Comparison of vitamins K_1, K_2 and K_3 effects on growth of rat glioma and human glioblastoma multiforme cells in vitro

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Abstract

Glioblastoma multiforme is characterized as highly invasive and rapidly growing astrocytomas, and scientists have sought for efficient treatment against malignant gliomas for a long time. Therefore, we compared the respond of rat glioma (C6) and glioblastoma multiforme cells derived from two patients to vitamins K_1, K_2 and K_3. The cells were exposed to 100, 250, 500, 750 and 1000 μM of vitamins K_1 and K_2, and 1, 10, 25, 50, 75 and 100 μM of vitamin K_3 for 24 hours in an incubator atmosphere of 5% CO_2, 37°C and 100% humidity. Cell viability was estimated by MTT assay. Vitamin K_1 showed no growth effect on all the glioma cells examined. Vitamin K_2 did not cause any change in number of C6, however induced growth inhibition in a dose-dependent manner on glioblastoma multiforme. The IC_{50} values of vitamin K_2 were 960 μM and 970 μM for glioblastoma multiforme, respectively. Vitamin K_3 had also growth inhibitory effect in a dose-dependent manner on both C6 and glioblastoma multiforme. The IC_{50} values were 41 μM, 24 μM and 23 μM for vitamin K_3, respectively. We concluded that vitamin K_3 is more effective than vitamin K_2 for inhibition of cancer cell growth, and might have an alternative value as an anticancer drug against glioblastoma multiforme.

Key words : Glioma ; K vitamins ; cell proliferation ; MTT assay ; in vitro.

1. Introduction

Glioblastoma multiforme is characterized as rapidly growing and highly invasive astrocytomas (Louis and Cavenee, 1997). It is more common in older adults and affects more man than woman. These tumor types can be treated with surgery, chemotherapy and radiation therapy. Since cells of the tumor stray into other areas of the brain, surgery alone rarely controls the glioblastoma multiforme (Mikkelsen, 1998). Several classes of drugs are used for the chemotherapy of this type of tumors such as alkylating agents, nitrosoureas or polyamine inhibitors, however clinical resistance to chemotherapy and side effects have been major problems in treatment (Salford, 2002). The mean survival time after diagnosis for patients with glioblastoma multiforme is less than a year in spite of surgery, radiotherapy and chemotherapy (Kimmel, 1987). For a long time scientists have sought for efficient treatment against malignant gliomas.

Vitamin K exists in at least three different forms : vitamin K_1 (phylloquinone ; 2-methyl-3phytyl-1,4 naphthoquinone) mostly found in green leafy vegetables, vitamin K_2 (menaquinone ; 2-methyl-3(all-trans-difarnesyl)-1,4 naphthoquinone) synthesized by the intestinal flora, and vitamin K_3 (menadione ; 2 methyl-1,4 naphthoquinone) a synthetic derivative of vitamin K_1 and K_2 (Suttie, 1995).

Previous studies demonstrated that vitamin K_3 inhibits cell growth of various rodent and many types of malignant human tumor cells in vivo and in vitro (Nishikawa *et al.*, 1995 ; Ngo *et al.*, 1991). Vitamin K_1 and K_2 also have been shown to have growth inhibitory effects in vitro, but these effects are much weaker than those of vitamin K_3 (Prasad *et al.*, 1981 ; Wu *et al.*, 1993). Beside a broad spectrum of antitumor activity vitamin K_3 was shown to enhance the cytotoxic effect of some clinically useful anticancer agents, including action against multidrug resistant human cancer cell lines, and it was thought to have less serious toxic effects (Nutter *et al.*, 1991 ; Wang *et al.*, 1995). We noticed that there are insufficient studies related to the effect of K vitamins on growth of glioma cells in vitro. In addition there isn't any study comparing the antiproliferative effect of K vitamins on both rat glioma cell line and human glioblastoma multiforme cells. Therefore, we have attempted to compare the possible inhibitory effect of vitamin K_1, K_2 and K_3 based on MTT assay using both C6 and low passage human glioblastoma multiforme cells obtained from two different patients tumor tissue in culture.

2. Methods and materials

2.1. REAGENTS AND CELL CULTURE

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). We used C6 and cultured low passage human glioblastoma

multiforme which were derived from primary cultures of two patients' tumor tissue as described previously by Durmaz et al. (Durmaz et al., 1999).

All the cells were maintained in 75 cm^2 flasks, and incubated in 1:1 mixture of Dulbecco's Modified Eagle's Medium and F-12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air (Unterman et al., 1991). The medium was changed 2-3 times within a week until the cultures approached confluence. For experimentation, monolayer cultures were washed with Hank's balanced salt solution (without calcium and magnesium) and then incubated in the presence of 0,25% 3 ml trypsin-EDTA solution for 10-15 minutes at 37°C. After the cells were dispersed, trypsin activity was inhibited by adding growth medium and the cells were centrifuged at 1000 rpm for 5 minutes at 4°C, and then counted with a Coulter counter. Cell viability was accessed by trypan blue dye exclusion and found to be higher than 98%. Vitamin K$_1$ and K$_2$ were dissolved in absolute ethanol at 1000 μM concentration. Further dilutions were made at ratio of 1:10 in F-12 and the maximum concentration of ethanol was adjusted to be 0,01%. This amount was added to the culture medium together with the highest concentrations of vitamins K$_1$ and K$_2$ (1000 μM), and found to have no effect on cell viability when used alone (data not given). Water soluble form of vitamin K$_3$ (menadione sodium bisulfate) was dissolved in F-12 medium. For experimental protocol, the cells at exponential growth phase were seeded in 3 x 10^4 cells/well in 96 well microtiter plates, 10 wells for control (included growth medium only) and 10 wells for each tested K vitamins doses, and incubated for 24 hours. Vitamin K$_1$ and K$_2$ at the concentrations of 100, 250, 500, 750, 1000 μM ; vitamin K$_3$ at the doses of 1, 10, 25, 50, 75, 100 μM were added to the growth medium for 24 hours. All the test compounds were prepared immediately prior to use and protected from light.

2.2. MTT COLORIMETRIC ASSAY

After 24 hours, drug cytotoxicity screening was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay originally described by Mossmann and modified by Alley et al. (Mosmann, 1983 ; Alley et al., 1988). MTT was prepared freshly before each test as 5 mg/ml in phosphate buffered saline and filtered to sterilize with 0,22 μm filter. Then, 25 μl MTT solution was added to the each well and incubated for 4 hours at 37°C. The MTT solution was converted into blue formazan by mitochondrial dehydrogenase activity of viable cells (Alley et al., 1988). Therefore, the amount of formazan produced is proportional to the number of living cells (Abe and Matsuki, 2000). After the
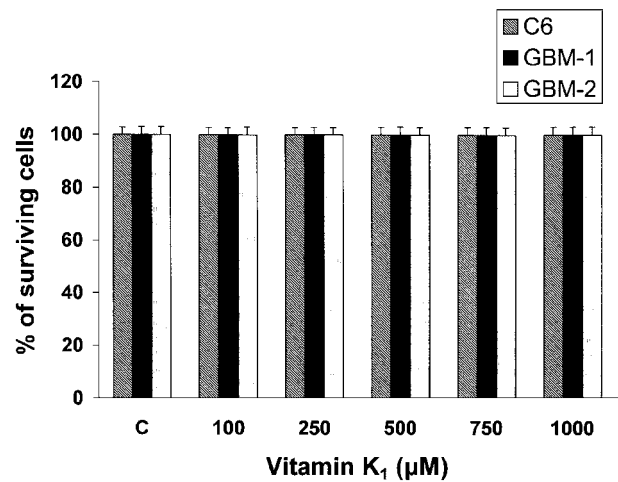

FIG. 1. — Action of vitamin K$_1$ on C6 cells, low passage human glioblastoma multiforme cells from the first patient (GBM-1) and human glioblastoma multiforme cells from the second patient (GBM-2).

medium was removed from the wells, 100 μl dimethyl sulphoxide was added to each well and the crystal formazan particles produced in viable cells was dissolved for 5 minutes at room temperature with a shaker. The absorbance of formazan dye was read at 550 nm using a microplate reader (Bio-Tek Instruments), and cell survival percentages were calculated according to the following formula ; absorbance of treated cells in each well x 100 / mean absorbance of control cells (Durmaz et al., 1999 ; Loveland et al., 1992). The dose response curves were calculated for three vitamin K forms at the above-mentioned concentrations and expressed as the mean percent fraction of control ± SEM. Fifthy % growth inhibitory concentration (IC$_{50}$) was determined by plotting the logarithm of the drug concentration against the mean percentage of living cells at the each dose of K vitamins. All statistical analyses were performed using one-way analysis of variance (ANOVA) and followed by Tukey's multiple comparison tests. A p value less than 0.05 was considered to be significant. Each experiment was repeated at least three times.

3. Results

When compared to the control, vitamin K$_1$ did not show any activity on C6 and the low passage human glioma cells proliferation (Fig. 1). K$_2$ also had no effect on C6 cell proliferation, however it inhibited cell survival in a dose-dependent manner in the human glioma cells. Vitamin K$_2$ at the doses of 100, 250 and 500 μM had no effect on the survival of the human glioma cells, but inhibitory effect was observed at 750 μM and reached down to 56% reduction at 1000 μM (p < 0.001). IC$_{50}$ of vitamin K$_2$ for the human glioma cells were esti-
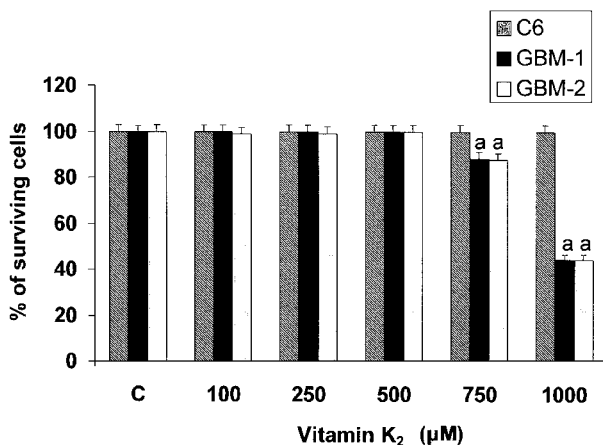
FIG. 2. — Effects of vitamin K_2 on C6, GBM-1 and GBM-2 cells. A means p < 0.001 when compared to the control.
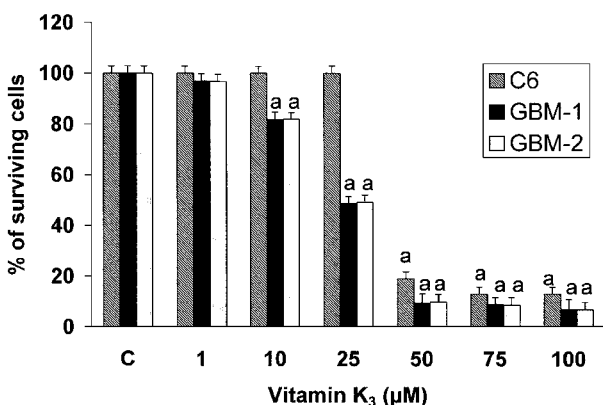

FIG. 3. — Cytotoxic effects of vitamin K_3 on C6, GBM-1 and GBM-2 cells.

mated as 960 μM and 970 μM, respectively (Fig. 2).

Vitamin K_3 at the concentrations of 1,10 and 25 μM (Fig. 3) did not influence C6 cell viability when compared to the control, but decreased the cell number starting from 50 μM and reached to almost 100% reduction at 100 μM (P < 0.001). IC_{50} of vitamin K_3 for C6 cells was calculated as 41 μM. In contrast, vitamin K_3 reduced the cell number starting from 10 μM in the human glioma cells (Fig. 3). The decline in cell viability reached down to 90% at 50 μM (p < 0.001). IC_{50} of vitamin K_3 for the human glioma cells was calculated as 24 μM and 23 μM, respectively. When compared to each other, there was no difference in terms of response of two different sources of human glioma cells to three different K vitamins at every dose studied.

4. Discussion

Vitamins of group K have pronounced cytotoxic properties leading to their application in the therapy of various neoplasms. Wu et al. compared the growth inhibitory effects of vitamins K_1, K_2 and K_3

against on five hepatoma cell lines, nasopharyngeal carcinoma, leukemia, oral epidermoid carcinoma and breast carcinoma cells. For all these cells, the ID_{50} values of vitamin K_1 ranged from 6 to 9 mM, and the ID_{50} of vitamin K_2 ranged from 1 to 2 mM. However, the respective ID_{50} values of vitamin K_3 for these nine different cells were between 15 and 42 μM (Wu et al., 1993). Nishikawa et al. reported that K vitamins inhibit growth of human hepatoma cells and 50% inhibitory doses were 1000 μM for vitamin K_1, 112 μM for K_2 and 10 μM for K_3 (Nishikawa et al., 1995). Markovits et al. also evaluated the action of K vitamins on another human hepatoma cells and found IC_{50} values approximately 500, 150 and 5 μM for vitamin K_1, K_2 and K_3, respectively (Markovits et al., 2003). Sata et al. found that menadione at 1 and 2 μM doses, and time dependently inhibited proliferation of rat pancreatic acinar cell line (Sata et al., 1997). Our results demonstrate that vitamin K_1 showed no growth effect on both C6 and human glioblastoma multiforme cells. Vitamin K_2 also did not show any growth inhibitory effect on C6, however induced growth inhibition in a dose-dependent manner on human glioma cells. Vitamin K_3 had antiproliferative effect on both C6 and human glioma cells. IC_{50} of vitamin K_3 were estimated as 41 μM for C6 cells, 24 and 23 μM for human glioblastoma multiforme cells. All these previous and our results demonstrate that the growth inhibitory effect of vitamin K_3 is stronger as much as 1000-fold than vitamin K_1 or K_2 on different cells in vitro.

Generation of reactive oxygen species (ROS) by redox cycling is generally accepted as a mechanism of vitamin K_3 cytotoxicity (Buckman et al., 1993). Recent reports indicated that especially astrocytes appear to be more resistant to ROS and play an important role in reducing ROS level in brain to protect neurons. Desagher et al. showed that the protective effect of astrocytes depend on the astrocytes/neurons ratio, and estimated that only one astrocytes has the capacity to significantly protect 20 neurons against the toxicity induced by a 30 min application of 100 μM hydrogen peroxide on mouse neuronal cultures (Desagher et al., 1996). Moreover, Abe and Saito examined the effect of 100 μM K_3 in cultured rat cortical astrocytes and found apparent morphological changes 2 hours later, and all cells were killed in 12 hours (Abe and Saito, 1996). This concentration can be very high for treatment or combination. Our results suggested that vitamin K_3 inhibits cell growth at low concentrations on glioma cells. The amount of ROS formed by low levels of vitamin K_3 may not be enough to injure neurons. Meanwhile Sun et al. results suggested that when antioxidant enzymes were added prior to addition of vitamin K_3, toxicity was markedly attenuated (Sun et al., 2000).

Both in vitro and in vivo studies for over half a century showed a synergistic or additive effect

when vitamin K_3 was combined with chemotherapeutic agents of about 20 different drugs on about 10 different tumors or cell lines (Lamson and Plaza, 2003). It is also concluded that vitamin K_3 has antitumoral activity on multi-drug resistant human cancer cell lines and thought to have less serious toxic side effects (Nutter *et al.*, 1991 ; Wang *et al.*, 1995). All these findings suggest that vitamin K_3 may be useful in combination treatment with antitumor drugs and some types of drug-resistant brain tumors. Although diffusion of chemotherapeutic drugs within the blood-brain barrier is fairly poor, vitamin K_3 has increased the permeability of rat brain capillary endothelial cells in an in vitro model of the blood brain barrier (Lagrange *et al.*, 1999).

In conclusion our results indicated that vitamin K_3 has potential antiproliferative effect on both C6 and the low passage human glioma cells. When the two cell types compared, antiproliferative effect of vitamin K_3 was stronger on the human glioma cells than on C6 cells. All these findings suggest that vitamin K_3 might be a good candidate in the clinical treatment of glioblastoma multiforme. For this reason, a study about the possible effects of vitamin K_3 on normal rat tissues is underway in our laboratory.

List of abbreviations

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
EDTA : Ethylenediaminetetraacetic acid.
IC_{50} : Fifty % growth inhibitory concentration.
ANOVA : Analysis of variance.
ID_{50} : Fifty % growth inhibitory dose.
ROS : Reactive oxygen species.

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