Acid and bile salts induce DNA damage in human oesophageal cell lines

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Barrett’s oesophagus is an acquired precancerous condition that develops from mucosal injury incurred due to chronic gastro-oesophageal reflux. The aim of this study was to determine if bile and/or acid components of the refluxate can induce DNA damage in vitro. The oesophageal cell lines FLO-1 and HET1-A were exposed to primary bile salts, individually or as a mixture, and the secondary bile salt sodium deoxycholate, in neutral or acidified media. Cells were then examined in the comet assay to measure DNA strand breaks. Cell viability was also monitored. Acidified media induced DNA damage in a pH- and time-dependent manner. The primary bile compounds sodium glycocholate, glycocholic acid, sodium taurocholate and taurochenodeoxycholate, as an equimolar mixture (100 µM), caused a small but significant ($P < 0.028$) elevation in DNA damage, but only at neutral pH in FLO-1 cells. Sodium deoxycholate (100 µM) caused a significant ($P < 0.008$) elevation in DNA damage in both cell lines, but again only at neutral pH. These data suggest that specific components of gastro-oesophageal refluxate are capable of causing DNA damage and may participate in the genesis and progression of Barrett’s oesophagus via this mechanism.

Introduction

Over the last two decades the incidence of adenocarcinoma of the oesophagus (AO) has increased in Western countries (Devesa et al., 1998; Botterweck et al., 2000; Wild and Hardie, 2003). The tumour is normally detected at an advanced stage, therefore prognosis is particularly dismal, with average 5 year survival rates of 15% (Chen and Yang, 1999) at 5 years (Chen and Yang, 2001). AO is frequently found in association with specialized intestinal metaplasia of the distal oesophagus, commonly termed Barrett’s oesophagus (BO). Indeed, BO is recognized as a precancerous lesion and patients with BO are at over 40-fold increased risk of developing AO when compared with the general population (Altorki et al., 1997). Gastro-oesophageal reflux disease (GORD) is a major risk factor for the development of both BO and AO (Lagergren et al., 1999; Wild and Hardie, 2003). GORD is a common condition that affects >10% of individuals in Western populations (Petersen, 1995; Locke et al., 1997). Despite the association of GORD with AO and BO, the biological mechanism underlying this association is poorly defined.

The acidic component of reflux has been considered the principal mediator of GORD-induced oesophageal injury. This is because ambulatory pH monitoring studies reveal that patients with BO have increased oesophageal exposure to gastric acid and experimental studies demonstrate that acid damages the oesophageal mucosa and can promote regeneration with a columnar rather than a squamous epithelial phenotype (DeMeester and DeMeester, 2000; Falk, 2002).

Acid exposure has a range of biological effects in oesophageal tissue and cell lines; stimulating MAP kinase activation and cyclooxygenase-2 expression and modifying proliferation and differentiation rates (Fitzgerald et al., 1996, 1998; Ouatu-Lascar et al., 1999; Kaur et al., 2000; Shirvani et al., 2000; Souza et al., 2002). Despite the introduction of acid suppressive therapy, a reduction in the risk of neoplastic progression has yet to be observed (Fitzgerald et al., 2001) and it is possible that factors in addition to acid may be important determinants in the pathogenesis of GORD.

Considerable attention has focused on the role of bile acids during alkaline duodenal gastro-esophageal reflux (DGOR), in view of their role as tumour promoters in colorectal carcinogenesis and the fact that BO patients have more bile-associated reflux compared with esophagitis and control groups (Stein et al., 1998; DeMeester and DeMeester, 2000; Debruyne et al., 2001). Bile acids have a diverse spectrum of biological activity; being able to stimulate proliferation and tumour invasiveness, inhibit apoptosis and modify the promoter function of genes involved in DNA synthesis, DNA repair and oxidative stress (Bernstein et al., 1999; Debruyne et al., 2001; Lechner et al., 2002). Some of these effects may be mediated through the bile acid-specific orphan nuclear receptor, FXR, and protein kinase C activation (Wang et al., 1999; Debruyne et al., 2001). Again, limited information exists concerning the molecular response to bile within the oesophagus, but ex vivo exposure of BO tissue to bile salts has been shown to up-regulate cyclooxygenase-2 expression and modify cell proliferation rates (Kaur et al., 2000; Shirvani et al., 2000).

Characterizing DGOR has proved difficult to date, but recent oesophageal aspiration studies in humans estimate total bile acid values to be in the micromolar range (0–820 µM) and suggest that the predominant bile acids reaching the oesophagus are cholic acid and its taurine and glycine conjugates (Nehra et al., 1999). Interestingly, the secondary bile salt, deoxycholic acid, and its conjugates have also been reported in micromolar concentrations in oesophageal aspirates from BO patients (Nehra et al., 1999).

The development of BO is associated with the accumulation of genetic and epigenetic alterations that lead to increased malignant potential (Chen and Yang, 2001; Wild and Hardie, 2003). One mechanism by which these molecular changes could arise is via DNA damage stimulated directly by components of DGOR. Currently there is little evidence to support the formation of DNA damage directly by bile acids. In

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addition there are no studies of the effect of low pH conditions on DNA damage in oesophageal cells. Therefore, in this study we tested the hypothesis that low pH or bile salt conditions, consistent with reflux episodes in BO patients, stimulate DNA damage in oesophageal cell lines.

Materials and methods

Materials

The Cytotox 96 non-radioactive cytotoxicity kit for the measurement of lactate dehydrogenase (LDH) release was obtained from Promega UK. Epithelial cell medium (ECM) and Dulbecco’s MEM with l-glutamine (DMEM) were obtained from Promocell GmbH (Heidelberg, Germany) and Life Technologies UK Ltd, respectively. All other reagents were obtained from Sigma UK Ltd.

Cell culture

Normal oesophageal cells or Barrett’s epithelia are not readily obtained in sufficient quantities for detailed laboratory analysis. Therefore, we used the SV40-transformed, squamous oesophageal epithelial cell line HET1-A and the Barrett’s associated adenocarcinoma cell line FLO-1 as models to investigate the genotoxic potential of bile and acid in the oesophagus. HET1-A is non-tumourigenic in athymic mice and expresses cytokeratin markers demonstrating the epithelial origin of these cells (Stoner et al., 1991).

HET1-A was cultured in ECM without antibiotics; FLO-1 was cultured in DMEM supplemented with 10% bovine serum, gentamicin (50 µg/ml) and amphotericin B (2.5 µg/ml). Both cell lines were grown in 75 cm² flasks at 37°C in 5% CO₂. Prior to experimental treatments, cells were detached from culture vessels with trypsin and 5 × 10⁵ cells aliquoted into wells of a 24-well tissue culture plate with appropriate medium for 16 h.

Acid and bile salt treatments

For acid conditions, serum-free culture medium was acidified with 1 N HCl (~3% v/v) to achieve the required pH. The pH was stable over the treatment period. To control for changes in osmolality, sterile water was added to control cells at a volume equal to acid-treated cells (~3% v/v). The primary bile acids or salts, sodium glycolate, glycocholic acid, sodium taurocholate and sodium taurochenodeoxycholate, were dissolved in serum-free medium. These preparations were added as an equimolar mixture (0–100 µM) for 3 h if cultured at pH 7 or for 15 min if cultured at pH 4.5. This composition of bile salts is physiologically relevant in BO patients based on observed concentrations in reflux fluids (Nehra et al., 1999) and has been used by other workers for in vitro treatments of squamous oesophageal biogels (Kaur et al., 2000). The secondary bile salt sodium deoxycholate (NDC) dissolved in ethanol was also studied (0–500 µM), but was not included in the initial composition. The ethanol concentration in contact with cells never exceeded 0.02%. After bile and/or acid exposure, the medium was carefully removed and stored for LDH analysis.

Cell viability

Cells were rinsed twice in phosphate-buffered saline (PBS), trypsinized and processed for the comet assay. Cell viability was also assessed by trypan blue exclusion.

Comet assay

Applied under alkaline conditions, the comet assay, also known as the single cell gel electrophoresis assay, is a useful technique for detecting single-strand breaks and alkaline-labile sites in DNA at the single cell level (Singh et al., 1988; Tice et al., 2000).

Following treatment with bile and/or acid, cells were resuspended in 1 ml of 0.8% low melting point (LMP) agarose in PBS at 37°C. Onto a slide precoated with 1% normal melting point agarose, 100 µl of cell suspension was applied with a coverslip and briefly incubated on ice to allow the cell suspension to solidify. A further 80 µl of LMP agarose was applied as above. Slides were placed in cold lysis buffer (2.5 M NaCl, 1 mM EDTA, 10 mM Tris, adjusted to pH 10, made to 10% DMSO, with 1% Triton X-100 added immediately prior to use) at 4°C in the dark for 1 h. Slides were then incubated in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) at room temperature for 40 min to facilitate DNA unwinding. This was immediately followed by electrophoresis at 23 V, 300 mA for 20 min. Slides were washed in neutralization buffer (0.4 M Tris, pH 7.5), then stained with 20 µg/ml ethidium bromide. Comets were visualized at 250× magnification by fluorescence microscopy. For each treatment, the percentage of DNA present in the tail of 125 cell comets was quantitated using an image analysis system (Komet 4; Kinetic Imaging Ltd, Liverpool, UK).

Statistical methods

Comet assay data were not normally distributed, therefore the median value from each treatment was calculated to provide an appropriate quantitative measure of the data in preference to the mean, which is sensitive to a small number of extreme measures (Lovell and Thomas, 1997). Except where indicated, experiments were repeated on three separate occasions and the data were expressed as the mean per cent release of total LDH. Analysed by ANOVA. The statistical significance of individual treatment groups in comparison with controls was subsequently determined with Dunnett’s multiple comparison post-test. Significant P values were accepted at the 0.05 level (SPSS version 11; Chicago, IL).

Results

Cell viability

Both acid and bile salts can be directly cytotoxic, thus cell viability, as judged by membrane integrity, was assessed in treated cells prior to the analysis of DNA damage.

At pH 3.5, >80% of FLO-1 cells continued to exclude trypan blue in excess of 210 min exposure, contrasting with HET1-A cells which excluded trypan blue for only 60 min in medium at pH 3.5, indicating greater acid sensitivity in this cell line (Figure 1A). For all acid and bile conditions utilized in the DNA damage studies below, trypan blue dye exclusion indicated that >85% of cells remained viable immediately post-treatment.

The release of LDH is a sensitive indicator of membrane integrity and was employed to provide an additional measure of cell viability. At neutral pH LDH leakage (>20%) was elicited in HET1-A cells by NDC and the bile mixture when present at 250 µM and above, but in FLO-1 only when NDC was present.
at 500 μM (Figure 1B and C). The maximal LDH release observed did not exceed 40% even at the highest concentrations used. These findings were consistent with the visual observation of loss of cell adhesion in cells incubated in NDC at concentrations of 500 μM.

Acid conditions interfered with the LDH assay, therefore an additional trypan blue dye exclusion test was applied following an extended incubation of the cells immediately after acid treatment, for 10 h in fresh medium at neutral pH. Results revealed no further loss of cell viability post-treatment (data not shown).

Together these data confirm that DNA damage was measured in viable cell populations following acid and/or bile salt treatment.

**DNA damage is induced by acid in a pH- and time-dependent manner**

DNA damage significantly increased when HET1-A cells and FLO-1 cells were exposed to acid conditions of ≲pH 4.5 for 30 min, in comparison with control cells at pH 7.5 (P < 0.001) (Figure 2A). The extent of DNA damage was dependent on the exposure time (Figure 2B). In both cell lines, DNA damage increased 2-fold compared with control values even after a 10 min acid pulse (pH 3.5).

In an additional experiment, no loss of cell viability and relatively moderate tail DNA damage (range 7–14%) was incurred at pH 4.5 with an exposure period of 15 min (data not shown). These conditions were therefore selected for subsequent experiments with bile salts at low pH to permit detection of any additional DNA damage stimulated by bile salts, superimposed on the effect of acid alone. At pH 4.5 primary bile salts are in a mixed ionized state, capable of entering cells (Nehra et al., 1999; DeMeester and DeMeester, 2000).

**Primary bile salts cause small increases in DNA damage levels at neutral but not acid pH**

Preliminary experiments exposing FLO-1 cells to individual primary bile salts (1–500 μM) under neutral and acid conditions induced only small increases in DNA damage (<1.5-fold), with the exception of sodium glycocholate, which caused a 2-fold increase in median per cent tail DNA at 500 μM (data not shown). In order to study potential effects of combinations of primary bile salts both cell lines were exposed to an equimolar mixture (1–100 μM) at neutral (pH 7, 3 h) or acidic (pH 4.5, 15 min) pH. At pH 7 mean DNA damage was raised 1.5- to 2-fold by the addition of an equimolar mixture of bile salts to both HET1-A and FLO-1 cells, respectively (Figure 3A). However, the increase in DNA damage was only significant (P < 0.028) in FLO-1 cells exposed to 100 μM bile salt mixture. At pH 4.5 addition of the bile salt mixture did not stimulate DNA damage above the level induced by low pH alone (Figure 3B).
However, this study provides in vitro evidence to suggest that exposure of the oesophageal epithelium to acid or bile reflux may also stimulate tumourigenesis via a directly genotoxic route.

The mechanism by which acid and bile salts stimulate DNA damage in oesophageal cells is currently unclear. Xiao et al. (2003) demonstrated that induction of topoisomerase II mediated DNA damage at acidic pH in a mouse skin model. An alternative mechanism could be via the induction of oxidative stress. Both acid and bile salts have been shown to induce reactive oxygen species (ROS) (Bagchi et al., 1999; Lechner et al., 2002). The comet assay employed in this study detects DNA strand breaks and alkali-labile sites, consistent with forms of DNA damage stimulated by ROS. However, alternative forms of DNA damage may also be stimulated directly by bile; for example, 32P-postlabelling techniques have shown that human bile can induce DNA adducts in vitro (Scates et al., 1996, 1997).

Our observations of different effects of bile salts on DNA damage at acidic and neutral pH, with effects of both conjugated and unconjugated bile acids apparent only under conditions of neutral pH, are informative. At neutral pH bile salts are in the ionized form, but under conditions of lower pH, increasingly dissociate into free acid forms. Previous studies have suggested that bile salts may be most injurious to cells between pH 3 and 6 when in solution as free acids. It is proposed that being lipophilic, free acid forms are able to enter the cell, become trapped by intracellular ionization, accumulate and exert toxicity on mitochondria (DeMeester and DeMeester 2000). In contrast, it would appear that DNA-damaging effects observed in this study do not require the free acid forms of bile salts. It is possible that the lack of DNA damage observed at acidic pH in the current study could reflect some precipitation of bile acids. This may have been a factor for deoxycholic acid, as precipitation of unconjugated bile acids starts to occur below pH 5, but is unlikely to apply for the glycine and taurine conjugates which precipitate at less than pH 4 and 2, respectively (Schweitzer et al., 1986; Bechi et al., 2000).

Uncontrolled DGOR will normally occur in an acidic milieu (pH < 4) (Nehra et al., 1999), where our results indicate that DNA damage would be stimulated by the acid component of reflux, but not bile salts. However, following pharmacological acid suppression of reflux symptoms, gastric pH values commonly reach 6–7. Under these conditions, acid suppressive therapy may actually enhance the DNA-damaging effects of reflux components such as bile salts, which are normally rendered innocuous by a lower pH milieu. A better understanding of the potential for DNA damage at higher pH will be informative in balancing any detrimental effects against the benefits of controlling acidity in reflux.

The induction of strand breaks by sodium deoxycholate has been reported previously in colonic cells (Powolny et al., 2001; Glinghammar et al., 2002). However, sodium deoxycholate has also been noted to cause cellular DNA fragmentation in a number of cell lines, at similar concentrations to those employed in this study (Martinez et al., 1998; Larue et al., 2000), therefore, it could be argued that the increased DNA damage measured in this study is a result of direct cytotoxicity or is incidental to the onset of apoptosis. Concerning cytotoxicity, Hartmann et al. (2001) have shown that extensive DNA fragmentation induced by cytotoxicity does not lead to elevated migration values (false positives).

The secondary bile salt NDC increases DNA damage at neutral but not acidic pH

At neutral pH exposure of both cell lines to NDC caused a significant increase in DNA damage. In HET1-A cells there was a dose-related increase that was evident above 1 μM and significant at 10 μM (P < 0.006) (Figure 4A). FLO-1 cells appear less sensitive to NDC but significant increases were observed at 100 μM and above compared with control cells (P < 0.008) (Figure 4A). When NDC was applied to cells at acidic pH there was no significant induction of DNA damage compared with acid medium alone (Figure 4B).

Discussion

Under physiologically relevant conditions, this study has demonstrated that low pH and both primary and secondary bile salts under neutral pH conditions can cause DNA damage in oesophageal cell lines. Previous studies on bile exposure have been suggestive of a promoting function in colon cells because proliferation is stimulated, apoptosis inhibited and tumour invasiveness increased (Debruyne et al., 2001). Potentially promoting effects reported in oesophageal tissue and cell lines for bile and acid include increased rates of proliferation, reduced differentiation and elevated cyclooxygenase-2 expression (Fitzgerald et al., 1996; Ouatu-Lascar et al., 1999; Kaur et al., 2000; Souza et al., 2002). However, this study provides in vitro evidence to...
in the comet assay, a finding independently supported by Roser et al. (2001). Furthermore, Henderson et al. (1998) suggest that only when cell viabilities fall below 75% may false positive results arise in the comet assay; in this study, cell viability always exceeded 85%. Therefore, our observation of increased DNA damage in oesophageal cells exposed to sodium deoxycholate is not attributable to a cytotoxic effect of the treatment.

The present study did not address the fate of oesophageal cells which sustained DNA damage following exposure to deoxycholate. Other studies have reported apoptosis in colon cells, but typically only after extended periods of exposure and/or exposure to higher concentrations of sodium deoxycholate (Glinghammar et al., 2002; Milovic et al., 2002) compared with conditions which stimulated DNA damage in the present study.

BO patients are distinguished by frequent acid reflux and increased levels of bile salts in the refluxate (Stein et al., 1998; DeMeester and DeMeester, 2000; Wild and Hardie, 2003). In conditions of chronic reflux and tissue injury this is likely to result in the selection of cell populations that are resistant to that reflux. The two oesophageal cell lines utilized in this study exhibited different sensitivities to acid and bile pulses. HET1-A consistently exhibited more DNA damage under acid conditions and rapidly lost viability when exposed to low pH conditions for >60 min. This contrasted with the FLO-1 cell line which maintained high cell viability even after 3 h exposure to low pH. The HET1-A cell line also exhibited more DNA damage when exposed to the primary bile salt mixture and sodium deoxycholate compared with FLO-1. Derived from a primary oesophageal adenocarcinoma, FLO-1 cells have possibly adapted to an environment dominated by reflux and may possess cyto-protective responses to both acid and bile, contrasting with the HET1-A cell line derived from normal squamous epithelium. Indeed, Kokoska et al. (1998) have reported that brief exposures to physiologically relevant concentrations of sodium deoxycholate induced a cyto-protective response effective against subsequent exposure in a gastric cell line.

Although modest increases in DNA damage were induced by acid and bile exposure in these oesophageal cell lines, it is noteworthy that the current study involved a single pulsed exposure; in contrast, it is common for patients with BO and AO to have reflux episodes extending over many years. Thus, under conditions of repeated exposure, stem cells in the oesophagus may accumulate biologically significant DNA damage via this route. Such damage, acting in concert with the promoting effects of bile and acid, would clearly provide a molecular basis to enhance tumourigenesis. Further studies to examine the effect of chronic, sub-lethal reflux exposure on DNA damage levels in vitro and to correlate reflux history with DNA damage levels in Barrett’s patients would clearly be of interest. It is of note that increased levels of DNA strand breaks in Barrett’s mucosa have been reported, compared with squamous epithelium (Oliver et al., 2004), as has a reduction in level of the oxidative DNA adduct 8-hydroxydeoxyguanosine in Barrett’s metaplasia following acid suppression therapy (Carlson et al., 2002).

In conclusion, we have demonstrated that low pH and bile salts, consistent with reflux conditions, cause DNA damage in oesophageal cells and could contribute to the malignant transformation of BO.

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