The novel selenium heteropoly compound $(NH_4)_4H_4[Se_2Mo_2V_4O_{24}]\cdot 7H_2O$ induces apoptosis of K562 cells

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Abstract. The purpose of this study was to investigate the antitumor effects and mechanism of the selenium heteropoly compound $(NH_4)_4H_4[Se_2Mo_2V_4O_{24}]\cdot 7H_2O$ (SeMoV) in K562 cells. The results showed that 0.313-10 mg/l SeMoV significantly inhibited the proliferation of K562 cells *in vitro* in a time- and concentration-dependent manner as determined by a microculture tetrazolium assay; the IC_{50} values were 7.69 and 4.06 mg/l following 48 and 72 h of treatment with SeMoV, respectively. Analysis of the cell cycle indicated that the proportion of cells in the G0/G1 phase was decreased at 48 h whereas the proportion of cells in the S phase was increased following treatment for 24 and 48 h. A significant sub-G1 peak was observed at 5 mg/l for 24 h. Morphological observation revealed typical apoptotic features. SeMoV significantly caused the accumulation of Ca^{2+}, Mg^{2+} and ROS, and a reduction in the pH value and the mitochondrial membrane potential (MMP) in the K562 cells compared with the control ($p<0.01$), as shown by confocal laser scanning microscopy. Experiments also showed that the expression of Bcl-2 was significantly inhibited by 20 mg/l SeMoV, while Bax expression increased. Meanwhile, the amount of cytochrome C and IκB in K562 cells was increased, while NF-κB expression was significantly decreased, following treatment with SeMoV for 24 h. The experiment implied that SeMoV had antitumor activity and its mechanism was attributed partially to apoptosis, which was induced by the elevation of the intracellular Ca^{2+}, Mg^{2+} and ROS concentration, a reduction in the pH value and MMP, and the NF-κB/IκB signaling pathway.

Introduction

Selenium is an essential dietary component and has been used as an anticarcinogenic or preventive chemical against tumors (1,2). It has been known for a number of years that populations with a low selenium intake and low plasma selenium levels have an increased incidence of cancer (3-9). Therefore, investigators have focused their attention on searching for potent selenium compounds, which possess higher antitumor efficacy and lower tissue toxicity.

Heteropoly complexes represent a class of polyanionic compounds with a variety of significant biological activities including antiviral or antitumor effects (10,11) and lower tissue toxicity. Heteropoly complexes have attracted attention in view of their potential application as antiviral and antitumoral agents (12). Recently, certain evidence showed that heteropoly complexes were promising anti-fungal compounds (13,14) and candidates for Alzheimer's disease treatments (15). However, little has been reported for selenium heteropoly complexes. In order to obtain a selenium heteropoly complex that has higher antitumor efficacy and lower tissue toxicity, a series of compounds were synthesized (16) with Selenium, vanadium, molybdenum, butyl, amido and hydroxyl.

Earlier studies performed *in vitro* have suggested that induction of apoptosis and/or inhibition of cell growth accounts for the cancer prevention by selenium compounds (17-19). Evasion of apoptosis is one of the hallmarks of human cancer. Apoptosis evasion is responsible for tumor promotion and progression, as well as for treatment resistance (20). Investigators have reported that genetic mutations culminating in the disturbance of apoptosis or derangement of apoptosis-signaling pathways appear to be an essential factor of carcinogenesis (20,21), and induction of apoptosis in cancer cells is one of the most significant methods for cancer treatment (21). A number of anticancer agents have been reported to induce apoptosis of cancer cells, and apoptosis induced by a change in intracellular ion concentrations such as Ca^{2+}, Mg^{2+} and H^+ is one of the mechanisms of cytotoxicity (22-24). Therefore, the aim of this study was to test whether $(NH_4)4H_4[Se_2Mo_2V_4O_{24}]\cdot 7H_2O$ (SeMoV) (16), a novel selenium compound, has antitumor activity in K562 cells and to explore its mechanism of action.

Materials and methods

Drugs and chemicals. $(NH_4)_4H_4[Se_2Mo_2V_4O_{24}]\cdot 7H_2O$ brown crystal was kindly provided by the Laboratory of Chemistry, Lanzhou University (Lanzhou, China). MTT and SDS were purchased from Sigma (St. Louis, MO, USA). Fluo-3/AM,

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Mag-Fluo-4/AM, carboxy SNARF-1/AM, 2',7'-dichlorofluorescein diacetate and MitoTracker Green FM were purchased from Molecular Probes Co. (Eugene, OR, USA). Monoclonal antibodies to Bcl-2 and Bax and the Bio-Rad protein assay kit were purchased from Molecular Probes Co. Mouse anti-human cytochrome C, IκBα antibody, anti-rabbit IgG antibody and anti-goat IgG antibody were purchased from Sigma. Anti-mouse IgG antibody was purchased from Amersham. RPMI-1640 medium was obtained from Gibco BRL (Grand Island, NY, USA). Bovine serum was purchased from Hangzhou Sijiqing Biotechnology Co. (Hangzhou, China). Other chemicals were of analytical purity.

Cell cultures and the MTT assay in vitro. The K562 cell line was purchased from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). Cells were grown in complete RPMI-1640 medium containing 10% heat-inactivated bovine serum, 2 mmol L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2, and were routinely passaged every other day.

Cytotoxicity was measured by MTT assay (25). Briefly, exponentially growing cells were resuspended in complete RPMI-1640 medium to a concentration of $1x10^8$ cells/l. A total amount of 100 μl aliquots of cells containing 0.313-10 mg/l SeMoV were seeded in quadruplicate into a 96-well flat bottom microculture plate (Costar, Corning, Lowell, MA, USA) for the designated amount of time. At the end of the incubation period, MTT was added for the last 4 h, and then SDS was added and mixed thoroughly at 37˚C. Optical density was read on a microplate reader (Elx800, BioTek Instruments, Inc., USA) at 570 nm after agitating the plates for 5 min.

Flow cytometry. The percentage of K562 cells in each phase of the cell cycle was analyzed by flow cytometry. Following treatment with 0.625-5 mg/l SeMoV for the designated time, cells were fixed in ice-cold 70% ethanol at 4˚C for at least 24 h, and then stained with propidium iodide (PI) solution (containing PI 50 mg/l and RNase 50 mg/l, respectively) at room temperature in the dark for 30 min. The samples were read on a Coulter Epics XL flow cytometer (Beckman-Coulter Inc., Fullerton, CA, USA). The percentage of cells in the sub-G1, G0/G1, S and G2/M phase were calculated by Multicycle software (Phoenix Flow System, San Diego, CA, USA).

Morphological features of apoptosis

Inverted microscopy. K562 cells ($1x10^5$ cells/ml) were grown in complete medium containing 0.313-10 mg/l SeMoV for 24 h, then stained with right-Giemsa and observed under inverted microscopy.

Electron microscopy. K562 cells treated with 5 mg/l SeMoV for 24 h were prefixed in cacodylate-buffered glutaraldehyde (2%), post-fixed in 1% osmium tetraoxide, dehydrated in a graded series of alcohol and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate and examined with an EM-1230 electron microscope (Japan).

Confocal laser scanning microscopy assay. Following treatment as described above, K562 cells were washed twice in ice-cold phosphate-buffered saline (PBS), then loaded with Fluo-3/AM (5 μmol/l), Mag-fluo-4 (5 mmol/l), carboxy SNARF-1/AM (10 μmol/l), 2', 7'-dichlorofluorescein diacetate and MitoTracker Green FM (1.25 μmol/l), respectively, for 45 min at 37˚C, then washed twice in PBS. The fluorescence intensity changes of intracellular Ca^{2+}, Mg^{2+}, ROS, pH value and mitochondrial membrane potential (MMP) were measured by confocal laser scanning microscopy.

Immunohistochemistry assay. Following treatment with 20 mg/l SeMoV for 24 h, cells were washed in cold PBS three times, smeared, air dried and fixed, then processed according to the manufacturer's instructions and examined with a microscope.

Western blot assay. Cells were treated for 24 h with SeMoV and resuspended in lysis buffer. The protein concentration was determined by the Bradford assay (Pierce, Rockford, IL, USA). A total amount of 20 μg protein extract/lane was loaded onto SDS-PAGE (20 MA) and separated under denaturing conditions. Protein samples were then transferred onto nitrocellulose membranes (180 MA, 90 min) and the individual target protein was immunoreacted with the appropriate dilution of its primary antibodies (cytochrome C, NF-κB, IκB) at 4˚C overnight. The protein was then incubated with an appropriate dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2,000 dilution), anti-mouse IgG antibody (1:670 dilution) or anti-goat IgG antibody (1:1,000 dilution) as a secondary antibody. Enhanced chemiluminescence (ECL, Amersham) was used to reveal antibody binding.

Statistical analysis. The two-tailed Student's t-test was employed to assess the significance of the data. The data were presented as the mean ± SD. $P<0.05$ was considered to be statistically significant.

Results

Anti-proliferation activity of SeMoV in vitro. As shown in Table I, 0.313-10 mg/l SeMoV significantly inhibited the proliferation of K562 cells *in vitro*. Following a 24 h treatment, inhibition rates (IRs) were 13.18, 16.24, 17.91, 18.64, 20.36 and 40.66%, respectively. Following treatment with SeMoV for 48 and 72 h, proliferation of K562 cells was significantly inhibited in a time- and dose-dependent manner, with IRs of 13.29, 22.92, 26.79, 27.76, 37.35 and 63.85%, respectively, after a 48 h treatment, and 17.62, 17.71, 24.42, 28.09, 50.29 and 78.15%, respectively, after a 72 h treatment.

Effect of SeMoV on apoptosis and cell cycle progression of K562 cells. The cell cycle distribution was analyzed by flow cytometry in K562 cells treated with various concentrations of SeMoV for 24 and 48 h, as shown in Fig. 1. The proportion of cells in the S phase was increased with the 24 and 48 h treatment, while the proportion of cells in the G0/G1 phase was decreased with the 48 h treatment. A significant sub-G1 peak was noted at 5 mg/l for 24 h. This indicates that SeMoV may induce changes in the cell cycle distribution and apoptosis.

Morphological features of apoptosis. Typical apoptotic characteristics were present in the K562 cells treated with

Table I. Antitumor effect of SeMoV in K562 cells *in vitro* following 24, 48 and 72 h treatment.

Groups	Concentration (mg/l)	24 h (OD_{570})	48 h (OD_{570})	IC_{50} (mg/l)	72 h (OD_{570})	IC_{50} (mg/l)
Control	0	0.692±0.010	0.956±0.116	7.69 (2.34-25.26)	1.316±0.115	4.06 (1.95-8.47)
SeMoV	0.313	0.601±0.014[b]	0.824±0.042	7.69 (2.34-25.26)	1.081±0.039[a]	4.06 (1.95-8.47)
SeMoV	0.625	0.579±0.016[b]	0.730±0.079[a]	7.69 (2.34-25.26)	1.073±0.108	4.06 (1.95-8.47)
SeMoV	1.25	0.563±0.019[b]	0.693±0.049[a]	7.69 (2.34-25.26)	0.992±0.073[a]	4.06 (1.95-8.47)
SeMoV	2.5	0.568±0.027[b]	0.682±0.035[a]	7.69 (2.34-25.26)	0.942±0.006[b]	4.06 (1.95-8.47)
SeMoV	5	0.551±0.033[b]	0.593±0.006[b]	7.69 (2.34-25.26)	0.652±0.027[b]	4.06 (1.95-8.47)
SeMoV	10	0.411±0.025[b]	0.345±0.031[b]	7.69 (2.34-25.26)	0.288±0.035[b]	4.06 (1.95-8.47)

OD, optical density. An MTT assay was used to measure the cytotoxic effect of SeMoV in the K562 cells. Results were expressed as the mean ± SD of three experiments. [a]$p<0.05$, [b]$p<0.01$ vs. the control. The potency of the drug was determined by the IC_{50} (50% growth-inhibition concentration) value.

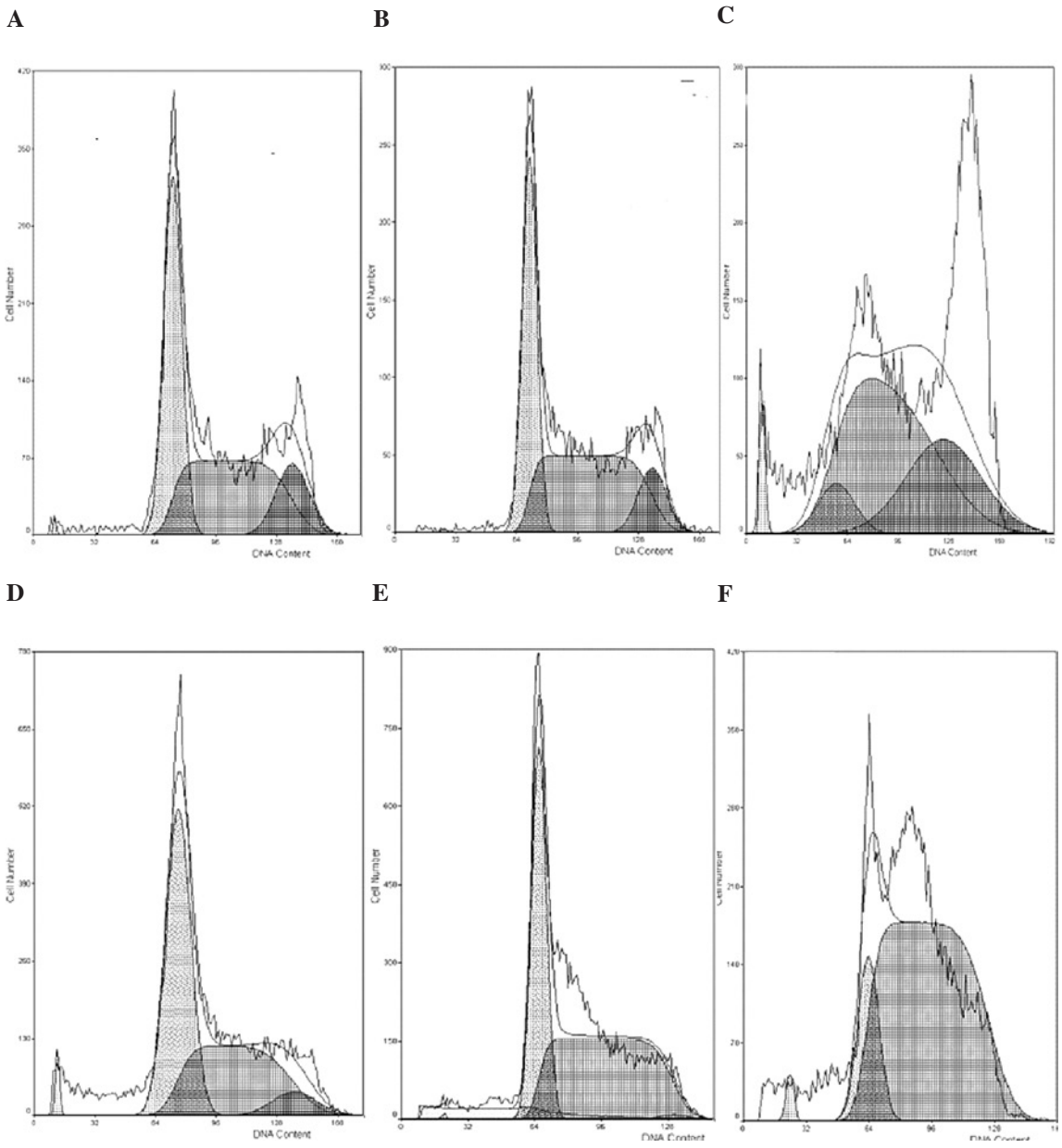

Figure 1. Apoptosis of the K562 cells treated for 24 and 48 h as determined by flow cytometry. (A and D) Control cells. (B and E) Cells treated with SeMoV 0.625 mg/l. (C and F) Cells treated with 5 mg/l. (A-C) Cells treated with SeMoV for 24 h. (D-F) Cells treated for 48 h. (A) The proportion of cells in the G0/G1, G2/M, S and sub-G1 phase is 37, 11.2, 51.8 and 0.4%, respectively; (B) 37.5, 12.2, 50.3 and 0.2%, respectively; (C) 8.2, 31.3, 60.5 and 3.6%, respectively; (D) 50.8, 7.0, 42.2 and 1.8%, respectively; (E) 39.2, 1.0, 59.8 and 0.2%, respectively; (F) 15.3, 0,48.7 and 1.6%, respectively.

0.313-10 mg/l SeMoV for 24 h. Nuclear condensation, chromosome fragmentation and apoptotic bodies were observed using an inverted microscope (data not shown). The electron microscopic observation also revealed typical apoptotic features, including shrinkage of cellular and nuclear membranes, condensed heterochromatin around the nuclear periphery and cytoplasmic vacuolation in the K562 cells treated with 5 mg/l SeMoV for 24 h (Fig. 2).

Effect of SeMoV on the intracellular Ca^{2+}, Mg^{2+} and ROS concentration, pH value and MMP. The changes in intracellular ion homeostasis may induce mitochondrial apoptosis and lower its membrane potential. The experiment showed that the fluorescence intensity of intracellular Ca^{2+}, Mg^{2+} and ROS was greatly increased following treatment with SeMoV as compared with the control group. However, the fluorescence intensity of intracellular pH value and MMP was markedly lowered as shown in Fig. 3.

Effect of SeMoV on the expression of Bcl-2 and Bax. The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic proteins. The expression of Bcl-2 and Bax were used as symbolic genes of apoptosis. The study showed that the expression of Bcl-2 was significantly inhibited, while the expression of Bax was increased by 20 mg/l SeMoV (data not shown).

Effect of SeMoV on the content of cytochrome C, NF-κB and IκB. Release of cytochrome C induced by perturbations of MMP may be one of the most conspicuous manifestations of apoptosis, and NF-κB is also closely related to apoptosis. Fig. 4 shows that the content of Cytochrome C and IκB in the K562 cells was increased and NF-κB expression was decreased markedly following treatment with various concentrations of SeMoV for 24 h.

Discussion

Our results clearly demonstrated that SeMoV significantly inhibited the proliferation of K562 cells *in vitro* in a concentration-dependent manner. Cell cycle analysis showed that SeMoV decreased the proportion of cells in the G0/G1 phase, and increased S phase cells following treatment for 24 and 48 h. A significant sub-G1 peak was also observed at high concentrations of SeMoV for 24 h, which was similar to the report that a number of selenium compounds are capable a arresting cells in either phase (26). SeMoV also induced typical apoptotic features.

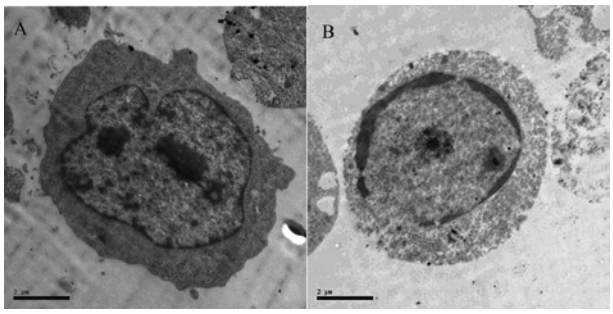

Figure 2. Morphological observation by electron microscopy of K562 cells treated with SeMoV 5 mg/l for 24 h. (A) Control cells (x8000). (B) Treatment cells (x8000).

It is known that apoptosis is not only a genetically controlled mechanism essential for development and the elimination of unwanted or damaged cells such as tumor cells (9) but is also a commonly accepted cellular event that may account for the cancer preventive effects of selenium compounds (27). In addition, mitochondrial disruption plays a major role during apoptosis induction, resulting in membrane permeability transition and the release of mitochondrial apoptogenic factors such as mitochondrial cytochrome C, which is released into the cytosol leading to activation of caspase 9 and the caspase cascade in response to apoptotic signals (28). Evaluation of the mitochondrial function during induction of apoptosis is recorded through decreased MMP, which when monitored by fluorescent probe has generally been adopted as an indicator of cell apoptosis (28,29). The Bcl-2 proteins are a family of proteins also involved in the response to apoptosis. A number of the pro-apoptotic and anti-apoptotic members, including Bax and Bcl-2, regulate apoptosis through the mitochondria either by their interaction, or their direct actions on the mitochondrial membrane.

Our results showed that SeMoV markedly induced the collapse of intracellular MMP and increased cytochrome C. The results were consistent with a report (30) that selenite

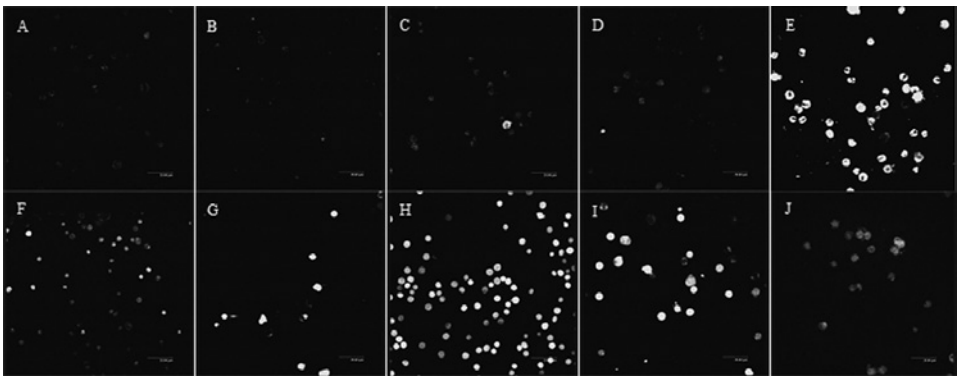

Figure 3. Fluorescent intensity of intracellular Ca^{2+}, Mg^{2+}, ROS, H^+ and MMP in K562 cells by confocal laser scanning microscopy following 24 h of treatment with SeMoV. (A-E) Control. (F-J) Treatment with SeMoV 2.5 mg/l. Fluorescent intensity of (A and F) intracellular Ca^{2+}, (B and G) Mg^{2+}, (C and H) ROS, (D and I) H^+ and (E and J) MMP, respectively. Scale bar,20 μm.

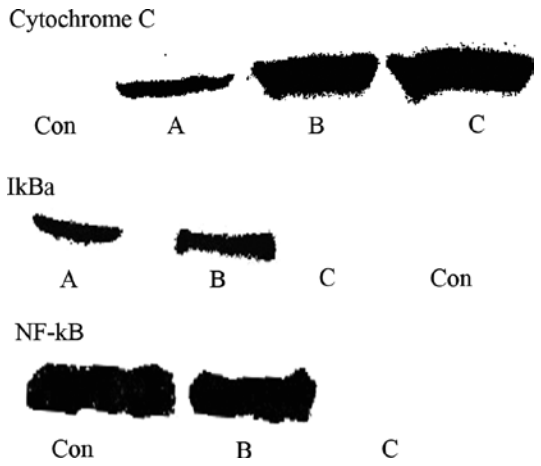

Figure 4. A Western blot assay was used to test the content of cytochrome C, IκBa and NF-κB in K562 cells after treatment with SeMoV at a concentration of (A) 1.25 (B) 5 and (C) 20 mg/l.

induces mitochondrial permeability transition and provokes the release of cytochrome C. The experiments also showed that the expression of Bcl-2 was significantly inhibited by 20 mg/l SeMoV, and that Bax expression increased. These results imply that the mechanism of SeMoV may relate to apoptosis induced by decreased MMP and the release of cytochrome C.

An excellent approach to apoptosis research has focused on changes in intracellular ion concentrations. It is presumed that perturbations of intracellular ion homeostasis could be the other conspicuous manifestation of apoptosis. The multitude of proteins activated in the apoptotic cascade invariably depends on the existence of certain intracellular ions. Munaron *et al* reported (31) that cell proliferation and differentiation is linked to the stimulation of the intracellular Ca^{2+} signal, and perturbations of intracellular Ca^{2+} appear to be a common mechanism of apoptosis. Particular emphasis has been placed on the influence of Ca^{2+} and Mg^{2+} ions (32). Ca^{2+} is one of the most significant intracellular messengers in modulating cell growth and differentiation, and plays an essential role in the induction of apoptosis. However, the role of Ca^{2+} as an intracellular messenger is incomplete without the coexistence of internal Mg^{2+} ions (33). Consistent with the above implication, the experiment showed that SeMoV markedly increased intracellular Ca^{2+} and Mg^{2+} concentrations. The present study suggests that Mg^{2+} may be adjunct to Ca^{2+} ions responsible for apoptosis induction, and the results were consistent with other research (34,35).

Changes in intracellular ion homeostasis, such as Ca^{2+} and Mg^{2+} accumulation, may induce mitochondrial apoptosis and lower its membrane potential. Ca^{2+} and Mg^{2+} play a crucial role in governing the morphological and biochemical changes attributed to apoptotic cell death (33). Therefore, the perturbations of intracellular ion homeostasis, pH value and MMP may be a conspicuous manifestation of apoptosis.

Another crucial factor, ROS, has been implicated as a main mediator of apoptosis in a number of different cellular systems (36). ROS may induce cell death by themselves or act as intracellular messengers during the cell death induced by various other types of stimuli (37). An excess of selenium was found to create an over-oxidized environment in cells and cause cell dysfunction and apoptosis (36,37). Our experiment showed that intracellular ROS was significantly increased by treatment with SeMoV, and suggests that apoptosis induced by SeMoV is closely correlated to the increase in intracellular ROS level through the affects of the intracellular redox status.

In general, these results are similar to another selenium heteropoly compound, $Na_5SeV_5O_{18}\cdot 3H_2O$, reported previously (38) in the same experimental system. Although there are different elements between selenium heteropoly $(NH_4)_4H_4[Se_2Mo_2V_4O_{24}]\cdot 7H_2O$ and $Na_5SeV_5O_{18}\cdot 3H_2O$, both of them contain the element selenium, as well as the same antitumor activity and mechanism. Thus, the element selenium may be a functional component of the compound.

In order to study further the apoptotic mechanism of SeMoV, we assessed the intracellular content of NF-κB and IκB. NF-κB is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation and various autoimmune diseases (39). The activation of NF-κB is thought to be part of a stress response as it is activated by a variety of stimuli that include growth factors, cytokines, agents and stress. Disturbance of the activation of NF-κB is related to a number of diseases, including chronic inflammatory arthritis and cancer (39), so investigators pay attention to the regulation of the activation of NF-κB. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the IκB family of inhibitor proteins. The various stimuli (TNF, IL-1) that activate NF-κB cause phosphorylation of IκB, which is followed by its ubiquitination and subsequent degradation. Degradation of IκB results in the exposure of the nuclear localization signals (NLS) on NF-κB subunits and the subsequent translocation of the molecule to the nucleus and the initiation of transcription (39). Our experiments showed that SeMoV markedly increased the intracellular content of IκB and clearly decreased NF-κB in a concentration-dependent manner.

Acknowledgements

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