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Secondary Bile Acid Induced DNA Damage in HT29 Cells: are Free Radicals Involved?

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Increased bile acid secretion, as a consequence of a high fat diet, results in the increased production of bile acids that may escape the enterohepatic circulation, and be subsequently metabolised by the colonic micro-flora to form the co–mutagenic and co–carcinogenic secondary bile acids. The potential of the secondary bile acids lithocholate (LOC) and deoxycholate (DOC), to induce DNA damage, in the colonocyte cell line HT29, at physiological concentrations both individually and in a 2:1 ratio was assessed. Results indicated significant levels of DNA damage induced by both bile acids, with LOC having the greater DNA damaging capacity. The potential role of vitamin A, and the antioxidant vitamin E, in reducing this damage was determined, over a range of vitamin concentrations. Both vitamins reduced the bile acid induced DNA damage. Vitamin A displayed a dose response relationship, whereas vitamin E reduced DNA damage close to negative control values at all concentrations above 50 μM. These results indicate a protective role for Vitamins A and E, against the DNA damaging capacity of LOC and DOC.

Keywords: SCGE assay, bile acid, DNA damage, antioxidants

INTRODUCTION

Bile acids in the colon exist in a micellar solution with phosphatidylcholine, lecithin and cholesterol. Their primary role is to facilitate the absorption of fats and oil soluble compounds across colonocyte membranes, into the cytoso.[1] This property confers a crucial role upon the bile acids, in the uptake of both potential pro-carcinogens such as bacterial menaquinones and iron complexes,[2] vitamin K_1, vitamin A, carotenoid pigments and the major antioxidant vitamin E.

Numerous case control studies have supported the relationship between colorectal cancer (CRC), bile acids and diet,[4–6] some have not.[7,8] A failure to support this relationship may ultimately depend upon the experimental design.

Many mechanistic studies involving both primary and secondary bile acids have indicated the presence of a tumour inducing and promoting capacity, either directly,[9–12] or indirectly, following administration of a carcinogen.[13–15] Bile acid mediated stimulation of colonocyte proliferation, which may play a role in tumour promotion, has been associated with bile acid induced production of reactive oxygen species,[16] protein kinase-C activation (PKC)[17]

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and metabolic products of the arachidonate cascade.[18] Formation of the products of action of the lipoxygenase enzymes, hydroperoxyeicosatetraenoic acid (HPETE), and hydroxyeicosatetraenoic acid (HETE), via the arachidonate cascade have been shown to be associated with bile acid induced increases in colonocyte proliferation.[16] HPETE and HETE have been shown to exhibit a clastogenic action,[19] possibly implicating bile acids in the direct formation of such factors. Kulkarni *et al.*,[20] have shown that lithocholate can cause single strand breaks in the DNA of whole cells and isolated nuclei. Kendall *et al.*[10] have shown DNA damage and unscheduled DNA synthesis in response to bile acids.

Several naturally occurring dietary components are known to counteract oxidative stress and to be potential anti cancer agents. These include, amongst a host of others, the antioxidant vitamins C and E, as well as vitamin A. In this study only vitamins E and A were included. Vitamin A is thought to act by interfering with PKC activation and possibly by reducing levels of superoxide production in cells exposed to tumour promoters.[17] Alternatively, vitamin A may interfere with PKC activation by disordering gel like regions of cell membranes and so interfering with signal transduction.[22] Vitamin E is known to be a "chain breaking" antioxidant, breaking the chain of events involved in lipid peroxidation leading to the formation of the more stable tocopheryl radical,[21] thus protecting the cell from lipid peroxidation and its consequences.

The aim of this study was to determine the DNA damaging effects of a range of concentrations of secondary bile acids and the potential role of vitamin A and the antioxidant vitamin E in modulating this damage, possibly implicating a role for reactive oxygen species.

A variety of techniques exist, used specifically for the detection of altered and/or damaged cellular DNA following exposure to deleterious substances. The technique used to assess DNA damage in the experiments performed in this study was the Single Cell Gel Electrophoresis Assay, or the Comet Assay. This is a simple visual and rapid technique and has recently been extensively reviewed.[23] The technique was developed by Ostling *et al.*[24] and refined by Singh *et al.*[25]

The DNA damaging effects of the secondary bile acids LOC and DOC to the colon carcinoma cell line HT29 were assessed using the comet assay. Protection against this bile acid mediated damage was attempted using vitamin A and the antioxidant vitamin E. Results indicate that preincubation and simultaneous incubation of HT29 cells with these vitamins provides a degree of protection from the DNA damaging effects of secondary bile acids.

MATERIALS AND METHODS

Chemicals

RPMI 1640, Retinol Acetate (tissue culture grade, and water soluble methyl β-cyclodextrin linked), D and L-α–Tocopherol, Lithocholic acid, Sodium Deoxycholate, Dimethylsulphoxide (DMSO), Trypsin-EDTA, Sodium Hydroxide, Lauryl sarcosine, EDTA (di-sodium salt), Methanol, Diethyl Ether, Hexane, Tetrahydrofuran (THF), Ammonium Acetate, Phosphate Buffered Saline (PBS), Sodium chloride, Menadione, β-cyclodextrin and Ethidium Bromide, were all purchased from Sigma, Poole, Dorset. Foetal calf serum was purchased from ICN Flow, High Wycombe. All reagent dilutions were freshly prepared biweekly, except when it was necessary to prepare reagents on the day of use.

Cell Culture

HT29 cells, from an adherent colon carcinoma cell line were maintained at 37°C in an atmosphere of 5% CO_2, 95% O_2. Culture medium RPMI was supplemented with 10% foetal calf serum. For experimental use, cells were treated with trypsin-EDTA to detach them from the tissue culture flask surface. Detached cells were plated

onto 35 mm petri dishes at approximately $2*10^5$/ml, and allowed to adhere to the petri dish surface overnight at 37°C. Following appropriate treatments and incubation periods, cells were removed from their adherent surface by use of a cell scraper to gently detach the cells from the surface of the petri dish. The methyl β-cyclodextrin linked retinol acetate was solubilised in culture media, α-tocopherol was solubilised in 70% ethanol. Sodium deoxycholate was solubilised in phosphate buffered saline, lithocholic acid was solubilised in 50\50 (v\v) DMSO and ethanol. The final concentration of solvent never exceeded 0.5% in any incubation.

Comet Assay

The comet assay was performed essentially as described by McKelvey-Martin *et al.*[23] 85 µl of molten 1% normal agarose in phosphate buffered saline was dropped onto a precoated microscope slide, covered with an 18*18 mm No 1 glass coverslip and left on ice to set. Once set the coverslip was removed, HT29 cells, following appropriate treatment were mixed with 85 µl of 1% low melting point agar and were immediately pipetted onto the layer of agarose on the slide. The coverslip was replaced and the slide placed on ice to allow the agar to set. Slides with coverslips removed were immersed in 150 ml of ice cold lysis buffer, (2.5M sodium chloride, 83mM EDTA, 10mM TRIS, 1% N-lauryl-sarcosine, and adjusted to pH 10 with sodium hydroxide pellets), containing 1% triton-X 100 (v\v) and 10% DMSO (v\v). The cells in lysis buffer were incubated at 4°C for 60–90 minutes. All steps following lysis were performed under diffuse light. On removal from lysis buffer, slides were placed into an electrophoresis tank containing, as the electrode buffer, 0.3M sodium hydroxide and 1mM EDTA. The slides containing the cells were incubated in the electrophoresis buffer for 20 minutes prior to electrophoresis. Electrophoresis was performed at 20 volts/32mA for 24 minutes. Slides were then transferred to an absorbent surface and washed three times in neutralising buffer, (100mM TRIS, pH7.5). Cell nuclei and any extra cellular DNA was stained using 10 µl of the fluorescent stain, ethidium bromide added to the agarose on the slide. A coverslip was then replaced and slides were analysed immediately or stored overnight in a humidified atmosphere at 4°C. The cells were scored using a Nikon fluorescence microscope and Kinetica Imaging image analysis and statistical calculation software. At present, in our laboratory, the comet assay is used in conjunction with computer aided image analysis and a range of relevant statistical analyses, to quantify the extent of DNA damage to cells, in a project concerned with the standardisation of results, irrespective of the type of treatment or cell type. The parameter used to measure the DNA damage in this study was "relative tail moment". This refers to the product of the amount of DNA in the tail and the mean distance of migration of the DNA in the tail, divided by the negative control value. Results represent mean data from three experiments, scoring 30 cells/slide or dose, ± S. E. M. and typical results are displayed here.

Vitamin Uptake Determined By HPLC

HT29 cells were pre-incubated with appropriate vitamin (A and E), in serum free media at appropriate concentrations for 15 and 30 minutes respectively. Following incubation, cells were washed three times with PBS. Cells were lysed with 1 ml of methanol for 20 minutes at 4°C. Proteins were precipitated with 1 ml of 1 M sodium chloride. The vitamins were extracted by the addition of 1.5 ml diethyl ether and 1.5 ml of hexane. The sample was mixed and the phases allowed to separate. The organic layer was removed and dried under a stream of nitrogen. The extract was resuspended in 20 µl THF and 180 µl of ethanol. 20 µl of the sample was injected onto a reverse phase ODS2 column (250 * 46 mm) via a Rheodyne valve. The vitamins were eluted using an isocratic solvent system, composed of

acetonitrile, THF, methanol and ammonium acetate (65:22:10:0.02). The flow rate was set at 1 ml/minute and the vitamins were detected at 290nm using a scanning wavelength detector, integration was performed using software provided by spectra physics. Appropriate controls were set up to determine endogenous vitamin levels in HT29 cells. Results obtained for vitamin uptake were adjusted to account for these endogenous concentrations.

RESULTS

Secondary Bile Acid Induced DNA Damage

HT29 cells incubated for 60 minutes at 37°C in the presence of the secondary bile acid (LOC), at a range of physiologically relevant concentrations (25–300 μM), and with the appropriate negative and positive controls (menadione 10 μM), showed extensive DNA damage as determined by the comet assay. The extent of the DNA damage induced by lithocholate appeared to be dose dependent with the highest concentration tested (300 μM), resulting in the largest relative tail moments (Fig. 1). This trend was repeated when the secondary bile acid (DOC), was assessed for its DNA damaging capacity using HT29 cells, with the same incubation period and bile acid concentrations as for LOC above. The extent of DNA damage induced by the highest concentration of DOC tested (300 μM), gave relative tail moments of 3.4 as opposed to LOC at 300 μM which gave relative tail moments of 6.3. The relative difference in the DNA damaging potential of these two secondary bile acids was maintained at all of the concentrations tested, indicating a greater DNA damaging potential for LOC compared to DOC at equivalent concentrations. DNA damage induced by these secondary bile acids in HT29 cells was assessed at 60 minutes only, and a linear increase in DNA damage was seen up to this time point. However, the DNA damaging potential of LOC and DOC to HT29 cells continued to increase above the 60 minute incubation point. Results, with respect to relative tail moments, appear to indicate a non linear increase in damage from 60 minutes up to 180 minutes incubation, (results not shown). The reason for this apparent reduced rate of DNA damage is due to solely to the limitations of the software application used to assess DNA damage, since it is unable to quantify severe damage, instead indicating a negative result, an observation confirmed by visual means. Sixty minutes was chosen as the incubation period as DNA damage increases linearly and is quantifiable around this point.

Owen *et al.*,[3] have suggested that a 2:1 ratio of LOC to DOC in the colon may predispose individuals to an increased risk of CRC. To investi-

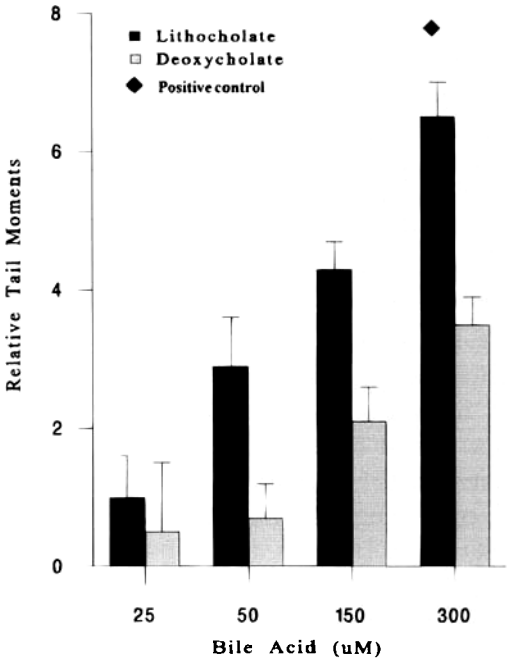

FIGURE 1 Dose response data from the Comet Assay, for the DNA damaging effect of the secondary bile acids lithocholate and deoxycholate, upon HT29 cells, an adherent colon carcinoma cell line. Relative tail moments, are displayed here, that is, the tail moment divided by the control value, (2.2) ± S.E.M, (S. E. M. values equal to or greater than 0.5 displayed in these results). Results represent the mean data from three experiments, in this set experiments 10 μM menadione was used as the positive control, producing relative tail moments of 7.7.

gate whether the presence of a 2:1 ratio of LOC : DOC may be responsible for an increase in risk of CRC, and may be due in some part to the concentration of the LOC in this ratio, experiments were performed using a 2:1 ratio in favour of either bile acids at a range of concentrations, from 12.5–300 μM. Results shown in Figure 2., indicate that DNA damage was greatest when LOC was the predominant bile acid in the ratio. DOC as the predominant bile acid, consistently, at all concentrations tested, lead to an increased level of DNA damage when compared to values obtained for DOC alone (Fig. 1). However, this DNA damage was approximately 50% less than that caused by LOC alone at equivalent concentrations.

Cell viability determined by trypan blue exclusion, as shown in Table I., indicated that DOC individually or in a ratio, at the highest concentration used in these experiments (300 μM), did not induce membrane damage (dye uptake), 2.4% ± 1.1 of cells failed to exclude the dye, compared to negative controls of 1.9% ± 1.2. LOC at 300 μM induced an increase in the number of membrane damaged cells, (12% ± 4.7) when compared to the negative control of 1.9% or DOC at equivalent concentration, (2.4% ± 1.1). This 12% of cells failing to exclude the dye on exposure to 300 μM LOC did not account for the number of DNA damaged cells detected in the comet assay, as up to 90% of HT29 cells exposed to 300 μM LOC displayed DNA damage of some kind, as assessed by the comet assay:

Reduction of Lithocholate Induced DNA Damage by α—Tocopherol

HT29 cells were incubated simultaneously with a known DNA damaging concentration of LOC

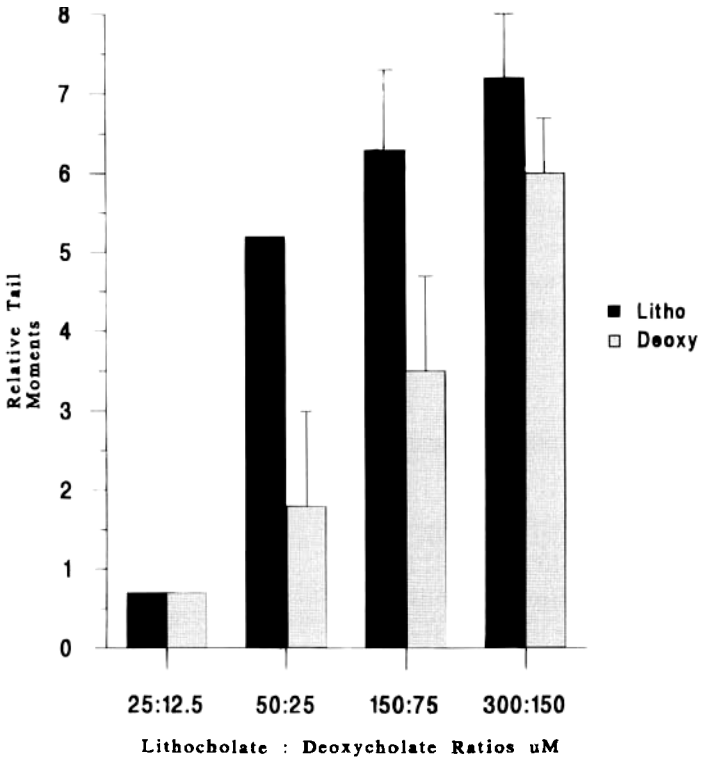

FIGURE 2 Dose response data from the Comet Assay, for the DNA damaging effect of a 2:1 ratio of lithocholate: deoxycholate and deoxycholate: lithocholate, bile acid concentration was the same at each experimental point. Experimental detail as Figure 1. (control relative tail moment = 2.25).

TABLE I Cell viability determined by uptake or exclusion of the vital stain trypan blue, by HT29 cells, following 60 minutes exposure of cells to the secondary bile acids lithocholate and deoxycholate. Control cell values indicated that 98.1% ± 1.3% of cells excluded the dye, with 1.9% ± 1.2% failing to exclude the dye. DNA damage to control cells never exceeded 2%. DNA damage was assessed by performing a comet assay.

Bile Acid Conentration, (μM)	Dye Exclusion, (% Cells)	Dye Uptake (% Cells)	Cells diplaying DNA damage
Lithocholate 300	88.0 ± 5.1	12 ± 4.7	>90%
Deoxycholate. 300	97.6 ± 3.2	2.4 ± 1.1	90%

(300 μM) and a range of concentrations of α-tocopherol (0–500 μM) for a period of 60 minutes at 37°C, with the appropriate controls. Results from these experiments indicate that protection against LOC induced DNA damage is conferred upon the HT29 cells by α-tocopherol, at a range of concentrations above 50 μM, (Fig. 3.). Below 50 μM the protective effect of α-tocopherol is not seen, and LOC induced DNA damage close to 300 μM LOC alone, in the presence of 10 μM α-tocopherol (relative tail moments of 6.5 ± 1 and 5.0 ± 1.25 respectively). Pre incubation of HT29 cells for 30 minutes with this range of α-tocopherol concentrations, followed by removal of α-tocopherol and exposure to 300 μM LOC for 60 minutes at 37°C, gave results similar to the simultaneous addition of these components for 60 minutes, (results not shown). That these data do not yield a linear dose response, may indicate a saturation of the α-tocopherol uptake mechanism at concentrations above 50 μM.

Retinol Acetate Reduces Lithocholate Induced DNA Damage

HT29 cells were pre incubated with a range of concentrations of retinol acetate (0–100 μM) for 15 minutes at 37°C, after which time the retinol acetate was removed by washing the cells three times in serum free media. Following this removal, the cells were incubated for 60 minutes at 37°C with a DNA damaging concentration of LOC (300 μM). Appropriate controls were included in these experiments, (see figure. 4). Results shown in figure. 4, indicated that the extent of DNA damage induced by 300 μM LOC was dependent upon the concentration of the retinol acetate used for the pre incubation step, with the highest concentration of retinol acetate used (100 μM) affording almost 100% protection to the HT29 cells from the DNA damaging effects of LOC, with relative tail moments of 0.75 ± 1. Moreover, a dose response relationship exists

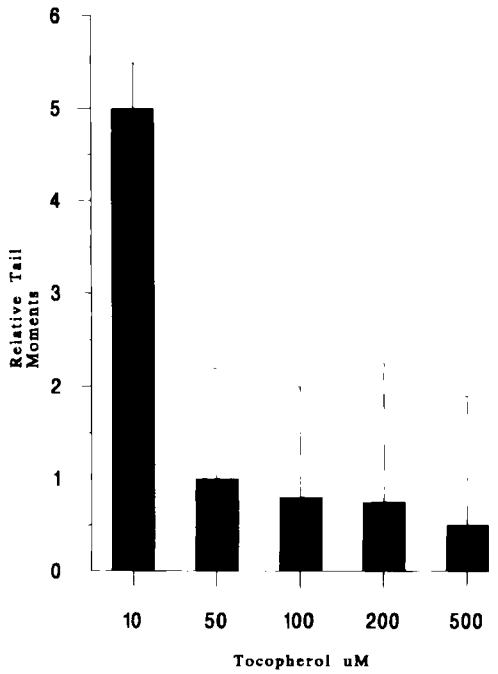

FIGURE 3 Dose response data from the comet assay, indicating the protection that α tocopherol confered upon HT29 cells, from the DNA damaging effects of 300μM lithocholate. Cells were incubated simultaneously with 300μM lithocholate and a range of α tocopherol concentrations. See Figure 1. for experimental detail. (control relative tail moment = 1.25).

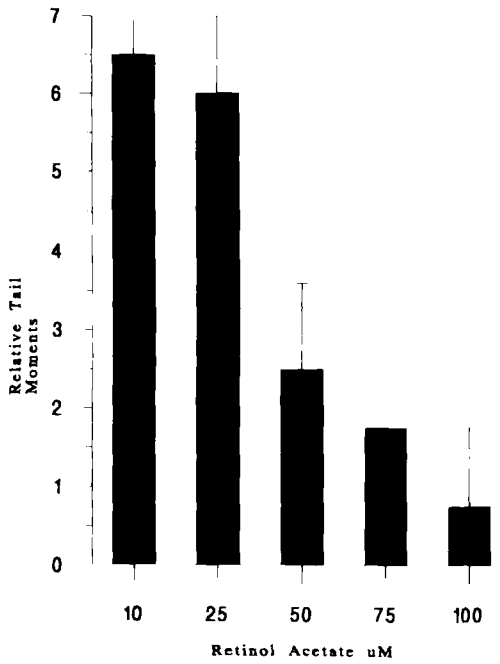

FIGURE 4 Dose response data from the comet assay, indicating the protective role of retinol acetate, from the DNA damaging effects of 300μM lithocholate, in HT29 cells. See Figure 1. for experimental detail. (control relative tail Moment = 4.0). Cyclodextrin control relative tail moment = 8.5 ± 1.3.

between DNA damage detected in response to LOC incubation and retinol acetate concentration. The lowest concentration of retinol acetate (10 μM), was apparently unable to afford any protection from the DNA damaging effects of 300 μM LOC, with relative tail moments of 6.5 ± 1 (300 μM LOC alone induces relative tail moments of 6.5–7).

Vitamin Uptake By HT29 Cells

Uptake of 100 μM α-tocopherol by HT29 cells, following a 30 minute incubation at 37°C was determined by HPLC to be 30 μM. This result was adjusted to account for endogenous vitamin E (10 μM), giving a total concentration of 40 μM vitamin E in the cell, prior to bile acid treatment. A total of 40 μM α-tocopherol was unavailable for uptake due to adherence to culture dish or vitamin containing vessel. Vitamin uptake was determined to be linear up to the 30 minute incubation period, (30 minutes was used as the pre-incubation step in work involving vitamin E).

Uptake of 100 μM retinol acetate by HT29 cells, following a 15 minute incubation at 37°C was determined by HPLC to be 48.5 nM. This result was adjusted to account for endogenous vitamin A (10.5 nM), giving a total concentration of 59 nM vitamin A in the cell, prior to bile acid treatment. Vitamin uptake was determined to be linear up to the 15 minute incubation period, (15 minutes was used as the pre-incubation step in work involving vitamin A).

DISCUSSION

Secondary Bile Acid Induced DNA Damage

The secondary bile acids LOC and DOC, at physiological concentrations (12.5–300 μm), were found to cause extensive DNA damage in HT29 cells, following a 60 minute incubation period at 37°C. Moreover, a previously unrecognised dose response relationship appears to exist for both of these secondary bile acids, with the highest concentrations inducing greater levels of DNA damage. However, although both bile acids were capable of inducing extensive DNA damage, LOC consistently at all concentrations tested produced the greater level of damage, when compared to DOC at equivalent concentrations, (Fig. 1). The demonstration of LOC induced cellular DNA damage is not unique. Kulkarni *et al.*[20] demonstrated LOC induced DNA damage (single strand breaks) in intact cells, isolated nuclei and mixed lysates. Kendall *et al.*[10] have indicated a DNA damaging effect of chenodeoxycholate and DOC in bacterial and mammalian cells. However, this is the first report to our knowledge, involving the use of the comet assay to detect and visualise DNA damage induced by secondary bile acids.

Owen *et al.*[3] indicated an increased risk of CRC to individuals with a 2:1 ratio of the sec-

ondary bile acids LOC:DOC in the colon. As we have demonstrated that LOC alone is capable of inducing greater levels of DNA damage than DOC (Fig. 1), the possibility was investigated that a 2:1 ratio of LOC:DOC may predispose individuals to an increased risk of CRC, and may be due in part to a concentration effect of LOC, hence LOC induces greater DNA damage as the predominant bile acid in the ratio. Results shown in Figure 2 indicated that it is the total concentration of LOC in the mixture at each experimental point that determines the level of DNA damage, rather than the ratio of bile acids. This point is emphasised by the observation that the 2:1 ratio favour of DOC consistently produced levels of DNA damage greater than with DOC alone. These results indicate that LOC and DOC do not appear to act synergistically to induce DNA damage in HT29 cells, rather the LOC concentration is responsible for determining the majority of the DNA damaging capacity of either ratio. These results may also indicate that the bile acid induced mechanism of DNA damage, may not principally be due to the detergent properties of the bile acids. Saturable sites may be involved, with a high affinity for LOC, compared to DOC. This possibility is currently under investigation.

α-Tocopherol Reduces Lithocholate Induced DNA Damage

α-Tocopherol, a potent chain breaking anti-oxidant, acts to break the chain of events involved in lipid peroxidation. Protecting the cell from lipid peroxidation and related effects, which may lead to membrane collapse and ultimately to cell death. Our results indicate that α-tocopherol at a concentration above 50 μM is able to protect HT29 cells from the DNA damaging effects of LOC at 300 μM, (Fig. 3.). Below a concentration of 50 μM α tocopherol, LOC induced DNA damage is present at or close to that produced by 300 μM LOC alone, with relative tail moments of 5.0 ± 1.25. Pre incubation of HT29 cells with this range of α-tocopherol concentrations, followed by removal of α-tocopherol and exposure to 300 μM LOC for 60 minutes at 37°C, produced similar results to the simultaneous additions, (results not shown). These results indicate that α-tocopherol is not protecting the cells from the DNA damaging effects of LOC by formation of a micellar solution with LOC. These data do not yield a linear dose response and may indicate a saturating of the α-tocopherol uptake mechanism at concentrations above 50 μM. The protective effects of α-tocopherol from the DNA damaging potential of LOC, may represent a simple antioxidant effect (prevention of lipid peroxidation). However, an alternative protective role for α-tocopherol has been indicated. Suzuki *et al.*[26] have shown that incorporation of α-tocopherol into membranes, modifies acyl chain ordering and reorientation dynamics within the membrane. Lipid mobility is increased in the gel phase, whereas the liquid crystalline phase order is increased, and the rate of acyl chain reorientation is decreased. The resultant effects upon cell membranes due to these changes may be to alter them in such a way as to reduce their susceptibility to damaging species. However, such changes in membrane lipids will also result in a high efficiency of α-tocopherol recycling, which will also play a part in protecting cell membranes from damage. It is quite clear that α-tocopherol has the ability to inhibit a wide range of cellular damage induced by chemicals and radiation and in some cases the α-tocopherol is clearly acting as an antioxidant,[27] but in the majority of studies the mechanism of α-tocopherol protection remains unclear. Clarification of the results presented above is currently being attempted.

Retinol Acetate Reduces DNA Damage Induced by Lithocholate

Retinol acetate at 100 μM was shown to reduce to negative control levels, LOC (300 μM), induced DNA damage in HT29 cells, (Fig. 4). The cells were pre-incubated with retinol acetate for 15 minutes, prior to incubation with LOC. A dose response relationship was found to exist, with

100 μM retinol acetate providing almost complete protection from the DNA damaging effects of 300 μM LOC, with relative tail moments of 0.75 ± 1 and 10 μM retinol acetate unable to protect from the damaging effects of LOC, leading to relative tail moments of 6.5 ± 1 (LOC alone at 300 μM induces tail relative moments of 6.5–7.5). A 15 minute pre-incubation of HT29 cells with 100 μM β-Cyclodextrin alone, followed by 60 minutes incubation with 300 μM LOC, indicated that β-cyclodextrin is unable to modulate LOC induced DNA damage, producing relative tail moments of 8.5 ± 1.3. A 15 minute incubation of HT29 cells with 100 μM β-Cyclodextrin only, indicated that β-Cyclodextrin alone is unable to damage DNA of HT29 cells, producing relative tail moments of 1.2 ± 0.5 (results not shown). Unlike the experiments with α-tocopherol, above, retinol acetate was not present in the incubation media with LOC. This suggests that a reduction in the level of DNA damage to HT29 cells may be due to some effect of the retinol acetate upon the cells prior to addition of LOC. Craven *et al.*,[17] have previously indicated that retinoids may inhibit or interfere with colonocyte signal transduction by reducing Protein Kinase C (PKC), activation in response to tumour promoters. Witz *et al.*[22] have shown a potential mechanism of action of retinoids, in neutrophils at least, is in altering cell membrane fluidity. The effects seen by Craven *et al.*,[17] may be due in part to a general effect of retinoids in altering the dynamic properties of plasma membranes. Similarly, the results seen in our study may be due in part to alterations in plasma membrane properties, stabilising the membranes or altering them in a way that confers a greater degree of protection from the membrane and DNA damaging effects of secondary bile acids. The alterations in signal transduction and inhibition of PKC by retinoids may indicate a role for superoxide in the DNA damaging potential of these bile acids, as PKC activation has been shown by Craven *et al.*[16,17] to be involved in the bile acid induced production of superoxide, potentially indicating a role for free radicals in the DNA damaging potential of secondary bile acids. However, retinoids do not scavenge superoxide to affect a reduction in DNA damage, as Witz *et al.*,[22] and others have shown no decrease in cytochrome-C reduction in a xanthine-xanthine oxidase system, in the presence of retinoids. Therefore, any protection from the DNA damaging mechanism of bile acids, is not due to a scavenging effect of retinol acetate. However, retinol acetate may in conjunction with a stabilising effect upon the plasma membrane, prevent, or reduce formation of what ever amount of superoxide is produced in response to exposure to secondary bile acids. The possible involvement of free radicals in LOC induced DNA damage is currently under investigation.

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