
Abnormal NGF/TrkA signal transduction has been significantly associated with a poor prognosis in the cases of NB (12,13). VK1 exhibited cytotoxicity and VK3 exerted DNA damage in normal cells. VK3 analogs with a thioether group have been reported to inhibit PTPase activity to cause hydrolysis of phosphate groups in autophosphorylated tyrosine residues on the epidermal growth factor (EGF) receptor, producing sustained phosphorylation (8,9). It has also been reported that signals transmitted to MAPK from TrkA are normal in NB cells expressing trkA (14,15). Moreover, the NGF/TrkA signal transduction also resulted in induction of early genes (e.g. c-fos) and late genes (e.g. GAP-43) in the nucleus.

To determine the maximum effective concentrations of the VK1, VK3 and VK3 analogs, we analyzed the expression levels of c-fos mRNA at 2 h after the addition of the analogs at concentrations of 1.0 × 10^{-14}–1.0 × 10^{-6} M. Significant increase in the c-fos mRNA expression was noted in the presence of VK1, VK3 and all of the eight synthesized types of VK3 analogs (VK3 oxide, OH, OCH3, MOM, COOH, diol, acetal and THP) when compared with that in the control containing neither VK3 analogs nor NGF. Maximum c-fos expression was detected in the presence of 1.0 × 10^{-10} M for OH, 1.0 × 10^{-6} M for OCH3, 1.0 × 10^{-12} M for MOM, 1.0 × 10^{-10} M for COOH, 1.0 × 10^{-12} M for diol, 1.0 × 10^{-10} M for acetal and 1.0 × 10^{-10} M for THP, and these maximum effective concentrations were very low. In addition, seven of the VK3 analogs with a thioether group, except for VK3 oxide, induced a higher amount of c-fos mRNA expression than only NGF (Fig. 2E–K). The expression levels of c-fos mRNA at the maximum effective concentration at each reaction time are shown in Fig. 3. For VK1, VK3 and seven of the VK3 analogs except THP, the maximum peak was obtained at 4 h after the addition of each compound. Moreover, the expression peaks obtained following the addition of OH, OCH3, MOM, diol and COOH were statistically more significant than the peak obtained with the addition of only NGF (Fig. 3E–J). Therefore, in this preliminary experiment, we found that these five types of VK3 analogs (OH, OCH3, MOM, diol and COOH) were effective even at concentrations of <1.0 × 10^{-6} M.
For the requirement of more higher expression of c-fos mRNA, we examined the experiment by administration with both of VK3 analogs and NGF. Each of the five VK3 analogs at the maximum effective concentration was added with NGF, and the expression levels of c-fos mRNA peaked at 0.5–1 h and the levels observed following the addition of OH, OCH3 and MOM were gradually falling with time (Fig. 4B–D). In contrast, the expression level of c-fos mRNA continued to increase even after 0.5 h for COOH and diol (Fig. 4E–F). The finding that the expression of c-fos continued after the addition of COOH or diol with NGF may indicate that the signal between NGF/TrkA and c-fos mRNA expression still continued in NB cells. Normal NGF/TrkA signal transduction is required in NB cells to induce neuronal differentiation from undifferentiated cells (16).

The next question that needed to be answered was whether VK3 analogs might also induce the morphological differentiation of NB cells. The morphological changes in neural outgrowth, as assessed by method of Tanaka et al. (11), are shown in Fig. 5. Figure 5A shows the photographs obtained at 48 h after the addition of five of the VK3 analogs (OH, OCH3, MOM, diol and COOH) along with NGF. Figure 5B shows the proportion of cells showing neurite outgrowth of 2 or more times the length of the cell body. This result indicates that OH, OCH3, diol and COOH are very effective at inducing neural outgrowth. These results suggest that VK3 analogs induce c-fos gene expression and induce NB cell differentiation.

We conducted further investigation of the diol and COOH that effectively induced c-fos mRNA expression and morphological cell differentiation. Because OH was effective at activating the EGF receptor, we used it for comparison with diol and COOH (9,17).

In regard to the third question, we examined whether NB cell differentiation, as shown in Fig. 5, induced by the NGF/TrkA intracellular signal transduction system also increased the expression level of GAP-43 mRNA as an indicator of axonal differentiation. GAP-43 is known to be one of the late genes and to be involved in axonal outgrowth and synapse formation (4). Both of OH and diol in the presence of NGF induced the maximum expression level of GAP-43 at 4 h after addition. After 4 h, the expression level decreased gradually to the baseline. However, with COOH and NGF, the expression level continued to increase or was maintained for >4 h after addition. Therefore, addition of OH or diol resulted in a temporary increase in the expression of GAP-43 mRNA, and adding of COOH produced a more persistent increase (Fig. 6).

Moreover, to determine whether or not these analogs inhibit PTPases, we used TC-PTPase. TC-PTPase regulates tyrosine phosphorylation on receptor (TrkA) and transmits signals downstream, and it also activates neurite growth signals (7,8). Diol, COOH and OH were observed to exert inhibitory activity against TC-PTPase (OH 85.3%, diol 95.2% and COOH 49.1%). Especially, COOH exerted strong inhibitory activity against TC-PTPase (Fig. 7).

In addition, we examined the effects of VK3 analogs, by ELISA, on the auto-phosphorylation of TrkA as a tyrosine kinase. Measurement of the kinase activity indicated that the kinase activity induced by COOH was 80.3% when compared with that in the samples without the VK3 analogs, 68.2% for diol and 55.3% for OH. COOH had little effect on the kinase activity in regard to TrkA phosphorylation. However, addition of OH or diol inhibited TrkA kinase activity (Fig. 8).

These results suggest that COOH, one of the VK3 analogs, inhibits PTPase activity with little inhibitory activity on TrkA kinase activity. Therefore, the induction of intracellular NGF/TrkA signal transduction by VK3 analogs and NGF may affect the downstream molecules and regulate the expression of c-fos mRNA as an early gene. This may result in the induction of GAP-43 as a late gene and the differentiation of undifferentiated NB cells.

OH inhibited both PTPase and TrkA kinase activity. Therefore, the signal to c-fos mRNA expression was not transmitted adequately downstream, resulting in only transient expression of GAP-43 mRNA. Diol weakly inhibited TrkA kinase activity, but scarcely inhibited TC-PTPase activity. However, the expression level of c-fos mRNA was maintained, which led to transient expression of GAP-43 mRNA. We think that because of these reactions for PTPase and TrkA kinase action.

**Figure 7.** TC-PTPase inhibitory effect of VK3 analogs. The TC-PTPase inhibitory effect of COOH, diol and OH was examined (n = 3). Statistical comparison of the effect between VK3 analogs and control was conducted by two-way ANOVA (*P < 0.05, ***P < 0.001). TC-PTPase, T-cell protein tyrosine phosphatase; ANOVA, analysis of variance.

**Figure 8.** Effect of the VK3 analogs on the TrkA kinase activity. The effect of COOH, diol and OH on the TrkA kinase activity was examined (n = 3). Statistical comparison of the effect between the VK3 analogs and control was conducted by two-way ANOVA (*P < 0.05, ***P < 0.001). ANOVA, analysis of variance.
activity, diol may lead to maintained c-fos expression and transient GAP-43 expression after stimulating by NGF. In contrast, COOH inhibited PTPase activity but not TrkA kinase activity, and after stimulating by NGF, the duration of c-fos mRNA expression caused and its signal fully transmitted the downstream. Thus, sustained GAP-43 mRNA expression was noted, resulting in morphological differentiation of the NB cells (18).

From the above results, the duration of the effective sustained c-fos mRNA and GAP-43 mRNA expression is induced by both inhibition of TC-PTPase and stimulation of NGF, which can lead to differentiation of NB cells.

Moreover, it has been reported that VK3 analogs inhibit PTPase activity, because the SH group in PTPase forms a disulfide bond with the S group at the 2-position of the VK3 analogs (7). As it is at the center of the enzyme activity within the SH group of cysteine, competition between the phosphate group and VK3 analogs at this position is one of the most potent mechanisms of inhibition. It has been another report for VK3 analogs that inhibit tumor cell growth that the presence of thioether or O-ether on the side chain of quinone and the length of its side chain are very crucial (10). COOH with a carboxyl group may provide an appropriate side chain for this reason. Thus, we think that the VK3 analog with a COOH group strongly inhibits TC-PTPase activity without affecting TrkA autophosphorylation; this leads to sustained c-fos mRNA expression as an early gene and GAP-43 as a late gene by NGF stimulation, and differentiation of undifferentiated NB cells. Therefore, compounds targeting PTPases, especially VK3 analog with a COOH group, may be important therapeutic drugs against NB.

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Conflict of interest statement

None declared.

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