RESEARCH ARTICLE

Menadione (Vitamin K3) Induces Apoptosis of Human Oral Cancer Cells and Reduces their Metastatic Potential by Modulating the Expression of Epithelial to Mesenchymal Transition Markers and Inhibiting Migration

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Abstract

Oral cancer is one of the most commonly occurring cancers worldwide, decreasing the patient's survival rate due to tumor recurrence and metastasis. Menadione (Vitamin K3) is known to exhibit cytotoxicity in various cancer cells but the present study focused on its effects on viability, apoptosis, epithelial to mesenchymal transition (EMT), anchorage independent growth and migration of oral cancer cells. The results show that menadione is more cytotoxic to SAS (oral squamous carcinoma) cells but not to non-tumorigenic HEK293 and HaCaT cells. Menadione treatment increased the expression of pro-apoptotic proteins, Bax and p53, with a concurrent decrease in anti-apoptotic proteins, Bcl-2 and p65. Menadione induced the expression of E-cadherin but reduced the expression of EMT markers, vimentin and fibronectin. Menadione also inhibited anchorage independent growth and migration in SAS cells. These findings reveal and confirm that menadione is a potential candidate in oral cancer therapy as it exhibits cytotoxic, antineoplastic and antimigratory effects besides effectively blocking EMT in oral cancer cells.

Keywords: Apoptosis - colony formation - epithelial to mesenchymal transition - menadione - oral cancer

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Introduction

Oral cancer is the most common in men and women in India, due to ethnic or geographical factors and increased addictive habits such as tobacco chewing, smoking and alcoholism Byakodi et al. (2012). Oral cancer is known to exhibit multiple primary tumor formation, a phenomenon known as 'field cancerization', which leads to low survival rate in patients Hsu et al. (2004). In addition to this, oral cancer cells undergo EMT and exhibit metastatic behavior that is characterized by changes in morphology, decrease in E-cadherin and increase in vimentin and fibronectin expression, resulting in increased mobility, invasiveness and metastatic potential (Krisanaprakornkit and Iamaroon, 2012). The limited survival of oral cancer patients is also due to a high proportion of patients with advanced disease stages, lack of suitable markers for early detection, and failure to respond to available chemotherapy (Forastiere et al., 2001; Mao et al., 2004). Menadione (2-methyl-1, 4-naphthoquinone/vitamin-K3), a naphthoquinone and a precursor in the synthesis of vitamin K, is supplemented to neonatal patients with low prothrombin levels to prevent hemorrhagical disorders (Hassan, 2013). It is also used as an anti-inflammatory compound and as a component in multivitamin drugs. Menadione is shown to exhibit anti-neoplastic effect in various cancers such as breast, liver and nasopharyngeal carcinomas and rat models but also has side effects like allergic reactions and cytotoxic effects in liver cells (Vermeer et al., 2004; Cranenburg et al., 2007; Oh et al., 2013). The present study is focused on the anti-proliferative effects of menadione and elucidates its role in regulating EMT, migration and anchorage independent growth in oral cancer cells.

Materials and Methods

Chemicals

Menadione is a generous gift from Dr. Sreelatha, CLRI, Chennai. MTT reagent was purchased from Sigma, and Resazurin from Hi-media.

Cell culture

SAS cell line was procured from Japanese Collection of Research Bioresources Cell Bank, Japan. All the cell lines were maintained as adherent monolayers in a tissue culture flask fed with complete DMEM supplemented with 10% (v/v) Fetal Bovine Serum (FBS) containing 100mg/L penicillin and 66.6mg/L streptomycin. Cells were incubated in a humidified incubator set at 37°C and 5%CO_2.

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Cytotoxicity assay

The cell viability was assessed using the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) dye. Briefly, 5000 cells/well were seeded in 96 well plates and allowed to adhere overnight. The cells were treated with different concentrations of menadione for 24h and MTT (5mg/ml) was added, incubated for 3.5h, followed by aspiration of the medium and solubilization of the formazan crystals by adding Dimethyl Sulfoxide (DMSO). After 15min, the color developed was read as optical density (OD) using a Bio-Rad 680 model colorimeter at 570nm with 655nm as background reference.

Immunoblotting

8×10^5 SAS cells/well were seeded in 6 well plates and treated with 5μM menadione, and DMSO as a vehicle control for 24h, after which cells were collected and lysed in a lysis buffer (20mM Tris, 150mM NaCl, 1mM EDTA, 1mM β-glycerophosphate, 1%Triton×-100, 2.5mM sodium pyrophosphate, 0.5% sodium deoxycholate, 1mM PMSF, 20mM NaF, 1% protease inhibitor) for 1h on ice. The lysates were centrifuged at 10,000rpm for 10min at 4ºC and the supernatants collected were estimated by Bradford assay. Equivalent amounts of protein (30-50µg) were resolved in 8-10% SDS-PAGE and transferred onto PVDF membrane (Amersham). After blocking with TBS buffer [500mM NaCl, 20mM Tris-HCl (pH 7.4)] containing 5% skimmed milk for 1h, the membranes were incubated overnight at 4°C with specific primary antibodies (1:1000): anti-β-actin (SantaCruz), anti-Bax, anti-Bcl-2 (Cell Signaling), anti-E-Cadherin, anti-Vimentin and anti-Fibronectin (Santa Cruz). The membranes were subjected to three washes of 10min each in TBS containing 0.1% TWEEN20 (Sigma) and further incubated with HRP-conjugated secondary antibodies (1:10000) (Jackson) at room temperature for 1h, and proteins were detected by chemiluminescence (Versadoc, Bio-Rad) with Enhanced Chemiluminescence kit (Amersham).

In vitro wound healing assay

Cells were grown to a confluent monolayer in 48 well plates and a wound was created with autoclaved pipette tips and cells were treated with menadione dissolved in serum free media and images were captured at 0 and 10h after treatment. The healed area was measured using ImageJ software and the relative cell migration was calculated as follows: **Relative cell migration (%)=(Area without cover at 0h–Area without cover at 10h) of sample/ DMSO×100.**

A parallel alamar blue assay was performed to assess the effect of menadione on cell viability at 0 and 10h. Briefly, alamar blue (10mg/ml) was added and incubated in 37ºC until color change from blue to purple and diluted 10 times in ultrapure water and optical density was measured at 570nm with a background reference of 595nm.

Soft agar assay

To determine anchorage independent growth of cells, 12 well plates were layered with a mixture 1% agarose and 2× cDMEM (1:1) forming a bottom layer. Cells treated with menadione (2 or 4µM) for 6h were suspended at a density of 2000 cells per well in a mixture of 2× cDMEM and 0.6% agarose layered over the bottom layer. After 15 days of incubation at 37°C with 5%CO_2, colonies formed in each well were visualized in an inverted phase contrast microscope and the sizes of colonies formed were measured using ImageJ software.

Statistical analysis

Values are expressed as means±SE of at least three independent experiments. Differences between control and treated samples were analyzed by student t-test. The p value of <0.05 was considered as statistically significant in each experiment.

Results

Menadione inhibits the viability of oral cancer cells in a concentration-dependent manner but is less toxic to non-tumorigenic cells

Cytotoxic effects of menadione on oral cancer (SAS) and immortalized/non-tumorigenic (HEK293 and HaCaT) cells were evaluated by MTT assay. Cells were treated with different concentrations of menadione or 0.1% DMSO (control) for 24h and the IC_{50} values are found to be 8.45, 98.5 and 74.5μM in SAS, HEK293 and HaCaT cells, respectively (Figure 1). The IC_{50} values of menadione for HEK293 and HaCaT cells were higher than that for SAS cells suggesting that menadione is relatively less toxic to immortalized/non-tumorigenic cells. In agreement with this, menadione-treated HaCaT and HEK293 cells show 90-95% cell viability at 8.45μM (Figure 1C and D). These results suggest that menadione effectively inhibits the viability of oral cancer cells but not that of non-tumorigenic cells.

Menadione up-regulates the expression of pro-apoptotic proteins and down-regulates that of anti-apoptotic

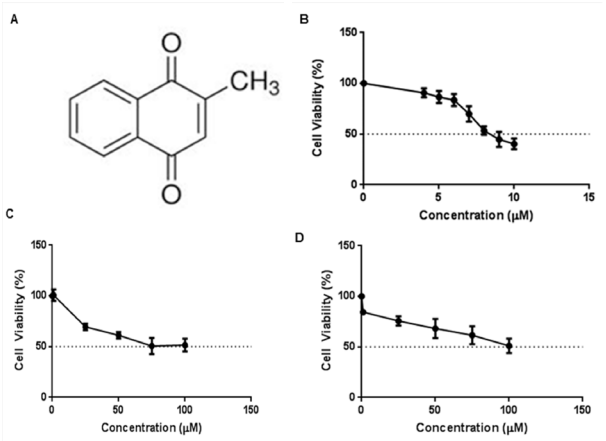

Figure 1. Effects of Menadione on the Viability of SAS, HaCaT and HEK293 Cells. A) Structure of menadione (vitamin K3); **B)** shows concentration dependent changes in cell viability of SAS cells upon menadione treatment (1-10μM) for 24h; **C)** and **D)** represent concentration dependent changes in cell viabilities of HaCaT and HEK293 cells, respectively, upon menadione treatment (1-100μM) for 24h. Data are expressed as mean±SD for at least 3 independent experiments

proteins in SAS cells

Bcl-2 protein family plays a vital role in the regulation of the mitochondria–mediated apoptotic pathway. To determine its involvement in menadione-mediated cell death in oral cancer cells, expression levels of the pro-apoptotic and anti-apoptotic Bcl-2 proteins were analyzed in SAS cells by immunoblotting. The expression levels of anti-apoptotic protein (Bcl-2) decrease while that of pro-apoptotic protein (Bax) increases upon treatment with menadione (Figure 2). Furthermore, treatment with menadione up-regulates the expression of tumor suppressor p53, and down-regulates the anti-apoptotic protein p65 (Figure 2). The decrease in Bcl-2 and p65 expression with a concomitant increase in Bax and p53 expression and high Bax to Bcl-2 ratio compared to the control strongly suggests that menadione induces cell death via apoptosis in SAS cells.

Menadione down-regulates the expression of vimentin and fibronectin (mesenchymal markers) and up-regulates that of E-cadherin (epithelial marker)

SAS cells are metastatic in nature Yamamoto et al. (2006) and the endogenous expression levels of E-cadherin were low whereas vimentin and fibronectin levels were high in SAS cells and hence it was of interest to study the effect of menadione on the expression of various markers regulating EMT. Immunoblotting revealed that menadione (5μM) decreases the expression level of mesenchymal markers, vimentin and fibronectin, and increases the expression of epithelial marker E-cadherin (~4 fold) compared to DMSO vehicle control (Figure 3). These results suggest that menadione modulates EMT by regulating the expression of epithelial and mesenchymal marker proteins.

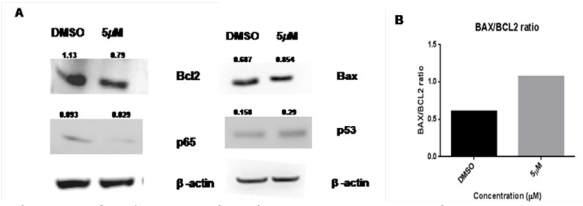

Figure 2. Alterationin the Expression Levels of Anti-Apoptotic and Pro-Apoptotic Proteins upon Menadione Treatment. A) Cells were treated with 5μM menadione for 24h and expression levels of pro-apoptotic proteins Bax and p53, and anti-apoptotic proteins Bcl-2 and p65 were analyzed by immunoblotting. The fold change in expression of the proteins is represented above the blot, after normalizing with β-actin; **B)** The graph represents the Bax/Bcl-2 ratio in DMSO control and menadione treated cells. The results are representative of two independent experiments

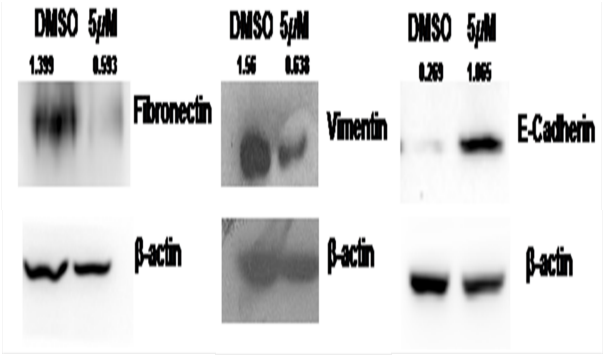

Figure 3. Menadione Modulates Expression Levels of EMT Markers. Cells were treated with 5μM menadione and the expression levels of mesenchymal markers (Fibronectin, Vimentin and epithelial marker E-cadherin) were analyzed by immunoblotting. The fold change in expression is represented above the blot, after normalizing with β-actin. The results are representative of two independent experiments

Menadione inhibits colony formation and migration of SAS cells

Anchorage independent growth of cells in soft agar is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth, with normal cells typically being incapable of growing in semisolid matrices (Franken et al., 2006). To assess the role of menadione in regulating anchorage independent growth, soft agar assay was carried out in SAS cells treated with menadione or DMSO (vehicle control). Upon treatment with 4μM menadione, size of colonies in SAS cells reduces to 70% compared to the control (Figure 4). Thus, menadione inhibits the anchorage independent growth and colony formation in SAS cells.

Cell migration is a multistep event during cancer cell

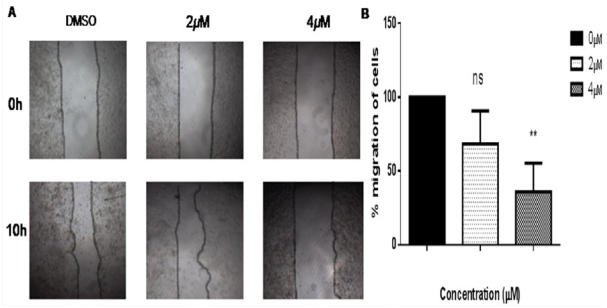

Figure 5. Menadione Inhibits the Migration of SAS Cells. Wound healing assay to study the effect of menadione treatment on migration. The migratory ability of the cells was observed under a phase contrast inverted microscope at 0h and 10h. **A)** is a representative figure of the images captured during wound healing assay at 0h and 10h; **B)** Shows the relative migration of cells analyzed and data expressed as mean±SD for 3 independent experiments (**Denotes p<0.01)

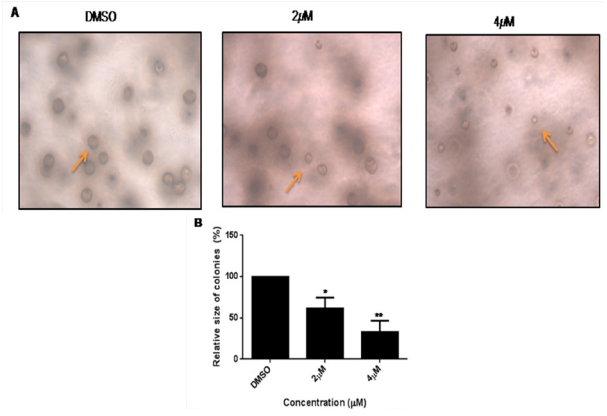

Figure 4. Menadione Inhibits Tumor Formation *in vitro*. Anchorage independent growth of SAS cells upon menadione treatment. **A)** is a representative figure of the colonies in soft agar on Day 15; **B)** After 15 days, the size of colonies formed was analyzed and all data are expressed as mean±SD for 3 independent experiments (*Denotes p<0.05, **p<0.01)

metastasis and *in vitro* wound healing assay can assess the directional cell migration Liang et al. (2007). The migratory ability of SAS cells upon menadione treatment was studied after 10h and the results show a significant decrease in migration at 4μM compared to DMSO control (Figure 5). A parallel alamar blue assay was performed to check the cell viability (data not shown). Menadione treated SAS cells show decreased wound closure without affecting the cell viability compared to the control (Figure 5). These results suggest that the decreased wound closure in menadione-treated cells is due to the anti-migratory effect of menadione but not due to cell death.

Discussion

Menadione (vitamin K3/2-Methyl-1, 4-naphthoqui none) is gaining importance because of its selective cytotoxic effects in cancer cells. Cytotoxic effects associated with menadione are thought to be mediated through various mechanisms including oxidative damage induced by reactive oxygen species (Chiou and Tzeng, 2000). A combination of menadione and ascorbate resulted in oxidative stress-mediated cell death in liver cancer cells (Veerax et al., 2004). A combination of menadione and sodium orthovanadate eliminated and inhibited migration of detached cancer cells (Delwar et al., 2012). Combination of menadione with ethacrynic acid inhibited angiogenesis by down regulating vascular endothelial growth factor expression and blocking HIF-α interaction with p300 thereby inhibiting hypoxia in cancer cells (Na et al., 2013). Analogs of menadione also exhibited selective cytotoxicity in neuroblastoma cells but not in normal cells (Kitano et al., 2012) which is in agreement with our data showing that menadione induces cell death in SAS but not in non-tumorigenic HEK293 and HaCaT cells. Similarly, menadione was found to inhibit proliferation in pancreatic cancer cells by activation of caspases and PARP cleavage (Osada et al., 2008). Our results showing a reduction in the protein levels of the anti-apoptotic proteins Bcl-2 and p65 by menadione are relevant as these proteins are known to be over-expressed and aid tumor progression, invasion and metastasis in oral cancer (Nakayama et al., 2001; Teni et al., 2002; Sulkowska et al., 2003). In addition, over expression of Bax in oral cancer correlates with long survival of oral cancer patients (Xie et al., 1999). Menadione also reduces the expression level of c-FLIP, an inhibitor of the extrinsic pathway of apoptosis in cancer cells (Wilkie-Grantham et al., 2013) and serves as a cancer cell chemo-sensitizing agent by acting as a substrate for P-glycoproteins (Oh et al., 2013). EMT in oral cell carcinoma is characterized by loss of adhesion molecules like E-cadherin (McConkey et al., 2009), increase in vimentin (Thompson et al., 2005) and fibronectin (Park and Schwarzbauer, 2013). EMT augments cell invasiveness, migration and stem cell like properties leading to metastasis (Alderton, 2013). In addition to these properties, EMT confers drug resistance to lung cancer (Nurwidya et al., 2012) and pancreatic cancer (Arumugam et al., 2009) cells. Given that menadione treatment up-regulated E-Cadherin (epithelial marker) and down-regulated vimentin and fibronectin (mesenchymal markers), and inhibited migration in SAS cells, it appears to be a potential candidate to modulate EMT in oral cancer cells.

In conclusion, the study reveals that menadione, a nutritional supplement, has a potential anti-proliferative property in oral cancer cells. It has selective cytotoxicity in oral cancer cells but has less toxic effects in non-tumorigenic HEK293 and HaCaT cells. Most importantly, we have demonstrated a novel role of menadione in targeting and modulating the expression levels of EMT markers. This makes it a promising candidate as a nutritional chemo-preventive as well as a potential chemotherapeutic compound to treat oral tumors.

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