Quinone-induced DNA single strand breaks in a human colon carcinoma cell line

Anna D’Odorico1,4, Giacomo C. Sturniolo1, Rodney E. Bilton2, Antony L. Morris3, Ian T. Gilmore3 and Remo Naccarato1

1Division of Gastroenterology, Padua University, Italy; 2School of Biomolecular Sciences, Liverpool John Moores University; 3Department of Gastroenterology, Royal Liverpool University Hospital, Liverpool, UK
4To whom correspondence should be addressed at: Division of Gastroenterology, Ospedale Civile, Via Giustiniani, 2, 35128 Padova, Italy

It has been demonstrated that synthetic quinones, such as menadione, cause DNA damage in different cell systems, possibly being mediated by free radicals generated during redox cycling. It has been suggested that the damage caused could be related to tumor induction in different sites. To our knowledge it has not yet been demonstrated that the natural quinones, vitamin K1 and K2, exert the same activity. Using a colon carcinoma cell line, HT-29, we examined the extent of DNA damage induced by menadione, vitamin K1 and K2. Menadione caused significant DNA damage at low concentrations (25–200 μM) with a linear correlation of r = 0.95. In the presence of dicoumarol, a DT-diaphorase inhibitor, the damage was detected at concentrations five times lower indicating that free radicals generated during the redox cycling play a key role. Neither vitamin K1, incorporated in micelles, nor K2 caused detectable single strand breaks with respect to the controls either in the presence or in absence of dicoumarol. Our results demonstrate that, despite their redox cycling properties, the natural forms of vitamin K do not cause DNA damage in HT-29 cells as menadione does in the experimental conditions used.

Introduction

Quinones are widely distributed in nature and form an important group of substrates for flavoenzymes. Many substances, such as vitamin K and clinically important drugs (doxorubicin, mitoxantrone and mitomycin) contain the quinone nucleus (1–3). They can be toxic and may exert their toxicity by a number of mechanisms including oxidative stress caused by redox cycling (4,6) direct interaction with cellular macromolecules (7–9) and inhibition of mitochondrial electron transport (10). The cytotoxic and antitumor properties of quinoid drugs are thought to be mediated through the one-electron reduction to semiquinone free radicals by flavoenzymes such as NADPH–cytochrome P450 reductase (5).

For its redox cycling properties, vitamin K has been included in an hypothesis presented to account for the dietary induction and promotion of colorectal cancer (11). Secondary bile acids, the vitamin K group and ferrous ion complexes may interact to subvert the normal free radical generating mechanisms involved in mucosal defense. These effects on the colonic crypt stem cells can lead ultimately to semiquinone and hydroxyl radical mediated DNA damage and tumor induction. As reported by Babbs (12), the intracolonic free radical formation may explain the high incidence of cancer in the colon and rectum, compared with other regions of the gastrointestinal tract, as well as the observed correlations of a higher incidence of colon cancer with excessive fat in the diet, which may increase the fecal content of procarcinogens and bile acids and with red meat which increases both stool iron (involved in Fenton reaction) and Bacteroides fragilis strains (vitamin K2 producers).

Recently a National Cohort Study performed in the United Kingdom also implicated vitamin K, injected to the newborn, in an increased incidence of cancers in childhood (13).

In this study we tested the DNA damaging effect of different forms of vitamin K (K1, K2 and K3), on a colon carcinoma derived cell line (HT-29). This clone retains many of the morphological features and enzyme levels characteristic of normal colonocytes.

Materials and methods

Chemicals

Menadione (2-methyl-1,4-naphthoquinone), vitamin K1 (2-methyl-3-phytyl-1,4-naphthoquinone) and K2 (menatetrenone), dicoumarol (3,3'-methylene guanosine 5'-triphosphate), sodium deoxycholate, phosphatidylcholine and dimethylsulfoxide (DMSO*) were obtained from Sigma Chemical Co. (St Louis, MO).

Cell line

HT-29, human colon carcinoma cells, were routinely cultured in a monolayer on plastic culture dishes at 37°C, in a humidified atmosphere (5% CO2/air), using Dulbecco’s minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and l-glutamine (29.2 mg/ml) (Gibco). The medium was changed every alternate day.

Chemical treatment

Menadione, dicoumarol and vitamin K2 were dissolved in DMSO immediately before treatment of cell culture. Control cell cultures were treated with DMSO alone. DMSO concentration, in either treated and untreated cells, was constant (1% v/v). Vitamin K1 was incorporated in mixed micelles with phosphatidylcholine and sodium deoxycholate as described by Nagata et al. (14). In all assays, cells were exposed to chemicals for 30 min at 37°C in an atmosphere of air/5% CO2 and the treatments were terminated by aspiration of the drug-containing medium and replacement with fresh medium.

Analysis of DNA damage

Analysis of DNA damage was performed with the procedure of ‘in situ nick translation’ as described by Nose and Okamoto (16). Briefly, after 2 days of culture on multiwell dishes, the cells were incubated with increasing concentrations of menadione, vitamin K1, vitamin K2, micelles with and without vitamin K2, in the presence or absence of dicoumarol (0.03 mM). After 30 min the cells were harvested with trypsin and then collected by sedimentation in a plastic tube for 1 min and suspended with gentle pipetting in a solution containing 0.25 M sucrose, 0.1M Tris–HCl (pH 7.4), 10 mM MgCl2 and 0.5 mM dihydrothreitol. Aliquots of the cell suspension, containing 4×10^7/ml, were prepared in triplicate for each substance tested and for controls (untreated cells). Triton X-100 (0.02% final concentration) was added
as permeabilizing agent just before use. The cell suspension was kept in ice for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, for 10 min then spun down. The cell pellet was resuspended in the nick translation assay mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂

Detection of vitamin K absorption

HT-29 cells were separately incubated with vitamins K₁, K₂ and K₃ for 30 min, as previously described. After the incubation the medium was removed and the cell monolayers were washed twice with PBS and then, following the trypsinization the cells were counted, aliquoted into siliconized culture tubes and lysed following the method described by Downs and Wilfinger (17). The exposure of cultured cells to 200–800 \( \mu \text{M} \) vitamin K₃ (linear correlation \( r = 0.95 \)) triggered a dose-dependent increase in single strand breaks, with a maximum response being observed at 200 \( \mu \text{M} \) (Figure 2). No damage was detected at concentrations below 25 \( \mu \text{M} \). In the presence of quantities of menadione of over 200 \( \mu \text{M} \), the cells showed a progressive decrease of the damage detected (at 300 \( \mu \text{M} \) the single strand breaks were compared with those induced by 100 \( \mu \text{M} \)). The reduction of DNA damage at above 200 \( \mu \text{M} \) was not related to a loss of cell viability as demonstrated by the MTT assay, which showed the same viability of controls in the cells treated with up to 500 \( \mu \text{M} \) of menadione (Figure 2).

To test the involvement of free radicals in the damage caused we added 30 \( \mu \text{M} \) dicoumarol, a specific inhibitor of DT-diaphorase, to the incubations with menadione as illustrated in Figure 3. We also observed a DNA damaging effect of menadione at very low concentrations with the same linear correlation (\( r = 0.90 \)) but five times lower (\( P < 0.001 \)). Above 100 \( \mu \text{M} \) the effect of dicoumarol was lost (165 ± 9.4 SE versus 162.6 ± 7.2 SE without dicoumarol). No damage was detected in the presence of dicoumarol alone (data not shown).

The exposure of cultured cells to 200–800 \( \mu \text{M} \) vitamin K₂ dissolved in DMSO, did not induce DNA damage (Table 1) and the addition of dicoumarol did not increase the number of single strand breaks detected.

The same results were obtained using vitamin K₁, from 250–600 \( \mu \text{M} \) transported in micelles (Table 1). It was not possible to increase the concentration of the two natural vitamins (K₁ and K₂) because of their liposolubility. Indeed, for vitamin K₂ the dissolution in DMSO, a well known hydroxyl
Vitamin K and DNA damage

**Fig. 4.** MTT test: dose–response curves using HT-29 cells following exposure vitamin K$_1$ in micelles (■) and micelles with only phosphatidylcholine plus sodium oxycholate (○). Values represent the means ± SEM of three experiments.

**Table I.** Single strand breaks induced on DNA from HT-29 cells by increasing concentrations of vitamins K$_1$ and K$_2$

<table>
<thead>
<tr>
<th>µM</th>
<th>Vitamin K$_2$ (%)</th>
<th>Vitamin K$_2$ + dicoumarol (30 µM/l) (%)</th>
<th>Vitamin K$_1$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>101.3 ± 8.5</td>
<td>100.3 ± 5.8</td>
<td>100.5 ± 1.0</td>
</tr>
<tr>
<td>250</td>
<td>-</td>
<td>-</td>
<td>100.7 ± 0.6</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
<td>-</td>
<td>98.0 ± 3.5</td>
</tr>
<tr>
<td>400</td>
<td>100.0 ± 3.6</td>
<td>122.6 ± 3.3</td>
<td>99.0 ± 6.3</td>
</tr>
<tr>
<td>500</td>
<td>-</td>
<td>-</td>
<td>108.2 ± 8.0</td>
</tr>
<tr>
<td>600</td>
<td>82.0 ± 4.8</td>
<td>95.3 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>700</td>
<td>100.6 ± 1.1</td>
<td>111.3 ± 2.1</td>
<td>-</td>
</tr>
<tr>
<td>800</td>
<td>92.6 ± 3.2</td>
<td>105.0 ± 3.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are percent of the controls and represent the mean ± SEM of three different experiments.

**Table II.** Vitamin K concentration in the medium and in the cells. The values are means ± SEM of three experiments

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (µmol/l)</th>
<th>Medium</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin K$_1$ (micelles)</td>
<td>600</td>
<td>75.6 ± 30.5</td>
<td></td>
</tr>
<tr>
<td>Vitamin K$_2$ (micelles)</td>
<td>600</td>
<td>74.7 ± 25.4</td>
<td></td>
</tr>
<tr>
<td>Vitamin K$_2$ (DMSO)</td>
<td>600</td>
<td>70.0 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Vitamin K$_3$ (DMSO)</td>
<td>500</td>
<td>136.7 ± 30.6</td>
<td></td>
</tr>
</tbody>
</table>

radical scavenger, was limited by the final concentration of the solvent in the medium (1% v/v). For vitamin K$_1$, the presence of sodium deoxycholate and phosphatidylcholine in the preparation of the micelles limited the use of higher concentrations of the vitamin compatible with cell viability, as shown in Figure 4 using the MTT assay.

Cell absorption of the three types of vitamin K are shown in Table II.

**Discussion**

Vitamin K has been recently implicated in the etiopathogenesis of several cancers (11,13) including colorectal cancer. It has been hypothesized that this effect could be related to its redox cycling properties. We have compared the different activity on DNA damage of the natural forms of vitamin K (K$_1$ or phylloquinone and K$_2$ or menaquinone) with the effects of the synthetic form (K$_3$ or menadione).

While synthetic and hydrophilic vitamin K (K$_3$) have been demonstrated to cause DNA damage in different cell systems (rat hepatocytes, K562, L1120, etc.) (18–21), to our knowledge no reports have been published demonstrating that the same effects are exerted on mammalian cells by the natural forms of vitamin K: K$_1$, present in vegetables and K$_2$, produced by different bacterial strains, some of which are located in the human colon (22–24). The structural analogies (Figure 5) of natural and synthetic vitamin K are with regard to the naphthoquinone nucleus that can undergo the ‘redox cycling’ process, with differences related to the length of the lateral side chain responsible for their solubility properties.

In the ‘redox cycling’ process is summarized in Figure 6 the semiquinone may regenerate the quinone through its reaction with molecular oxygen and in the process produce superoxide O$_2^{-}$-.

Similarly the hydroquinone, generated from quinone with a two-electron reduction, may produce O$_2^{-}$- by reaction with molecular oxygen to regenerate the semiquinone.

Oxidative stress following redox cycling often results from a disproportionate consumption of oxygen and cellular reducing equivalents [NAD(P)H], resulting in the generation of active oxygen species (O$_2^{-}$, H$_2$O$_2$ and HO*) (4). The toxicity of quinones is thus influenced by the relative contribution of one-electron or two-electron reduction. The flavoprotein DT-diaphorase, which increases the semiquinone formation, catalyzes the two-electron reduction directly to the hydroquinone, whereas NADPH cytochrome P450 reductase, NADH cytochrome b5 reductase and NADH ubiquinone oxido-reductase can catalyze the electron reduction of the quinone to the reactive semiquinone free radical (25). DT-diaphorase can be inhibited by dicoumarol and may therefore be able to protect the cells from the toxic effect of quinones by competing with the one-electron pathways (26). We investigated the extent of SSB in HT-29 cells exposed to these quinones and demonstrated MD induction of extensive SSB on DNA of enterocytes (HT-29) as reported on other cell systems (18,20,27). The damage was detected at low concentrations (25 µM) reaching a maximum effect at 200 µM. No loss of cell viability was demonstrated by the MTT test. The involvement of radical oxygen species (ROS) in this effect was assessed indirectly by adding, to MD treated cells, an inhibitor of DT-diaphorase, which increases the semiquinone formation...
via the one electron reduction of cytochrome P450 reductase. The results confirmed the role of ROS in DNA damage, as reported by Nutter et al. (27); a five-fold decrease in MD concentration was shown to cause detectable SSB with respect to controls. These results suggest that inhibition of DT-diaphorase mediated metabolism of menadione in HT-29 cells by dicoumarol increases the availability of menadione for single electron reduction and redox cycling resulting in enhanced free radical production.

Vitamin K$_2$ did not cause any appreciable DNA damage at high concentrations (800 µM) with and without dicoumarol present in the culture medium. The same results were obtained using vitamin K$_1$, which was incorporated in micelles to improve its cellular uptake. We chose to deliver vitamin K$_1$ into the cell with micelles because of its elevated liposolubility and because mixed micelles are the natural means for the absorption of all liposoluble substances by the gut (28). In these experiments the cells of control groups were treated with micelles composed of the same substances, but in the absence of vitamin K.

Akman et al. (21) found that phylloquinone (670 µM) raised the GSH pool to 150 ± 21% of the control unlike equitoxic concentrations of MD (72 µM), which actually reduced it to 20 ± 18% of the control. These results confirmed that structurally similar quinones have different modalities of action. Unfortunately we could not reach higher concentrations of the natural quinones (vitamins K$_1$ and K$_2$) in the culture medium because of the concentrations of solvating substances: DMSO, which is a HO$_2^-$ scavenger and NaDOC plus PC for K$_1$, which cause loss of cell viability as demonstrated by the MTT assay. Therefore the lack of effect on DNA damage of vitamins K$_1$ and K$_2$ could be related to a low concentration of these vitamins entering the cells. We evaluated the absorption amount of different types of vitamin K entering the cells: the micelles are a useful carrier for the two liposolubel vitamins as shown in Table II. Nevertheless, vitamin K$_1$ and K$_2$ absorption is about 50% lower compared with vitamin K$_3$, which reaches concentrations at which MD causes DNA damage.

Our results show that the natural forms of vitamin K (phyloquinone and menaquinone) cannot cause DNA damage in HT-29 cells as MD did in the experimental conditions used.

Acknowledgements

The authors are grateful to Ms Chiara Carlotto for her technical assistance. This work was supported by a grant from Consiglio Nazionale delle Ricerche.

References