Transcriptional regulation of human mucin MUC4 by bile acids in oesophageal cancer cells is promoter-dependent and involves activation of the phosphatidylinositol 3-kinase signalling pathway

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Abnormal gastro-oesophageal reflux and bile acids have been linked to the presence of Barrett's oesophageal premalignant lesion associated with an increase in mucin-producing goblet cells and *MUC4* mucin gene overexpression. However, the molecular mechanisms underlying the regulation of *MUC4* by bile acids are unknown. Since total bile is a complex mixture, we undertook to identify which bile acids are responsible for *MUC4* up-regulation by using a wide panel of bile acids and their conjugates. MUC4 apomucin expression was studied by immunohistochemistry both in patient biopsies and OE33 oesophageal cancer cell line. *MUC4* mRNA levels and promoter regulation were studied by reverse transcriptase–PCR and transient transfection assays respectively. We show that among the bile acids tested, taurocholic, taurodeoxycholic, taurochenodeoxycholic and glycocholic acids and sodium glycocholate are strong activators of MUC4 expression and that this regulation occurs at the transcriptional level. By using specific pharmacological inhibitors of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, protein kinase A and protein kinase C, we demonstrate that bile acid-mediated up-regulation of MUC4 is promoter-specific and mainly involves activation of phosphatidylinositol 3-kinase. This new mechanism of regulation of *MUC4* mucin gene points out an important role for bile acids as key molecules in targeting MUC4 overexpression in early stages of oesophageal carcinogenesis.

Key words: bile acid, mitogen-activated protein kinase (MAPK), MUC4, mucin, oesophagus, phosphatidylinositol 3-kinase (PI3K), transcription.

INTRODUCTION

About 5000 new cases of oesophageal cancer are diagnosed each year in France with high incidence in Northwestern regions [1]. Although epidermoid carcinoma represents the main histological type, adenocarcinoma incidences have been increasing lately and, in recent times, represent half of the oesophageal cancers [2–4]. Most of the adenocarcinomas arise from Barrett's mucosa with a prevalence of 10–15 %, with a risk of cancer 30–50 times more frequent than in the general population [5]. Barrett's oesophagus corresponds to an abnormal repair process of lesions induced by gastro-oesophageal reflux and leads to metaplasia in which epidermoid mucosa is replaced by a glandular-like mucosa in a multistep process characterized by the metaplasia–dysplasia–adenocarcinoma sequence [1,6]. Gastro-oesophageal refluxes of acid and bile are the predominant initiating factors in Barrett's metaplasia and its degeneration into oesophagus adenocarcinoma by acting on the apical membrane of metaplastic cells [8–12], although the precise mechanism of cytotoxicity is unclear. Gillen et al. [13] have shown an increase in intragastric bile acid concentration in patients with Barrett's oesophagus and it has been suggested that incomplete intestinal-type metaplasia may be a response to reflux of gastroduodenal contents and, in particular, to bile acids [10]. Moreover, duodeno-oesophageal bile reflux has recently been identified as a predominant and sufficient carcinogen to induce oesophageal adenocarcinoma on Barrett's oesophagus in a rat model [14,15]. Dysplasia is the only biological tumoural marker for Barrett's mucosa surveillance. However, it remains unsatisfactory due to the absence of endoscopic translation and regular distribution, because of complex histological diagnosis and difficulty to predict its evolution [7]. Identification of new markers of early stages of oesophageal carcinogenesis is thus still an active area of research.

Human mucins are secreted in normal oesophagus to protect the underlying mucosa against potential injuries such as reflux of gastroduodenal contents including acid and bile acids [16]. Two main families of mucins are distinguished: secreted mucins that participate or not in mucus gel formation (MUC2, MUC5AC, MUC5B, MUC6, MUC7) and membrane-bound mucins that are involved in cell signalling and are thought to play important roles in tumour cell biology (MUC1, MUC3, MUC4) [17,18]. It is important to note that MUC4 is also considered to participate in mucus formation and mucosal defence after proteolytic cleavage of its extracellular domain [17]. In normal oesophagus, *MUC1* and *MUC4* are the main mucin genes expressed in the stratified squamous epithelium, whereas *MUC5B* is expressed in the submucosal glands [19,20]. Barrett's oesophagus corresponds to an abnormal healing process in which the normal squamous

Abbreviations used: CDC, chenodeoxycholic acid; CME, cholic methyl ester; DC, deoxycholic acid; DHC, dehydrocholic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, glycocholic acid; GNa, sodium glycocholate; LC, lithocholic acid; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; RT, reverse transcriptase; TC, taurocholic acid; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid.

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mucosa is replaced by a columnar epithelium that can be of three types: intestinal, cardial and fundic [21]. In high-grade dysplasia and adenocarcinoma of Barrett's oesophagus, down-regulation of *MUC2*, *MUC5AC* and *MUC6* is observed, whereas expression of *MUC1* and *MUC4* mucin genes is dramatically increased [16,19]. In consequence, altered expression/secretion of mucins will have dramatic impact on the mucus composition, on the biological properties of cancer cells and consequently on the maintenance of epithelium integrity. From these studies, several authors have proposed that mucin genes may be considered as reliable phenotypic markers to follow a metaplastic process that leads to Barrett's adenocarcinoma [19–21]. Since MUC4 is expressed at the surface of epithelial cells and possesses a soluble form, two mechanisms are proposed for MUC4 function in cancer progression: first, as an anti-adhesive or anti-recognition barrier at the epithelial surface and secondly as an actor in ErbB2 signalling [18]. However, at this time, nothing is known about the molecules and the downstream intracellular signalling cascades that are responsible for the overexpression of *MUC4* mucin gene observed in oesophageal cancer resulting from persistent gastro-oesophageal reflux.

Previous studies on gall-bladder and colon cancer cells have shown that bile acids activate mucin secretion via PKA (protein kinase A), PKC (protein kinase C) and intracellular Ca^{2+}-dependent signalling pathways [22–26]. It is, however, not known which mucin gene is activated and if regulation at the transcriptional level is involved. In relation to *MUC4* overexpression in oesophageal adenocarcinoma and the role of bile acids in Barrett's metaplasia and its consequent degeneration into oesophageal adenocarcinoma, we undertook to identify which bile acids were responsible for *MUC4* up-regulation.

MATERIALS AND METHODS

Cell culture

The oesophageal cancer cell line OE33 was purchased from ECACC (European Collection of Cell Cultures, Porton Down, Salisbury, Wilts., U.K.). The cell line was established from the adenocarcinoma of the lower oesophagus [27]. Cells were cultured in RPMI 1640 medium, supplemented with 2 mM glutamine and 10 % (v/v) foetal calf serum (Roche Diagnostics, Meylan, France) and maintained at 37 °C in an incubator with 5 % CO_2. Cells were treated with bile acids and their conjugates for 24 h with the following concentrations of bile acids: 0.1 mM CDC (chenodeoxycholic acid), 0.5 mM cholic acid, 0.05 mM DC (deoxycholic acid), 0.5 mM TCDC (taurochenodeoxycholic acid), 0.5 mM GNa (sodium glycocholate), 0.5 mM GC (glycocholic acid), 0.5 mM TC (taurocholic acid), 1 mM TDC (taurodeoxycholic acid), 1 mM DHC (dehydrocholic acid), 0.05 mM CME (cholic methyl ester) and 0.1 mM LC (lithocholic acid). Toxicity of each bile acid was evaluated, under the same experimental conditions, by Trypan Blue exclusion measurement on a haemocytometer. In inhibition studies, pharmacological inhibitors were incubated with the cells for 30 min, before the addition of bile acids, at the following final concentrations: PD98059 [30 μM, inhibitor of MAPK (mitogen-activated protein kinase)], KT5720 (1 ng/ml, inhibitor of PKA), GF109203X (10 μM, inhibitor of PKC) and wortmannin [2.5 nM, inhibitor of PI3K (phosphatidylinositol 3-kinase)]. All reagents were from Sigma unless otherwise indicated.

Reverse transcriptase (RT)–PCR

Total RNAs from OE33 cells were prepared using the RNeasy mini-kit from Qiagen (Courtaboeuf, France). Cells were harvested at 100 % of confluence and 1.5 μg of total RNA was used to prepare cDNA (Advantage™ RT-for-PCR kit, Clontech) as described before [28]. PCR was performed on 1 μl of cDNA using specific pairs of primers (MWG-Biotech, Germany) for *MUC4* mucin gene: forward primer 5′-CGCGGTGGTGGAGGCGTTCTT-3′ and reverse primer 5′-GAAGAATCCTGACAGCCTTCA-3′ (accession number AJ242546). PCRs were performed in 50 μl final solutions as described in [29]. Annealing temperature was 60 °C. PCR products were analysed on 2 % agarose gels run in 1 × Tris/borate/EDTA buffer. A 100 bp DNA ladder was purchased from Amersham Biosciences. GAPDH (glyceraldehyde-3-phosphate dehydrogenase; forward primer 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse primer 5′-CATGTGGGCCATGAGGTCCACCAC-3′) was used as the internal control. Expected sizes of *MUC4* and *GAPDH* PCR products are 596 and 980 bp respectively. RT–PCRs were performed on cDNAs from four different sets of experiments. Densitometric analysis of DNA bands was performed using the GelAnalyst-GelSmart software (Clara Vision, Orsay, France).

pGL3-*MUC4* promoter constructs

pGL3-*MUC4* deletion mutants covering both promoters of *MUC4* were described previously [30]. Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Transfections

Transfections were performed using Effectene® reagent (Qiagen) as described previously [31]. Cells were passed at 0.5×10^6 cells/well the day before the transfection. Total cell extracts were prepared after a 48 h incubation time at 37 °C using 1 × reagent lysis buffer (Promega France SARL, Charbonnières-les-Bains, France) as described in the manufacturer's instruction manual. Total protein content in the extract was measured using bicinchoninic acid protein assay (Pierce, PERBIO Science France SAS, Brebières, France). The relative luciferase activity is expressed as fold induction of the test plasmid activity compared with that of the corresponding empty vector (pGL3 basic; Promega). Each plasmid was assayed in triplicate in three separate experiments.

Immunohistochemistry

Confluent OE33 cells were trypsinized, centrifuged, washed once with 1 × PBS, the pellet was fixed in 4 % (w/v) paraformaldehyde, then embedded in paraffin and 3 μm sections were prepared. Morphological analysis was performed after Alcian Blue staining. Section pretreatment before immunostaining was performed as described before [32]. MUC1 and MUC4 apomucin expressions were also analysed on tissue obtained from patients with oesophageal adenocarcinoma. The surgical specimen was quickly immersed in 10 % (v/v) neutral formaldehyde solution (pH 7.4) in phosphate buffer. Samples from normal oesophageal mucosa and adenocarcinoma were processed for paraffin embedding. The diagnosis was assessed by two pathologists after staining the sections (4 μm) with haematoxylin–eosin–saffron. Immunohistochemistry was performed on serial sections of the same blocks using an automated immunostainer (ES, Ventana Medical System, Strasbourg, France). The pretreatment was carried out in a microwave for 20 min in citrate buffer (pH 6.0). The immunohistochemistry method used a three-step indirect process based on the biotin–avidin complex. Slides were counterstained with haematoxylin. Negative controls were run by omission of the primary antibody. Monoclonal anti-MUC4 antibody [33] was used at a 1:20000 dilution. Monoclonal MUC1

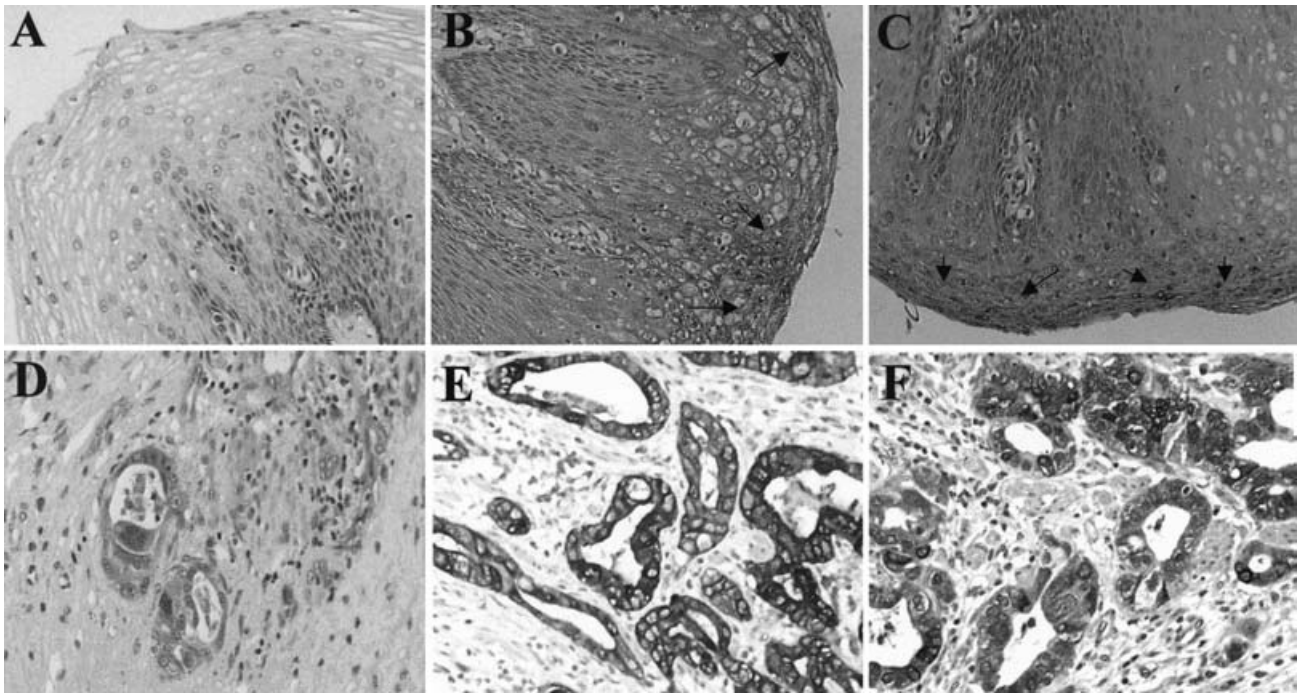

Figure 1 Expression of MUC1 and MUC4 apomucins in human oesophageal mucosa and adenocarcinoma

Immunohistochemistry was performed as described in the Materials and methods section. (**A**–**C**) Normal oesophageal mucosa and (**D**–**F**) oesophagus adenocarcinoma. (**A**) Haematoxylin–eosin–saffron staining of normal oesophageal mucosa, (**B**) MUC1 staining in normal oesophageal mucosa, (**C**) MUC4 staining in normal oesophageal mucosa, (**D**) haematoxylin–eosin–saffron staining of adenocarcinomatous oesophageal mucosa, (**E**) MUC1 staining in oesophageal adenocarcinoma, (**F**) MUC4 staining in oesophageal adenocarcinoma. Magnification × 250.

antibody (LICR-LON-M8, 1:50 dilution) was a gift from Dr D. Swallow (University College London, London, U.K.). Polyclonal anti-MUC5B LUM5B-2 antibody (1:50 dilution) was a gift from Dr I. Carlstedt (Lund University, Lund, Sweden).

RESULTS

MUC1 and MUC4 apomucin expression in human oesophageal mucosa and adenocarcinoma

MUC1 and *MUC4* mRNAs were previously detected in oesophageal adenocarcinoma tissues by *in situ* hybridization [19,20], but the apomucin expression has never been studied. Figure 1 shows the expression of MUC1 and MUC4 apomucins in normal oesophageal mucosa (Figures 1B and 1C) and in oesophageal adenocarcinoma (Figures 1E and 1F) respectively. In normal oesophageal epithelium (Figure 1A), expression of MUC1 (Figure 1B) and MUC4 (Figure 1C) is moderate and restricted to the cytoplasm. Haematoxylin–eosin–saffron staining of the tissue section from a patient with oesophageal adenocarcinoma shows that it is moderately differentiated with carcinomatous glands dispersed in a stroma reaction (Figure 1D). When the sample was processed for MUC1 (Figure 1E) and MUC4 (Figure 1F), the staining was more intense than in normal mucosa and was found both in the cytoplasm and in the cell membrane.

Regulation of *MUC4* mRNA expression by bile acids in OE33 cancer cells

Regulation of *MUC4* mRNA expression in OE33 cancer cells by bile acids was assessed by RT–PCR. OE33 cells were treated with bile acids or conjugates for 24 h as described in the Materials and methods section before cell lysis and RNA extraction. The results

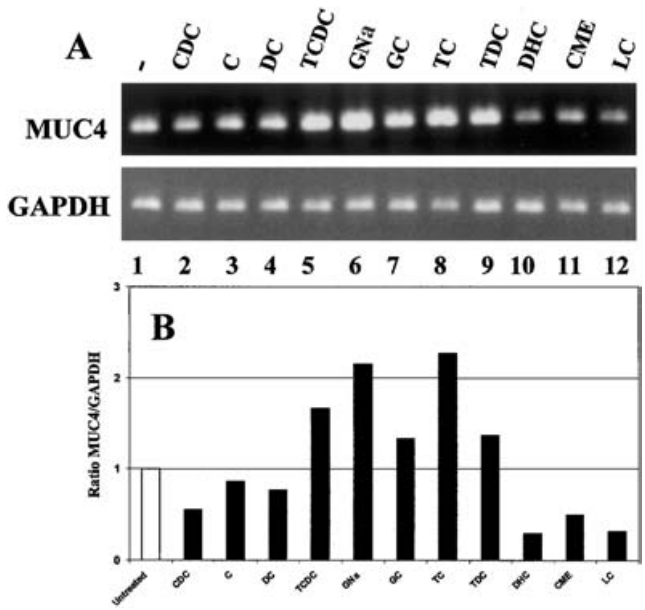

Figure 2 Study of the effect of bile acids and their conjugates on the expression of *MUC4* mRNA in OE33 cancer cells by RT–PCR

Cells were treated with the indicated bile acids for 24 h as described in the Materials and methods section. (**A**) *MUC4* and *GAPDH* PCR products (8 and 2 μl respectively) were separated on a 2 % agarose gel containing ethidium bromide after electrophoresis in 1 × Tris/borate/EDTA buffer. (**B**) Densitometric analysis of the DNA bands.

shown in Figure 2 indicate that *MUC4* mRNA levels are either modified slightly or not modified after cell treatment with CDC (lane 2), cholic acid (C, lane 3) and DC (lane 4). On the other

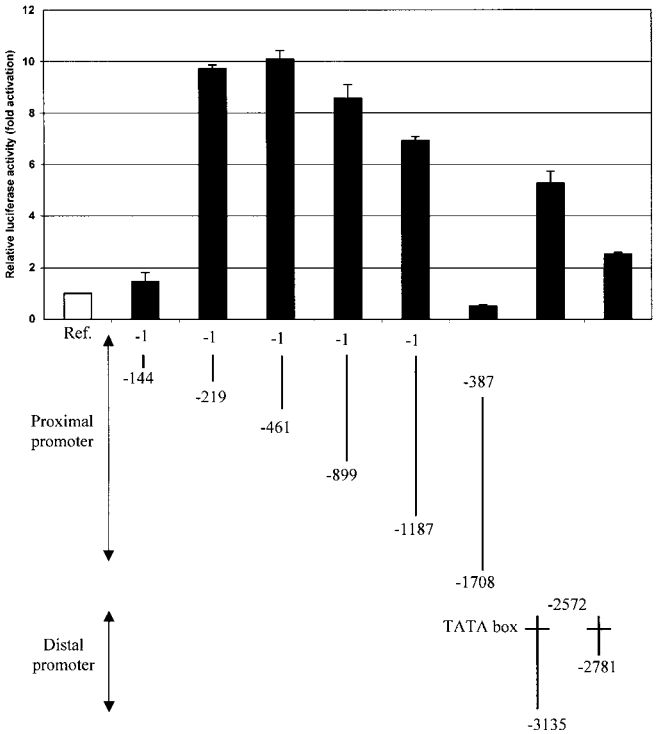

Figure 3 Characterization of the promoter activity of *MUC4* in OE33 cancer cells by transient transfection

Each pGL3-*MUC4* deletion mutant (1 μg) was transfected in OE33 cells as described in the Materials and methods section. Ref., transfected cell with the empty vector pGL3 basic. Means $\pm$ S.D. are calculated from the values obtained in triplicate from three separate experiments.

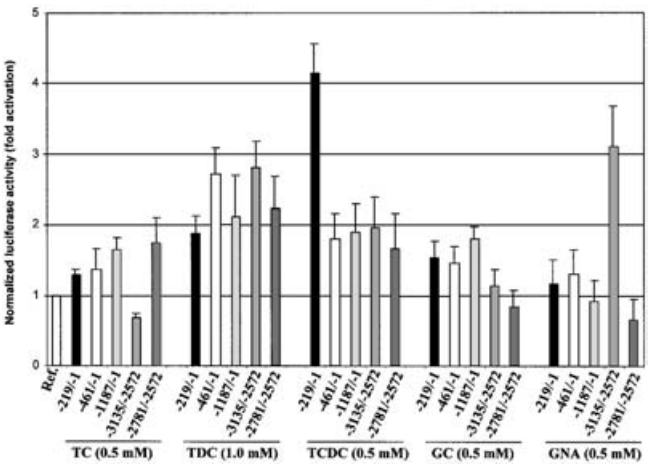

Figure 4 Effect of bile acids and their conjugates on the transcriptional activity of *MUC4* promoters in OE33 cancer cells

Effect on constructs representing the proximal promoter of *MUC4* ($-219/-1$, $-461/-1$ and $-1187/-1$) and the distal promoter ($-3135/-2572$ and $-2781/-2572$). Each pGL3-*MUC4* deletion mutant (1 μg) was transfected in OE33 cells as described in the Materials and methods section. Cells were then treated with bile acids for 24 h with indicated concentrations. Ref., the transfected cell without bile acid treatment (this value was defined as 1). Means $\pm$ S.D. were calculated from values obtained in triplicate from three separate experiments.

hand, a strong increase in *MUC4* mRNA levels is observed after cell treatment with TCDC (lane 5), GNa (lane 6), GC (lane 7), TC (lane 8) and TDC (lane 9). Finally, treatment with DHC (lane 10), CME (lane 11) and LC (lane 12) induced a slight decrease in *MUC4* mRNA levels in OE33 cells. These results indicate that bile acids and their conjugates can be classified into three families depending on their effect on *MUC4* mRNA expression: no effect (CDC, C and DC), activators (TCDC, GNa, GC, TC and TDC) or inhibitors (DHC, CME and LC).

Regulation of *MUC4* promoter activity by bile acids in OE33 cancer cells

The 5′-flanking region of *MUC4*, which we previously characterized, contains two transcriptional units [30]. Among the constructs covering the proximal promoter, the highest activity was obtained with fragments $-219/-1$ and $-461/-1$ (10-fold activation) (Figure 3), whereas the shortest fragment $-144/-1$ had no activity. Addition of longer DNA segments of *MUC4* promoter in the constructs ($-1187/-220$) did not lead to any significant changes in luciferase activity. This indicates that the $-219/-145$ region of the proximal promoter contains positive regulatory elements that confer maximal activity to *MUC4* proximal promoter in these cells. This is confirmed by the absence of luciferase activity of the fragment $-1708/-387$, which lacks the first 386 nucleotides of the proximal promoter. The distal promoter is also active and essential elements are present in the $-3135/-2572$ region (5-fold activation), since the activity of that promoter drops to 2-fold activation when the $-3135/-2781$ region is not present.

Having shown that the *MUC4* endogenous mRNA expression was up-regulated by TC, TDC, TCDC, GC and GNa, we next focused our studies on these bile acids. Bile acid-responsive regions within *MUC4* promoters were identified after performing transient transfection experiments in which transfected cells were treated with bile acids or conjugates under the same conditions as for RT–PCR experiments. Among the pGL3-*MUC4* constructs, we chose the five deletion mutants covering the two promoters of *MUC4* possessing the highest luciferase activity (see Figure 3). Among the tauric conjugates, TC induced a 2-fold transactivation of the distal fragment $-2781/-2572$ (Figure 4). TDC, the tauric conjugate of DC, transactivates both promoters of *MUC4* in the same proportion [3-fold activation of proximal region $-461/-1$ and 3-fold activation of distal region $-3135/-2572$]. TCDC, the tauric conjugate of CDC, strongly transactivates the $-219/-1$ region of *MUC4* proximal promoter (4-fold activation).

GC transactivates the $-1187/-1$ region of *MUC4* proximal promoter (2-fold). Since the shortest fragments do not show any transactivation by GC, one can conclude that GC-responsive elements are located within the $-1187/-461$ region of the proximal promoter. GNa strongly transactivates the distal region $-3135/-2572$ of the *MUC4* promoter (3-fold activation).

In conclusion, the reporter assays show that bile acid-responsive elements are present in both promoters of *MUC4* and that bile acid-mediated up-regulation of *MUC4* transcription is promoter-dependent.

Effect of bile acids on MUC4 apomucin expression in OE33 cells

Having shown that *MUC4* promoters contain responsive elements for bile acids that control *MUC4* expression in OE33 cells, we undertook to check whether transcriptional control had an impact on the expression of MUC4 apomucin. To this end, cells were treated with bile acids for 24 h under the same conditions as for RT–PCR and reporter assays. They were then trypsinized and processed for immunostaining with a specific antibody against MUC4 (Figure 5). Bile acid treatment under the conditions used in the present study neither induced any change in the morphology

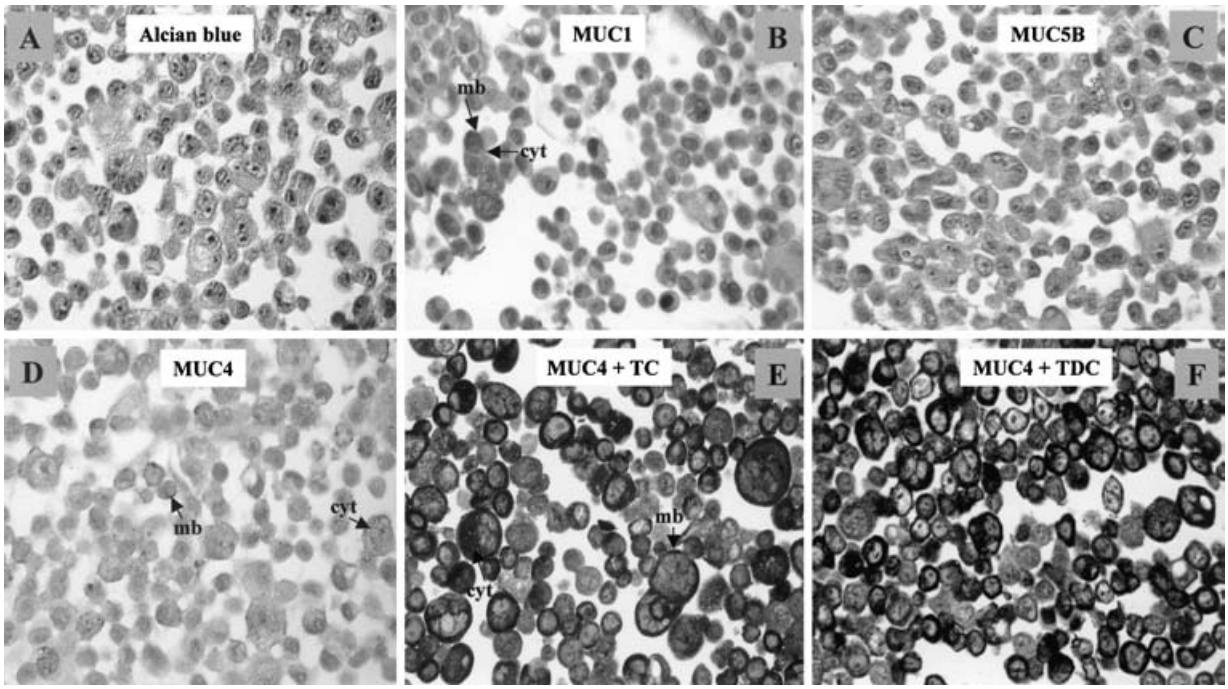

Figure 5 Effect of bile acids on MUC4 apomucin expression in OE33 cancer cells

Immunohistochemistry was performed as described in the Materials and methods section. (**A**–**D**) Untreated cells. (**A**) Alcian Blue staining, (**B**) MUC1 immunostaining, (**C**) MUC5B immunostaining, (**D**) MUC4 immunostaining, (**E**) MUC4 immunostaining after TC treatment, and (**F**) MUC4 immunostaining after TDC treatment. Magnification × 400. Staining of the membrane (mb) or cytoplasm (cyt) is indicated by arrows.

of the cells (cf. Figures 5E and 5F with 5D) nor did it induce cell death, as Trypan Blue-exclusion measurements were identical both in untreated and bile acid-treated cells. Untreated cells show a relatively low degree of differentiation as no vacuoles and a few grains of secretions were detected (Figure 5A). To characterize OE33 cells better, expression of MUC1 and MUC5B apomucins was also studied. A few cells express MUC1 and the expression is found both in the cytoplasm (cyt) and in the membrane (mb) (Figure 5B, arrows), whereas there is no expression of MUC5B (Figure 5C). When immunostained with anti-MUC4 antibody, 30 % of the cells were positively labelled (Figure 5D). The staining was seen both in the membrane and cytoplasm. The labelling became very intense and 100 % of the cells were positively stained for MUC4 when cells were treated with TC (Figure 5E) or TDC (Figure 5F). Cell treatment with TCDC, GNa and GC also resulted in a dramatic increase in MUC4 apomucin expression, with 100 % of the cells labelled. In conclusion, these studies indicate that TC, TDC, TCDC, GC and GNa are strong activators of MUC4 apomucin expression in OE33 cells.

Identification of the signalling pathways involved in bile acid-mediated up-regulation of MUC4 in OE33 cells

Having identified TC, TDC, TCDC, GC and GNa as the bile acids responsible for MUC4 up-regulation in OE33 cells, we undertook to identify the intracellular signalling pathways responsible for that phenomenon. To this end, specific inhibitors of MAPK (PD98059), PKC (GF109203X), PKA (KT5720) and PI3K (wortmannin) were used and preincubated for 30 min with the cells before the 24 h incubation with each bile acid. Figure 6(A) shows the results obtained with bile acids previously shown to activate the proximal promoter of *MUC4* (see Figure 4). Inhibition of PKC did not alter the transactivating effect of GC, TC, TCDC and TDC on *MUC4* promoter activity. Inhibition of PKA had no effect on the transactivating effect of GC, TC and TCDC, except for TDC for which it induced a mild transactivating effect (1.8-fold). Inhibition of PI3K by wortmannin led to a dramatic inhibition of bile acid-mediated up-regulation of *MUC4* proximal promoter. GC effect was inhibited by 50 %, whereas 70–75 % inhibition was observed for TC, TDC and TCDC. Inhibition of MAPK did not inhibit, but, surprisingly, activated the transactivating effect of GC, TC and TCDC by 2-fold. No effect was seen on TDC transactivation. These results indicate that GC, TC, TCDC and TDC transactivation of *MUC4* proximal promoter is mediated by PI3K signalling pathway, whereas PKA (TDC) and MAPK (GC, TC, TCDC) cascades are negatively controlling these effects.

The bile acids that were transactivating the distal promoter of *MUC4* are also triggering the PI3K signalling pathway, since pretreatment with wortmannin inhibited TDC, GNa and TCDC transactivating effect by 75–80 % (Figure 6B). MAPK is also involved in TDC-mediated up-regulation of *MUC4* distal promoter as 50 % of the activity is lost when cells are preincubated with PD98059 inhibitor. Pretreatment with the inhibitor of MAPK induced mild activation of the transactivation by TCDC (1.8-fold activation). Inhibition of PKA and PKC induced activation of the transactivating effect of TDC (approx. 2-fold activation) and, to a lower extent, of GNa (1.5–1.75-fold activation).

Importance of the PI3K signalling pathway in the bile acid-mediated regulation of MUC4 expression in OE33 cells

To confirm the importance of the PI3K signalling pathway in mediating bile acid effects on MUC4 expression in OE33 cells, RT–PCR and immunohistochemistry were performed on cells pretreated with the PI3K inhibitor (wortmannin) before bile acid

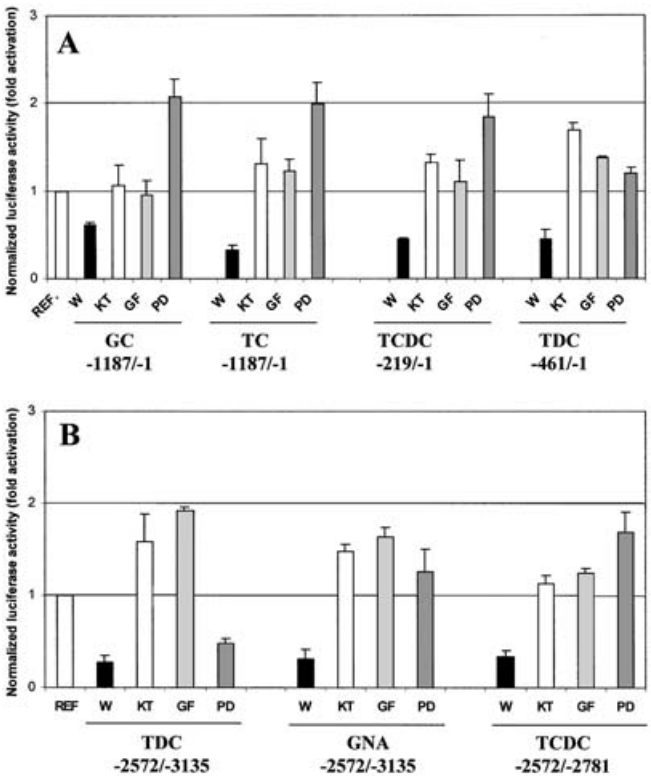

Figure 6 Identification of the signalling pathways triggered by bile acids to regulate *MUC4* promoter activity in OE33 cancer cells

(**A**) Bile acids transactivating the proximal promoter of *MUC4* and (**B**) bile acids transactivating the distal promoter of *MUC4*. Each pGL3-*MUC4* deletion mutant (1 μg) was transfected in OE33 cells as described in the Materials and methods section. Before bile acid treatment for 24 h, cells were pretreated for 30 min with the pharmacological inhibitor as indicated (W, wortmannin; KT, KT5720; GF, GF109203X; PD, PD98059). Ref., the bile acid-treated transfected cells without inhibitor pretreatment (this value was defined as 1). Means $\pm$ S.D. were calculated in triplicate from three separate experiments.

incubation. RT–PCR studies indicate that, as shown previously in Figure 2, TDC and TC increase *MUC4* mRNA levels in the cells (Figure 7A, lanes 2 and 4 respectively). Pretreatment with wortmannin inhibited TDC- and TC-mediated increase in *MUC4* mRNA levels which then returned to basal levels (Figure 7A, lanes 3 and 5 respectively). Immunostaining of the cells with anti-MUC4 antibody confirmed the involvement of PI3K in TDC-mediated up-regulation of MUC4 apomucin expression, as the labelling went from 72% in TDC-treated cells (Figure 7B) to 16% in wortmannin-pretreated cells (Figure 7C). The same conclusion was drawn regarding TC-mediated up-regulation of MUC4 apomucin expression, as 64% of TC-treated cells are positive for MUC4 (Figure 7D), whereas only 29% remain labelled when cells were pretreated with wortmannin (Figure 7E).

DISCUSSION

Human mucins are secreted in normal oesophagus to protect the underlying mucosa [16]. In normal oesophagus, *MUC1* and *MUC4* are the main mucin genes expressed in the surface epithelial cells, whereas *MUC5B* is expressed in the submucosal glands [19,20]. In high-grade dysplasia and adenocarcinoma of Barrett's oesophagus, expression of *MUC1* and *MUC4* mucin genes are dramatically increased [16,19]. Progression of oesophageal cells to cancer follows a multistep process characterized by the metaplasia–dysplasia–adenocarcinoma sequence [1]. Genetic defects have been identified such as loss of p16 and p53 expression or gain of cyclin D1 expression [34,35], but gastro-oesophageal reflux of acid and bile are the predominant initiating factors in Barrett's metaplasia, although the precise mechanism of cytotoxicity is unclear [36]. Lately, much attention has been focused on MUC4 for it could represent a new therapeutic target in epithelial cancers as it is often strongly overexpressed in carcinomas. It is a transmembrane protein that interferes with tumour cell properties and is involved in ErbB2 signalling [17,18]. It is also thought that MUC4 may participate in mucus formation after proteolytic cleavage of its extracellular domain and its release in the lumen [18]. However, at this time, not much is known about MUC4 regulation at the transcriptional level in oesophageal cancer. Recently, some evidence suggested that bile reflux may be considered as an important carcinogen in the development of oesophageal adenocarcinoma on a Barrett's oesophagus [14,15]. For that reason and because it was previously shown that (i) most bile acids are active in the refluxate, (ii) increased exposure to total bile correlates with worsening mucosal damage, (iii) bile acids promote goblet cells containing metaplasia in the oesophagus as well as in other epithelia, (iv) bile acids induce mucin secretion, and (v) *MUC4* mucin gene is overexpressed in oesophagus adenocarcinoma, we undertook to study the regulation of MUC4 by bile acids and conjugates both at the transcriptional and translational levels in an oesophageal cancer cell line.

In the present study, we have identified TC, TDC, TCDC, GC and GNa as strong inducers of MUC4 mucin expression and showed that the regulation occurs at the transcriptional level. Consequently, these conjugates may be considered as the most important factors in the bile to mediate MUC4 up-regulation in oesophageal cancer associated with bile acid refluxate. However, dramatic induction of MUC4 apomucin expression in immunohistochemistry studies also suggests that post-transcriptional mechanisms may also occur, which is not an uncommon feature for mucins. Since bile acids are known to act directly on the apical membrane of metaplastic epithelial cells and because MUC4 is a transmembrane mucin expressed at the surface of those same cells, which has a particularly long extracellular domain (2.12 μm) [37] that protrudes far away from cell membrane and glycocalix, one can envision that altered expression/secretion of MUC4 will have dramatic consequences on the biological properties of the oesophageal epithelial cancer cells.

Previous reports had essentially focused on studying the role of bile acids on mucin secretion in primary gall-bladder cells or colon cancer cells in relation to gall-stone formation and colon-cancer pathogeny respectively [22–26]. In an isolated vascularly perfused rat colon model, it was shown that mucin secretion and mucus discharge were induced by DC and CDC, whereas cholic acid, ursodeoxycholate or detergent Tween 20 had no effect [38]. Interestingly, these authors noticed that tauric conjugates of these bile acids were not as efficient. In the present study, we found that tauric conjugates were quite efficient on MUC4 up-regulation. This may indicate that bile acid action differs or that the composition of the bile is different in oesophageal and colonic or gall-bladder epitheliums. This hypothesis seems to be the most probable, as these authors showed in another study that the bile acid content of bile influenced its capacity to induce mucin secretion [23]. They concluded that a bile composed of hydrophobic bile acids, such as TCDC and TDC, favours mucin secretion when compared with a bile composed of more hydrophilic bile acids like TC and TDC [23]. Our results also

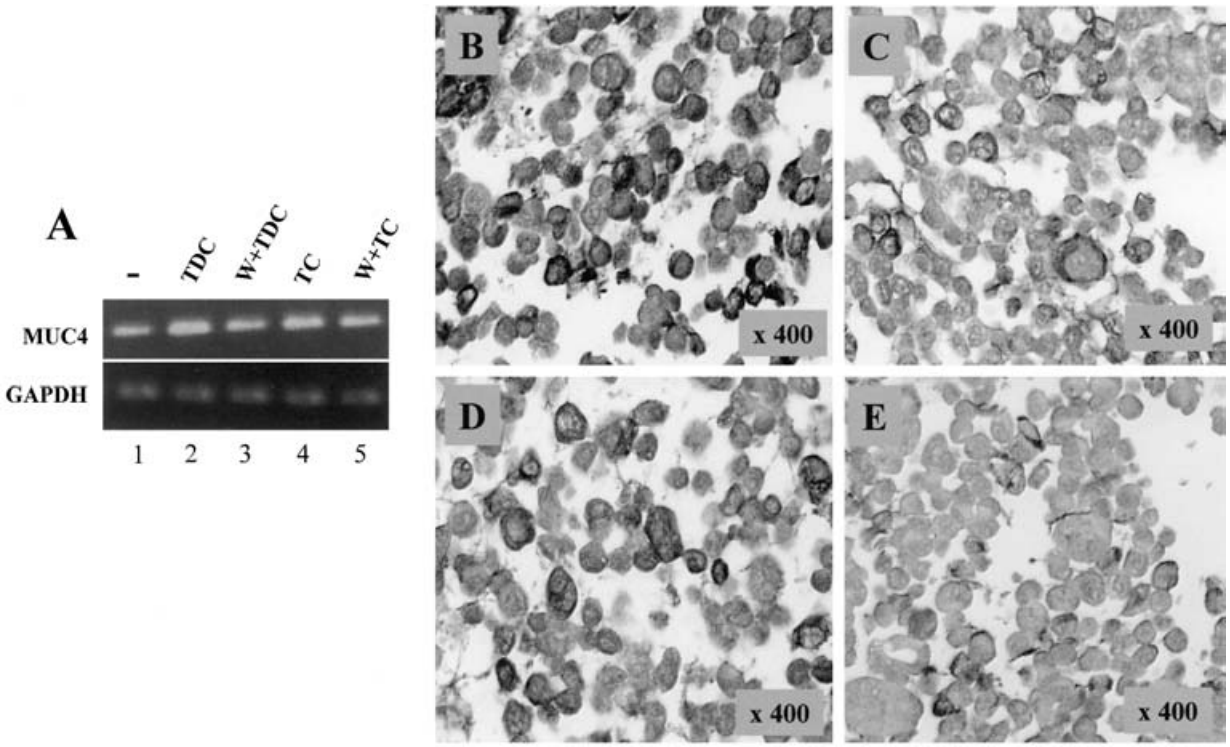

Figure 7 Importance of the PI3K signalling in TDC- and TC-mediated regulation of MUC4 expression in OE33 cancer cells

(**A**) Cell treatment with wortmannin and the indicated bile acids and RT–PCR were performed as described in the Materials and methods section. *MUC4* and *GAPDH* PCR products (8 and 2 μl respectively) were separated on a 2 % agarose gel containing ethidium bromide after electrophoresis in 1 × Tris/borate/EDTA buffer. Immunohistochemistry with anti-MUC4 antibody was performed as described in the Materials and methods section. (**B**) TDC-treated cells, (**C**) TDC-treated cells pretreated with wortmannin, (**D**) TC-treated cells and (**E**) TC-treated cells pretreated with wortmannin. Magnification was × 400.

suggest a major role for TDC and TCDC in mucin and more particularly MUC4 overexpression.

Bile salts mediate their biological effects via several signalling cascades (PI3K, MAPK, PKC, PKA) [38–43]. TC-, TDC-, TCDC-, GC- and GNa-mediated up-regulation of MUC4 mainly occurs through activation of PI3K. However, in some cases, inhibition of PKA, PKC or MAPK resulted in the activation of MUC4 expression by bile salts. Bile salts are thus capable of activating more than one signalling pathway with a different output on the expression of *MUC4*. Moreover, bile salt effects on *MUC4* expression are promoter-dependent and most probably involve different combinations of transcription factors. Bile acids are known to interact with farnesoid X receptor (FXR) and pregnane X receptor (PXR) to regulate transcription of their target genes [44,45]. Future experiments aiming at deciphering the molecular mechanisms used by TC, TDC, TCDC, GC and GNa and, more particularly, at exploring the role of FXR and PXR transcription factors in the regulation of *MUC4* expression will be performed to show their direct or indirect involvement. We hypothesize that the diversity of activated pathways and transcription factors binding to *MUC4* promoter in a sequence-specific manner may explain the differences seen in the responses between the different bile salts. Consequently, the balance between PI3K and MAPK, PKA and PKC pathways will be important and will result either in MUC4 activation (PI3K) or MUC4 inhibition (MAPK, PKA, PKC) and will determine the specific outcome of each bile acid on MUC4 expression.

A better understanding of MUC4 regulation in Barrett's oesophagus will allow a demonstration of whether MUC4, as a target of bile acids, which are deleterious factors in Barrett's oesophagus associated with gastro-oesophageal reflux and its consequent evolution into oesophagus adenocarcinoma, may be considered as a possible marker in the early steps of oesophageal carcinogenesis.

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