The role of secondary bile acids in neoplastic development in the oesophagus

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Abstract

Bile acids have been demonstrated, through the use of animal models and clinical association studies, to play a role in neoplastic development in Barrett’s metaplasia. How specific bile acids promote neoplasia is as yet unknown, as are the exact identities of the important bile acid subtypes. The combination of bile subtype with appropriate pH is critical, as pH alters bile acid activity enormously. Hence glycine-conjugated bile acids are involved in neoplastic development at acidic pH (pH ∼4), and unconjugated bile acids are involved in neoplastic development at more neutral pH (∼6). Bile acids (at the appropriate pH) are potent DNA-damaging agents, due to the induction of ROS (reactive oxygen species), which are mainly induced by bile-induced damage to mitochondrial membranes, allowing leakage of ROS into the cytosol. These ROS also induce pro-survival signalling pathways [e.g. via PKC (protein kinase C)-dependent NF-κB (nuclear factor κB) activity]. Interestingly, NOS (nitric oxide synthase), through induction of NO may exacerbate this NF-κB activity and form a positive-feedback loop to amplify the activation of NF-κB by deoxycholic acid in particular. This combination of induced DNA damage and cell survival by bile acids is of major importance in neoplasia. Antioxidants and the tertiary bile acid UDCA (ursodeoxycholic acid) can block bile-induced DNA damage and bile-induced NF-κB activity, and should be considered in chemopreventative strategies.

Barrett’s oesophagus

Barrett’s oesophagus is a pre-malignant condition of the oesophagus strongly linked to chronic GORD (gastro-oesophageal reflux disease) [1]. This metaplastic condition is increasing in incidence in the West [2], affecting 1–2% of unselected Western populations [3], and is accompanied by an increased risk of OAC (oesophageal adenocarcinoma), a type of cancer that is also increasing in the West [4] and which is associated with very poor survival rates (http://info.cancerresearchuk.org/cancerstats/types/oesophagus/survival/). Finding the molecular basis for neoplastic progression in Barrett’s metaplasia is urgently required in order to produce biomarkers of cancer risk and to identify targets for intervention studies to slow the neoplastic process.

A role for bile acids?

There is much evidence that bile acids present in DGOR (duodenogastro-oesophageal reflux) contribute to the neoplastic progression in Barrett’s metaplasia, not least the fact that current anti-acid approaches have failed to prevent year-on-year increases in OAC incidence. Bile acids are natural detergents produced from cholesterol to aid in the absorption of dietary fats. There has been increasing evidence that bile acids are multi-faceted molecules capable of inducing signalling events in cells which affect cell proliferation. These signalling events also include well-orchestrated feedback loops to control bile acid biosynthesis and export. Hence, much attention has been paid to the potential role of bile acids in neoplastic processes in the gastrointestinal tract.

There are two major strands of evidence linking bile acids to neoplastic progression in Barrett’s oesophagus: that from animal models and that from clinical studies measuring bile acids in Barrett’s metaplasia patients. By constructing anastomoses in rats between the duodenum and the oesophagus, several groups reported the induction of OAC by DGOR [6,7]. These studies also proposed that bile acids (duodenal contents) were more carcinogenic than stomach acid in comparative surgical models (gastroplasty) [7]. A complication of these studies, however, was the need for the addition of an alkylating agent to induce the tumours. However, a seminal study in 1998, using a jejuno-oesophageal anastomosis, showed that jejunal contents could induce OACs without the need for the addition of an alkylating agent [8]. This study also confirmed that bile acids (duodenal contents) were carcinogenic independently of stomach acid, as tumours were evident whether the stomach was retained or removed.

Clinical studies have emphasized further the importance of oesophageal bile exposure by demonstrating the widespread presence of specific bile acids in the oesophagi of refluxing
patients [9,10] (Table 1). It is interesting to note that bile acid reflux into the oesophagus is more common in the supine position compared with the upright position, and this raises the possibility of pools of bile acids lying in the oesophagus after nocturnal DGOR [9,10]. From the aspiration studies, one can see that bile exposure to the oesophagus is common and enhances the severity of reflux and complications such as Barrett’s metaplasia [9–12]. Furthermore, ‘mixed’ bile + acid reflux exacerbates the damage to the oesophageal epithelium compared with acid reflux alone [13].

A major question mark surrounding the labelling of bile acids as oesophageal carcinogens has been how do they function as carcinogens? A recent study has shed some light on how bile acids actually get into oesophageal cells in the first place: Dvorak et al. [14] have shown that Barrett’s oesophageal cells possess the active bile-transport apparatus, common in the ileum and capable of transporting both conjugated and unconjugated bile acids. Hence, refluxed bile acids can actively be transported and accumulated in Barrett’s metaplasia tissues. Key hallmarks of carcinogens are that they are capable of inducing DNA damage and also concurrently promoting cell proliferation and survival. So, in the present review, we address the question can bile acids perform these functions?

**DNA damage induction by bile acids**

There are several reports of DNA damage induction by individual bile acids in colorectal cells [15,16] as well as by human faecal water containing bile [17]. DNA damage studies in oesophageal cells and tissues are, however, rarer. In one such report, Dvorak et al. [18] showed elegantly that not only is there abundant oxidative DNA damage [measuring 8-OHdG (8-hydroxydeoxyguanine) levels] present in Barrett’s metaplasia tissues (relative to matched normal tissue), but also that *ex vivo* exposure of Barrett’s metaplasia biopsies to a cocktail of bile acids, in combination with acid, induced further 8-OHdG formation. In this study, acid alone, or the bile cocktail alone, failed to induce DNA damage in the *ex vivo* model. The bile cocktail used in this study was representative of the bile acids found in previous aspiration studies and contained equimolar amounts of GCA (glycocholic acid), TCA (taurocholic acid), GDCA (glycodeoxycholic acid), GCDCA (glycochenodeoxycholic acid) and DCA (deoxycholic acid). As the majority of the bile acids in this cocktail were glycine conjugates which tend to have pKₐ values of ~4, it follows that the DNA damage observed was preferentially at acidic pH. Indeed, in a separate study of bile-induced ROS (reactive oxygen species) induction, it was shown that glycine-conjugated CDCA (chenodeoxycholic acid) (i.e. GCDCA) preferentially induced ROS at pH 4, once again reflecting its intrinsic pKₐ [19]. Another study of bile-acid-induced DNA damage employing the Het1A and Flo cell lines, studied comet induction as a marker of DNA damage [20]. In this study, Jolly et al. [20] showed that acid exposure (pH < 4.5) induced DNA damage as well as a cocktail of bile acids containing GCA, TCA and TCDCA (taurochenodeoxycholic acid). This latter result was only observed at neutral pH, not at acidic pH, although it should be noted that the neutral pH incubations were longer than the acidic incubations because of associated toxicity with the mixed exposures (3 h and 15 min respectively). Finally, Jolly et al. [20] also showed that the secondary bile acid DCA was DNA-damaging at neutral pH, but not at acidic pH, but the same caveat about incubation time translates as well.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method and sampling site</th>
<th>[Conjugated bile]</th>
<th>[Unconjugated bile] average</th>
<th>Peak [DCA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kauer et al. [10]</td>
<td>HPLC oesophagus</td>
<td>230 μM average total bile [98 μM GCA, 69 μM GDCA]</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Darragh et al. [12]</td>
<td>GC (gastric aspirates)</td>
<td>323–9069 μM</td>
<td>31–1507 μM</td>
<td>115 μM</td>
</tr>
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bile-induced DNA damage and demonstrated the abrogation of DCA-induced DNA damage by pre-incubation of the cells with equimolar amounts of the antioxidant vitamin C [23]. It is well accepted that bile acids induce ROS in epithelial cells through the role of mitochondrial membrane disruption [24–26], as well as perhaps through the involvement of NOX (NADPH oxidase) [19]. Figure 1 collates some of the mechanisms whereby bile acids can induce DNA damage. It is essential to point out that only at the appropriate pH will particular bile acids be soluble, non-ionized and capable of entering the epithelial cells and inducing DNA damage.

**NF-κB activation by bile acids**

Cells burdened with DNA damage pose no threat if those cells are removed by apoptosis, autophagy or necrosis. However, the concurrent activation of survival factors in the same damaged cells, blocking apoptosis and causing those cells to persist in situ, represents a major threat in terms of neoplasia. The activation of the oncogenic transcription factor NF-κB is one such survival factor that can prevent apoptosis [by up-regulating genes such as Bcl-X<sub>L</sub> and XIAP (X-linked inhibitor of apoptosis) which inhibit the apoptotic process]. However, is there evidence that bile acids activate survival factors such as NF-κB?

There are numerous reports linking bile acid exposure to NF-κB activation in colorectal cells and hepatocytes [27,28], but the reports of NF-κB activation in oesophageal cells by bile acids are far fewer. Muhl Bauer et al. [27] showed DCA-induced NF-κB activation in HT29 colon cells leading to specific up-regulation of IL-8 (interleukin 8). In the same study, TDCA activated NF-κB weakly, requiring a much higher concentration (6–10-fold higher). We reported in 2004 that DCA could activate NF-κB in OE33 cells as measured with a luciferase assay and by analysis of NF-κB-dependent gene expression [IL-8 and IκB (inhibitor of NF-κB) in particular] [29]. Other reports have shown that DCA (and stomach acid) can activate NF-κB in OE33 cells by mobility-shift assays [30]. Further studies have shown that unconjugated bile acids preferentially up-regulate IL-8 at neutral pH (through NF-κB) in primary oesophageal epithelial cells, whereas conjugated bile acids preferentially up-regulate IL-8 at acidic pH [31]. Other groups have similarly shown that neutral pH DCA can activate NF-κB and cause NF-κB-dependent expression of cell adhesion genes such as Muc2 (mucin 2) [32] and homeobox genes such as Cdx1 (Caudal-type homeobox 1) [33]. Hence, there is very good evidence that bile acids (DCA in particular) can activate NF-κB in oesophageal cells. This activation appears to be PKC (protein kinase C)-dependent [31,32]. The fact that this activation of a survival pathway occurs in parallel, in terms of dose, pH and timeframe, to DNA damage induction by the same bile subtype in similar cell types suggests that bile acids pose a significant neoplastic threat.

**Antioxidants as chemopreventative agents**

As bile acids mediate much of their toxic and carcinogenic effects through the generation of ROS/RNS (reactive nitrogen species), it is plausible that antioxidants could effectively
block many bile-mediated events. Indeed, epidemiological evidence suggests that Barrett’s metaplasia patients with the highest intakes of antioxidants have >40% reduced risk of OAC [34]. We have shown that DNA damage induction by DCA in OE33 cells is effectively blocked by equimolar amounts of the antioxidant vitamin C [23]. Furthermore, we have also shown that other antioxidants [resveratrol, vitamin C and the tea polyphenol EGCG (epigallocatechin gallate)] can block the NF-κB-dependent gene expression induced by DCA [35]. Other groups have shown that antioxidants, e.g. the NO scavenger C-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], can abrogate DCA-induced DNA damage [21]. In colorectal cells, it has similarly been shown that dietary antioxidants β-carotene and α-tocopherol can protect cells against DCA- and CDCA-induced DNA damage [36]. Therefore it appears that several strands of evidence from *in vitro* and *in vivo* studies point to the fact that key bile acids are capable of inducing ROS/RNS and that these ROS/RNS are key mediators of both DNA damage and survival.

**UDCA (ursodeoxycholic acid), a bile-based chemopreventative agent**

The tertiary bile acid UDCA has been used in Chinese medicine for centuries as a treatment for a range of conditions. Current approaches using synthetic UDCA (rather than UDCA harvested from bears) have spread to Western countries, where UDCA is sometimes used to dissolve gallstones and treat cholestatic conditions. The mechanism of UDCA’s chemopreventative nature is not clearly known, but is believed to be involved with its ability to protect mitochondria. The hydrophilicity of UDCA may also underlie its protection, owing to the association between hydrophobicity and bile-induced toxicity/bioactivity. We have seen above that several bile acids mediate damaging effects by permeabilizing the mitochondrial membrane and causing the leakage of mitochondrial ROS. Hence, preventing this mitochondrial damage can ameliorate much of the toxic nature of bile acids. Indeed, there are several reports of UDCA supplementation preventing the deleterious effects induced by bile acids. For example, Shah et al. [37] showed convincingly that UDCA blocked DCA-induced NF-κB and AP-1 (activator protein 1) activity in colorectal HCT116 cells. Data from our group (shown in Figure 2A) also supports a role for UDCA in protecting oesophageal cells (OE33) from DCA-induced DNA damage (Figure 2A) and from DCA-induced NF-κB dependent gene expression (IL-8 in particular) (Figure 2B).

**Conclusions**

There is now a weight of evidence to suggest that bile acids are key oesophageal carcinogens and play an intricate role in the development of OAC; this evidence is particularly strong for secondary bile acids. Bile acids are a diverse group of chemicals with widely differing activities, pH preferences and latent bioactivities. We have to be cautious when making statements that ‘bile’ does this or does that, because of the diversity of this group of chemicals. For example, saying that bile induces DNA damage preferentially at acidic pH is both true and false, glycine-conjugated bile acids certainly do induce DNA damage optimally at ~pH 4, but other...
bile acids (e.g. DCA) optimally induce DNA damage at higher pH. In clinical terms, this is important because, owing to the interactions with differing levels of stomach acidity, different bile acids may cause different effects in different patients. The pH-dependence of bile subtypes is largely due to the intrinsic $pK_a$ of the different bile acids. Hence, taurine-conjugated bile acids (with $pK_a$ values <2) are only active (soluble, non-ionized) in the most acidic (mitogen-activated protein kinase), NF-$\kappa B$ activate numerous signalling pathways [e.g. involving MAPK and COX-2 (cyclo-oxygenase-2) [38]] in a similar ROS/RNS-mediated manner. The role of NO in amplifying these signalling cascades is also worthy of study, as we have shown NF-$\kappa B$ activation to be linked to iNOS (inducible NOS) activity (Figure 2C). Owing to the role of ROS/RNS in bile signalling, antioxidant therapies should abrogate many of the deleterious effects observed by bile acids to date (and certainly seem to be experimentally). Furthermore, Nature has supplied its own bile-specific chemopreventative agent in the form of UDCA (ursodiol). By protecting mitochondrial membrane integrity, UDCA can ameliorate many of the threats posed by certain bile acids. The success of UDCA in ameliorating many of the deleterious effects of bile acids adds strength to the argument that mitochondrial ROS are central to the bioactivity of bile acids. Synergizing the powerful effects of UDCA with the known chemoprotection afforded by dietary antioxidants should be considered.

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