The Utility of Vitamin K3 (Menadione) against Pancreatic Cancer

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Abstract. Background: To evaluate the efficacy of vitamin K3 (VK3) against pancreatic cancer, the molecular mechanism of VK3 or gemcitabine (GEM)-induced inhibition of proliferation was characterized. Materials and Methods: The cell viability was determined using the 3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium bromide (MTT) test method. The expressions of cellular proteins were evaluated by Western blot analysis. For morphological studies of the in vivo transplanted cancer cells, the tissues were stained with hematoxylin and eosin. Results: The IC50 of VK3 for pancreatic cancer cells was calculated for 42.1±3.5 μM. Western blot analysis showed that VK3 induced rapid phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) 30 minutes after application. ERK but not JNK phosphorylation was maintained for at least 12 hours. Activation of apoptosis by VK3, as shown by molecular weight shifts of the pro-activated 32-kDa form of caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage of the 112-kDa form, was found. Treatment with the thiol antioxidant, L-cysteine (>0.2 mM), completely abrogated the VK3-induced phosphorylation of ERK, but not the JNK, and inhibition of proliferation. A caspase-3 inhibitor antagonized caspase-3 activation, but had no inhibitory effect on the proliferative activity of VK3, as shown by molecular weight shifts of the pro-activated 32-kDa form of caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage of the 112-kDa form, was found. Treatment with the thiol antioxidant, L-cysteine (>0.2 mM), completely abrogated the VK3-induced phosphorylation of ERK, but not the JNK, and inhibition of proliferation. A caspase-3 inhibitor antagonized caspase-3 activation, but had no inhibitory effect on the proliferative activity of VK3. GEM at concentrations >0.1 μg/ml was found to inhibit cell proliferation after 24 hours. GEM also induced phosphorylation of JNK, activation of caspase-3 and accumulation of cyclin B1. Local application of VK3 was found to induce extensive tumor tissue necrosis, but slight hematemesis without necrosis was observed 48 hours after GEM injection. In Western blot, ERK, but not JNK phosphorylation, was clearly detected in response to VK3 injection into the tumor tissue.

Conclusion: The action of VK3 may lead to a favorable outcome against pancreatic cancer, and the detection of ERK phosphorylation in the tissue is important for predicting this effect.

Despite recent progress in diagnostic and therapeutic modalities, pancreatic carcinomas are usually detected at an advanced stage, with a low resectability rate of approximately 30% (1). Chemotherapy can provide symptom relief for some patients, but its impact on survival has been modest and it can lead to unacceptable levels of toxicity. To decrease unfavorable reactions to chemotherapeutic drugs, local delivery of drugs might be useful (2). In this regard, a new technique, endoscopic ultrasound-guided fine-needle injection, may be used. The technique involves the injection of antitumor agents directly into localized carcinomas (3). But, no obvious association between commonly used antitumor agents and patient survival has yet been detected (2). Vitamin K3 (VK3, menadione) has been reported to inhibit proliferation of several cancer cell lines, even some resistant to standard chemotherapeutic agents (4-6), such as pancreatic cancer cell lines (7). VK3 has been found to show unique mechanisms by activating cellular signal factors. In the present study, the efficacy of local VK3 injection therapy against pancreatic cancer was evaluated and compared with the standard chemotherapeutic reagent gemcitabine (GEM, Gemzar; Eli Lilly and Company, Indianapolis, IN, USA) (8). GEM has been approved for the treatment of advanced pancreatic cancer on the basis of improved clinical benefit and increased overall survival (9). In addition, the molecular mechanism of VK3 or GEM-induced inhibition of proliferation was characterized.

Materials and Methods

Cell lines. The rat pancreatic cancer cell line ARIP (American Type Culture Collection, Manassas, VA, USA) was grown in F12K medium (MP Biomedicals Inc.). The medium was supplemented with 10% fetal calf serum, penicillin, streptomycin, L-glutamine, and fungizone. The cells were harvested after trypsin-EDTA treatment, washed with Dulbecco's PBS, and re-suspended in serum-less medium for 24 hours.
Determination of cell viability. The cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test method. MTT (5 mg/ml) was dissolved in PBS and the solution was stored at 2–8°C for frequent use after filtration though a 0.2 µm filter. To determine the effects of VK3 or GEM on cell growth inhibition, the cells were treated with several concentrations of VK3 (from 0 to 100 µM), ethanol or GEM (from 0 to 10 µg/ml) for 24 hours. For the determination of cell viability, the medium was discarded and MTT solution was added, followed by incubation for 3 hours. At the end of the incubation period the MTT solution was removed and the cells’ dye crystals were dissolved by adding dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm in a spectrophotometer and the results were expressed as a percentage of the absorbance of the samples in comparison with the control (without VK3 or GEM), as described previously (10).

Western blot analysis. The cell lysate was isolated and homogenized in radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L Tris-HCl (pH 7.5) 150 mmol/L NaCl; 5 mmol/L EDTA; 0.5% SDS; 1.0% NP40; and 1.0% sodium deoxycholate-containing protease inhibitors) as described previously (10, 11). The homogenate was incubated on ice for 30 minutes and centrifuged for 15 minutes at 14,000 rpm at 4°C. The supernatant was recovered and kept at –80°C. Protein determination was performed with the Bradford assay (Bio-Rad Laboratories), and equal amounts of cell tissue lysate (40 µg for each lane) were mixed with SDS-PAGE sample buffer and boiled for 5 minutes before loading onto a discontinuous SDS-PAGE gel. After electrophoresis, the gel was incubated in 3-cyclohexylamino-1-propane-sulfonic acid (CAPS) buffer (10 mmol/L 3-cyclohexylamino-1-propane-sulfonic acid (pH 11.0) 10% methanol) for 5 minutes, and proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) in CAPS buffer. The blot was blocked by incubating in 5% non-fat dried milk/Tris-buffered saline (TBS)-Tween for 1 hour at room temperature and then incubated for 1 hour with the first antibody for phospho-tyrosine (Cell Signaling, Beverly, MA, USA; 1:2,000), phospho-extracellular signal-regulated kinase (phospho-ERK) (Calbiochem, La Jolla, CA, USA; 1:1,000) or phospho-c-Jun NH2-terminal kinase (phospho-JNK), p38, caspase-3 and poly(ADP-ribose)polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000) in 5% non-fat dried milk/TBS-Tween at room temperature. The membrane was washed 3 times for 20 minutes in TBS-Tween and additionally incubated with a secondary antibody mouse (Sigma, St. Louis, MO, USA; 1:1,000) for phospho-JNK and phospho-tyrosine and rabbit antibody (Sigma; 1:500) for phospho-ERK immunoglobulin conjugated with horseradish peroxidase in 5% non-fat dried milk/TBS-Tween for 1 hour at room temperature. After washing 3 times for 30 minutes in TBS-Tween at room temperature, the signal was detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). The loading of protein was normalized by reprobing with a β-actin polyclonal antibody.

Morphological studies. All procedures with animals were conducted according to the criteria approved by the Institutional Animal Care and Use Committees at the Gifu University School. Wister rats were housed in individual cages and fed a standard laboratory chow. Under general anesthesia with Nembutal, 1.0x10⁶ pancreatic cancer cells were transplanted into the spleen in order to easily demonstrate the effects of the chemotherapeutic agents. Three days later, when the transplanted cancer cells were completely established, in a maximum treatment volume of 10 µl (to avoid any problems such as back flow or bursting) reagents (VK3, 100 µM and GEM, 200 µg) in ethanol or ethanol alone as control were injected. After 1 hour, the spleen was removed from sacrificed animal, frozen in liquid nitrogen as soon as possible and crushed in 1 ml RIPA buffer for Western blot analysis (11). For the purpose of microscopic study, the animals were sacrificed 48 hours after the injection of the test reagents, the spleen was resected, processed and embedded in paraffin. Six micrometer thick sections were cut and the tissues were stained with hematoxylin and eosin (H&E). Five individual rats were used for each group.

Statistical analysis. At least four independent determinations of each parameter were compared to the control using the Student’s t-test. Differences were considered significant when p<0.05 was obtained.

Results

Vitamin K3-induced inhibition of proliferation. Following the 24-hour VK3 treatment, the IC₅₀ of the ARIP cells was calculated to be 42.1±3.5 µM (Figure 1). The vehicle (ethanol) alone had no effect on cell proliferation (data not shown). Western blot analysis showed that VK3 induced rapid phosphorylation of ERK and JNK within 30 minutes of application (Figure 2A). JNK was phosphorylated in response to treatment with 50 µM VK3 but the response diminished after 60 minutes. In contrast, ERK
phosphorylation was maintained over 60 minutes and lasted for at least 12 hours (data not shown). Phosphorylation of another mitogen-activated protein kinase (MAPK) family member, p38, was not detected in response to treatment with VK3 (Figure 2A). In response to 100 µM VK3, ERK phosphorylation was observed after 10 minutes, and tyrosine phosphorylation was observed after 60 minutes (Figure 2B). The activation of apoptosis, as shown by caspase-3 activation (molecular weight shifts pro-activated 32-kDa form) and PARP cleavage of the 112-kDa form within 6 hours of treatment with 50 µM VK3 is shown in Figure 2C. Pretreatment with the thiol antioxidant L-cysteine (>0.2 mM) completely abrogated the VK3-induced ERK, but not JNK phosphorylation and diminished the VK3-induced inhibition of proliferation (Figure 3A). A non-thiol antioxidant, catalase, antagonized JNK phosphorylation but did not affect the inhibition of cell proliferation. A caspase-3 inhibitor, Asp-Glu-Val-Asp-chloromethylketone (DEVD-CMK), antagonized caspase-3 activation, but had no inhibitory effect on the anti-proliferative activity of VK3 (Figure 3B).

**GEM-induced inhibition of proliferation.** GEM at concentrations >0.1 µg/ml was found to inhibit cell proliferation at 24 hours, and significant inhibition was observed at 48 and 72 hours (Table I). GEM also induced phosphorylation of JNK but phosphorylation of other MAPKs was not observed (Figure 4A). Phosphorylation of JNK was completely inhibited by the presence of catalase (data not shown). Caspase-3 was activated by exposure to >1.0 µg/ml of GEM for 24 hours (Figure 4B). In addition, accumulation of cyclin B1 was detected after 24 hours (Figure 4C).

**In vivo experiments.** On the basis of the anti-proliferative effect of VK3 and GEM in vitro, the localized application of these agents for cancer therapy was evaluated in vivo. VK3 and the ethanol vehicle were found to induce extensive tumor tissue necrosis. In response to VK3 rather than ethanol, a large necrotic area with severe hematemesis was
noted. However, slight hematemesis without necrosis was observed 48 hours after GEM injection (Figure 5). By Western blot, ERK phosphorylation was clearly detected only in response to VK3 injection into the tumor tissue (Figure 6). JNK phosphorylation was not induced by VK3 or GEM treatment. The total protein volumes of ERK and JNK in each sample were similar.

Discussion

Recent studies have shown the involvement of two pathways of GEM toxicity, cell cycle arrest (12) and apoptosis (13). Caspase activation via the MAPKs, JNK or p38 (14), depending on the cell type, has been suggested to be important in GEM treatment. In the ARIP pancreatic cancer cells in the present study, the activation of JNK and caspase-3 was observed in response to >0.1 µg/ml GEM (Figure 4), which also inhibited cell proliferation (Table I). Signal transduction pathways that modulate cell death are known to involve induction of reactive oxygen species (ROS) (15, 16). Because GEM is a strong inducer of ROS compared to other chemotherapeutic agents (17), GEM-induced JNK phosphorylation has been suggested to be closely related to ROS induction. Many enzymatic or chemical antioxidants have been reported to abrogate apoptosis (18). Thus, JNK phosphorylation may be critical for the cellular response to GEM resulting in inhibition of proliferation.

Protein tyrosine phosphorylation is regulated by a balance between action of protein tyrosine phosphatase (PTPase) and protein tyrosine kinase (PTK) activities. Ligand-mediated phosphorylation, such as that induced by growth factors, is well-known to activate PTK activity. Inhibition of PTPases also leads to protein tyrosine phosphorylation (19) by binding the active site of PTPases (20). VK3 has been suggested to inhibit PTPases resulting in the phosphorylation of cellular proteins, which then occurs in the absence of ligand binding proteins (4). In our time-course experiment (Figure 2B), ERK phosphorylation was detected earlier than the tyrosine phosphorylation of other proteins. Thus, ERK phosphorylation may be induced by a direct inhibitory effect of VK3 on PTPase activity.

Indeed, VK3 has been found to mediate ERK phosphorylation, but not the phosphorylation of upstream MAPK kinases (4, 5). Other mechanisms of ROS induction should also be considered because VK3 increases intracellular levels of ROS, and ROS themselves inhibit PTPase activity (21). In the present study, VK3-induced ERK phosphorylation was completely inhibited by the thiol antioxidant cysteine but not by the non-thiol antioxidant catalase (Figure 3A) suggesting that sulfhydryl arylation (20) is more critical for ERK phosphorylation than oxidative stress. In contrast, JNK phosphorylation by VK3 was induced by oxidative stress but not by sulfhydryl arylation. Arylation in relation to ERK phosphorylation appeared to be more critical than oxidative stress and JNK activation in the VK3-induced inhibition of proliferation. There is evidence that ERK plays an anti-apoptotic role and that JNK plays a pro-apoptotic role (22), which is inconsistent with the present study. However, recent reports have shown that ERK phosphorylation promoted apoptosis or cell-cycle arrest, inhibiting proliferation (23). Indeed, VK3 induced an intermediate type of phosphorylated ERK (5, 23), which inhibited proliferation (4, 6), supporting our results.

Table I. Gemcitabine-induced growth inhibition of pancreatic cancer cells.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Gemcitabine (µg/ml)</th>
<th>0</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
<th>10</th>
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<tr>
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<td>99.4±1.3</td>
<td>88.1±4.8</td>
<td>82.3±8.2</td>
<td>86.2±7.5</td>
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<tr>
<td>48</td>
<td>100</td>
<td>101.2±1.2</td>
<td>97.6±1.8</td>
<td>78.2±6.6**</td>
<td>77.4±4.4**</td>
<td>58.1±4.7**</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>100</td>
<td>94.3±3.6</td>
<td>96.3±5.4</td>
<td>61.2±4.3**</td>
<td>56.2±5.4**</td>
<td>51.2±3.6**</td>
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Means±S.D. calculated for percentage of control, **means p<0.01.
Surgical resection of the primary tumor remains the only potentially curative treatment for pancreatic cancer (24), and preoperative therapy has demonstrated the ability to convert locally unresectable pancreatic cancer to a respectable disease (25, 26), thereby increasing the number of patients potentially cured. The use of VK3 with a local drug delivery system may represent a favorable strategy in the curative treatment of unresectable pancreatic tumors in the near future.

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References


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