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Activation of MUC1 mucin expression by bile acids in human esophageal adenocarcinomatous cells and tissues is mediated by the phosphatidylinositol 3-kinase

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Background. In esophageal adenocarcinoma, MUC1 mucin expression increases in early stages of the carcinogenetic sequence, during which bile reflux has been identified as a major carcinogen. However, no link between MUC1 overexpression and the presence of bile acids in the reflux has been established so far, and molecular mechanisms regulating MUC1 expression during esophageal carcinogenetic sequence are unknown. Our aim was to identify (1) the bile acids able to upregulate MUC1 expression in esophageal cancer cells and mucosal samples, (2) the regulatory regions in MUC1 promoter responsive to bile acids, and (3) the signaling pathway(s) involved in this regulation.

Methods. MUC1 mRNA and mucin expression were studied by the means of real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry, both in the human esophageal OE33 adenocarcinoma cell line and in an ex vivo explant model. MUC1 promoter was cloned and transcription regulation was studied by transient cell transfection to identify the bile acid–responsive regions. Signaling pathways involved were identified using specific pharmacologic inhibitors and siRNA approach.

Results. Taurocholic, taurodeoxycholic, taurochenodeoxycholic, glycocholic, sodium glycocholate, and deoxycholic bile acids upregulated MUC1 mRNA and protein expression. The highest induction was obtained with deoxycholic and taurocholic acids in both cellular and explant models. The bile acid-mediated upregulation of MUC1 transcription occurs at the promoter level, with responsive elements located in the -1472/-234 region of the promoter, and involves the phosphatidylinositol 3-kinase signaling pathway.

Conclusions. Bile acids induce MUC1 mucin overexpression in human esophageal adenocarcinoma cells and tissues by activating its transcription through a process involving phosphatidylinositol 3-kinase. (Surgery 2007; ::=-=.)

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ADENOCARCINOMA OF THE ESOPHAGUS, the incidence of which has profoundly increased during the past few decades,^{1,2} has one of the poorest outcomes of all human malignancies. The major risk factors for the development of esophageal adenocarcinoma are gastroesophageal reflux disease and its sequela. Barrett's esophagus is a condition in which metaplastic columnar epithelium replaces the normal stratified squamous epithelium.³⁻⁵ Increased intragastric concentrations of bile acids 166

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111 have has been observed in patients with Barrett's 112 esophagus, and it has been suggested that incomplete 113 intestinal-type metaplasia may be a response to reflux of gastroduodenal contents and, in particular, to bile 114 acids.^{6,7} Moreover, duodenoesophageal bile reflux 115 has recently been identified as a predominant and 116 117 sufficient carcinogen to induce esophageal adenocar-118 cinoma in Barrett's esophagus in a rat model.^{8,9}

119 Endoscopic surveillance of patients with known 120 Barrett's esophagus is the current strategy to manage 121 the risk of cancer formation, targeted at the detec-122 tion of dysplasia. Unfortunately, data suggest that 123 dysplasia is an imperfect predictor of cancer risk in Barrett's esophagus.¹⁰ Thus, an earlier indicator of 124 cancer risk would be detection of DNA damage itself 125 126 before the histologic manifestations of dysplasia are 127 even apparent. The identification of molecular 128 biomarkers may offer easy reproducibility, standardi-129 zation, and truly early detection of neoplastic pro-130 gression. Among them, mucins appear as good 131 candidates to evaluate, because their expression is altered dramatically in Barrett's esophagus.^{5,11,12} 132

133 In normal esophagus, mucins protect the under-134 lying mucosa against potential injuries such as 135 reflux of gastroduodenal contents including bile acids.¹³ Among the members that compose the fam-136 ily of mucins, membrane mucins are thought to play 137 138 important roles in tumor cell biology, cell proliferation, tumor progression, and metastasis.¹¹ MUC1 is 139 the best-characterized membrane mucin.¹⁴ It is a 140 large O-glycoprotein with an extended, heavily gly-141 cosvlated extracellular domain that protrudes far 142 143 away from cellular membrane (200 to 500 nm). 144 This structural feature confers to MUC1 the ability 145 to participate in cell-cell and cell-extracellular matrix recognition processes.^{11,15} Consequently, over-146 expression of MUC1 membrane mucin at the cell 147 148 surface provides the cancer cell with new biologic properties by altering its interacting properties 149 150 with immune, epithelial, or endothelial cells or the extracellular matrix.^{14,15} Moreover, MUC1 is known 151 152 to interact with the EGF-receptor and participate in intracellular signaling.¹⁴ These peculiar structural 153 154 features and biologic activities put forward mucins 155 as important molecules to study in order to better understand their role in carcinogenesis and biology 156 of the cancer cell.^{11,16,17} 157

158In normal esophagus, MUC1 and MUC4 are the159main mucin genes expressed in the stratified160squamous epithelium.^{12,18} In the preneoplastic161Barrett's esophagus, secreted mucins are expressed162mainly (MUC2, MUC5AC, MUC5B, MUC6) associ-163ated with MUC1 and MUC4.^{5,18-20} In high-grade164dysplasia and adenocarcinoma of Barrett's esopha-165gus, downregulation of secreted mucins is observed,

whereas expression of *MUC1* and *MUC4* mucin genes is sustained at high levels.^{12,13,20,21} From these results, several groups^{18,19} have been considering MUC1 as a valuable phenotypic marker to distinguish between Barrett's esophagus patients with different origins and those at risk to develop neoplasia in Barrett's esophagus. Finally, bile acids can activate the expression of 2 mucins found in Barrett's esophagus, the secreted mucin MUC2²² and the membrane mucin MUC4,^{21,2} suggesting a relation between increased mucin expression and progression of esophageal cancer associated with bile reflux; however, at this time, no data are available to explain the upregulation of MUC1 in Barrett's esophagus associated with reflux.

In relation with MUC1 overexpression during the development of esophageal adenocarcinoma in Barrett's esophagus and the role of bile acids in Barrett's metaplasia and its consequent degeneration into esophageal adenocarcinoma, we undertook in this work to identify (1) the bile acids responsible for the upregulation of MUC1 mucin in esophageal cancer cells and explants, (2) the regulatory regions responsive to bile acids in *MUC1* promoter, and (3) the signaling pathway(s) involved in this regulation.

MATERIAL AND METHODS

Cell culture. The esophageal cancer cell line OE33 was purchased from the European Collection of Cell Cultures (ECACC) and cultured as described previously.²¹ Cells were treated with bile acids and their conjugates for 24 hours with the following concentrations as previously described²¹: taurocholic acid (TC), 0.5 mM; taurodeoxycholic acid (TDC), 1 mM; taurochenodeoxycholic acid (TCDC), 0.5 mM; glycocholic acid (GC), 0.5 mM; sodium glycocholate (GNa), 0.5 mM; and deoxycholic acid (DC), 0.05 mM. In inhibition studies, pharmacologic inhibitors were incubated with the cells for 30 minutes prior to the addition of bile acids at the following final concentrations: wortmannin (2.5 nM, inhibitor of PI3K), PD98059 (30 μ M, inhibitor of MAPK; Calbiochem, VWR International S.A.S., France), U0126 (10 µM, inhibitor of MEK; Promega, Charbonnières, France), GF109203X (10 µM, inhibitor of PKC; Calbiochem), and KT5720 (1 ng/ml, inhibitor of PKA; Calbiochem). All reagents were from Sigma (St Louis, Mo, USA) unless otherwise indicated.

Toxicity of each bile acid was evaluated under the same experimental conditions by trypan blue exclusion measurement on a hemocytometer. Effects of bile acids on cell proliferation and apoptosis were studied by immunochemistry, using

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221 monoclonal anti-Ki67 (dilution of 1:50; Dako/ 222 Dakocytomaton, Trappes, France) and polyclonal 223 anticleaved caspase-3 (dilution of 1:150; Cell Sig-224 naling, Ozyme, France) antibodies, respectively. 225 Level of caspase-3 mRNA was also evaluated by 226 reverse transcriptase-polymerase chain reaction 227 (RT-PCR) using a specific pair of primers (MWG-Biotech, Germany) for caspase-3 gene: forward 228 229 primer: 5'-TATGGTTTTGTGATGTTTGTCC-3', re-230 verse primer: 5'-TAGATCCAGGGGGCATTGTAG-3' 231 (GenBank accession no. G10724). Annealing tem-232 perature was 55°C. Expected size for caspase-3 PCR 233 product is 195 basepairs (bp).

234 RT-PCR. Total RNA from OE33 cells or esoph-235 ageal tissues were prepared using the RNeasy mini-236 [Q3] kit or midi-kit (Qiagen, Courtaboeuf, France), 237 respectively. To prepare cDNA ((Advantage RT-238 for-PCR kit; Clontech, Saint-Germain-en-Laye, 239 France), 1.5 μ g (0.4 μ g for tissues) of total RNA was used, as described previously.24 PCR was per-240241 formed on 2 μ l (5 μ l for tissues) of cDNA using a 242 specific pair of primers (MWG-Biotech, Germany) 243 for MUC1 mucin gene (GenBank accession 244 no. J05582): forward primer : 5'-GAACTACGGG 245 CAGCTGGACATC-3' (nucleotides 3639-3661), reverse primer : 5'-GCTCTCTGCGCCAGTCCTCC 246 TG-3' (nucleotides 4065-4086).²⁵ PCR reactions 247 were carried out in 50 µl final solutions as de-248 249 scribed previously.²⁶ Annealing temperature was 60°C. PCR products were analyzed on 1.5% aga-250 251 rose gels containing ethidium bromide run in 252 1× Tris-borate-ethylenediamine tetraacetic acid 253 (EDTA) buffer. A 100 bp DNA ladder was pur-254 **[Q4]** chased from Amersham Biosciences. Ribosomal

255 18S subunit (forward primer: 5'-GGACCAGAGGC 256 AAAGCATTTGCC-3'; reverse primer: 5'-TCAATCT 257 CGGCTGGCTGAACGC-3') was used as the inter-258 nal control. In siRNA experiments, GAPDH (for-259 ward primer: 5'- TGAAGGTCGGAGTCAACGGAT 260 TTGGT-3'; reverse primer: 5'- CATGTGGGCCATG 261 AGGTCCACCAC-3') was used as the internal con-262 trol. A specific pair of primers for PI3K p110 subu-263 nit (forward primer: 5'-ACAATGCCTCCAAGACC 264 ATCATC-3'; reverse primer: 5'-CATACATTGCTC 265 TACTATGAGGTG-3') was used to evaluate effectiveness of PI3K knockdown.²⁷ Expected sizes for 266 MUC1, 18S, GAPDH, and PI3K p110 PCR products 267 are 447, 496, 980, and 499 bp, respectively. RT-PCR 268 269 was carried out on cDNAs from 3 different sets of 270 experiments.

271Immunohistochemistry.After treatment with272bile acids, confluent OE33 cells were trypsinized,273centrifuged, washed once with 1x phosphate-buff-274ered saline (PBS). The final pellet was fixed in 4%275(w/v) paraformaldehyde and embedded in

paraffin; $3 \cdot \mu m$ sections were prepared and processed for immunostaining, as described before.²¹ Positive controls for MUC1 were run on normal alveolar pulmonary epithelium. Negative control was run by omission of the primary antibody. Cell counting for MUC1, caspase-3, and Ki67 positivity was performed by 2 independent pathologists. The monoclonal anti-MUC1 antibody (LICR-LON-M8, 1:50 dilution) recognizes the DTR epitope within the tandem repeat region of MUC1 and was a kind gift from Dr D. Swallow (MRC, London, England).²⁸

Tissue sample collection and explant studies. Surgical biopsy specimens were collected from patients who underwent curative esophagectomy by 1 surgeon (C.M.) for Barrett's adenocarcinoma without neoadjuvant treatment. Macroscopic adenocarcinoma mucosal samples of 1-mm thickness and weighing more than 20 mg were removed from the surgical specimen under sterile conditions using a frozen scalpel immediately after the vascular section. Consent was obtained from each patient included in this experimental study.

For the explant studies, tissues were placed immediately in supplemental medium and cultured under the same conditions used for the cell culture experiments. Tissues were exposed to [Q5] each bile acid for 30 minutes at the concentrations used in cell culture studies. In all experiments, a control sample was included by treating the explant with an identical volume of sterile water. Samples were then divided into 2 parts using aseptic technique. One part was immersed in 5 ml of a 4% neutral formaldehyde solution (v/v, pH 7.4) in phosphate buffer and processed for paraffin embedding. The diagnosis of adenocarcinoma mucosa was confirmed by 2 pathologists after staining the section $(4 \ \mu m)$ with hematoxylin-eosin-saffron. The second part was immersed overnight in 5 ml of RNA later solution (Qiagen) and processed for [Q6] RNA extraction as described above.

Western blotting. OE33 total cellular extract preparation, protein electrophoresis, and Western blotting were carried out as described previously.²³ Standard immunodetection procedure was then performed using the following specific antibodies: anti-p38 MAPK (1:1000 dilution), anti-stress-activated protein kinase/Jun-terminal kinase (SAPK/ JNK, 1:1000 dilution) rabbit polyclonal antibodies (Cell Signaling), anti-active p38 (1:2000 dilution), and anti-active JNK (1:5000 dilution) rabbit polyclonal antibodies (Promega). Secondary antibody consisted of antirabbit IgG alkaline phosphataseconjugate (Promega). Blots were then incubated with Nitro Blue Tetrazolium Chloride and

4 Mariette et al

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Position on the promoter	Primer sequence $(5' \rightarrow 3')$	Orientation	
-233/+33	CGC GGT ACC CAG GGA GTG GTT GGT GA	Sense	
	CGC GGT ACC GAT TCA GGC AGG CGC TGG	Antisense	
-616/+33	CGC GGT ACC CAA GGA GGG AAC CCA GGC	Sense	
	CGC GGT ACC GAT TCA GGC AGG CGC TGG	Antisense	
-1472/+33	CGC GGT ACC CTG CTC CCC AAA GGA TAG	Sense	
	CGC GGT ACC GAT TCA GGC AGG CGC TGG	Antisense	

331 Table I. Sequences of the pairs of primers used in PCR to produce deletion mutants of MUC1 promoter in 332 pGL3 basic vector

KpnI cloning site is italicized and underlined.

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343 5-bromo-4-chloro-3-indolyl phosphate substrate 344 (Life Technologies), and the reaction was stopped **[Q7]** in water. 345

346 Cloning of the MUC1 promoter. The pMAH-347 MUC1 vector, which contains 2.8 kb of MUC1 348 promoter in pT7-Luc vector, was a kind gift from 349 [Q8] Dr M. A. Hollingsworth (UNMC, Omaha, Neb, 350 USA). Internal deletion mutants were generated 351 by PCR using pairs of primers bearing KpnI restric-352 [T1] tion site at their 5'end (Table I). PCR reaction was 353 performed as described previously on 0.46 μ g of 354 plasmidic DNA with a specific annealing tempera-355 ture of 70°C. PCR products were digested with 356 KpnI restriction enzyme (Roche Biochemicals, Meylan, France), gel-purified (QIAquick gel ex-357 358 traction kit, Qiagen) and ligated into the promo-359 terless pGL3 Basic vector (Promega), which had 360 been linearized previously with KpnI using a 361 DNA rapid ligation kit (Roche Biochemicals). Positive clones containing the insert were sequenced 362 363 on both strands on an automatic LI-COR se-364 quencer (ScienceTec, Les Ulis, France) using infra-365 red-labeled RV3 and GL2 primers (Promega). 366 Plasmids used for transfection studies were pre-367 pared using the Endofree plasmid Mega kit 368 (Qiagen).

369 Transfections. Transfections were performed 370 using Effectene reagent (Qiagen), as described previously.²⁹ Cells were passed at 0.5×10^6 cells/ 371 372 well the day before the transfection. Luciferase 373 activity was corrected for transfection efficiency 374 by cotransfecting cells with pRL-TK vector (Prom-375 ega). Total cell extracts were prepared after a 48-hour incubation time at 37° C using 1× Passive 376 377 Lysis Buffer (Promega), per manufacturer's instructions. Luciferase activities were measured on 378 379 [Q9] a TD 20/20 luminometer (Turner Design). The 380 relative luciferase activity was expressed as fold in-381 duction of the test plasmid activity compared with 382 that of the corresponding empty vector. In bile 383 acids experiments, relative luciferase activity was expressed as fold activation of luciferase activity 384 in bile acid-treated cells compared with untreated 385

cells. All experiments were performed in triplicate and repeated 3 times.

Small interfering RNA (siRNA) assays. OE33 400 cell seeding $(15 \times 10^3 \text{ cells/well})$ and transfections 401 were performed as described previously 23 with 100 nM of PI3K SMARTpool siRNA, using 1 µl of 402 DharmaFECT1 transfection reagent (Dharmacon, 403 Brebières, France). Controls included mock trans-404 fected cells, cells transfected with siCONTROL 405 non-targeting siRNA, or siCONTROL GAPD 406 siRNA. Bile acid treatment was performed 24 407 hours after transfection and lasted 24 hours, as 408 described above. Total RNA was isolated 48 hours 409 410 after transfection, as described above. Each siRNA 411 was assayed in triplicate in at least 3 separate exper-412 iments. PCR was performed on 5 μ l of cDNA, as described previously. Densitometric analysis of 413 DNA bands was carried out using the GelAnalyst-414 GelSmart software (Clara Vision). Results were [Q10] 415 expressed as MUC1/GAPDH and PI3K p110/ 416 417 GAPDH ratio.

Statistics. When indicated, data were analyzed by the Mann-Whitney U test with differences of P < .05 considered significant.

RESULTS

Regulation of MUC1 mRNA expression by bile 423 acids in OE33 cancer cells. The level of MUC1 424 425 mRNA expression in OE33 cancer cells treated with different bile acids was assessed by RT-PCR 426 (Fig 1, A). The level of MUC1 mRNA in untreated [F1-4/C] cells (lane 1) was increased substantially after cell 427 treatment with taurocholic acid (TC, lane 2), taur-428 odeoxycholic acid (TDC, lane 3), taurochenodeox-429 ycholic acid (TCDC, lane 4), glycocholic acid (GC, 430 lane 5), sodium glycocholate (GNa, lane 6), and 431 deoxycholic acid (DC, lane 7). The highest induc-432 tion (4.5-fold) was obtained with DC. Other bile 433 acids (cholic acid, chenodeoxycholic acid, dehy-434 drocholic acid, and cholic methylester) previously 435 shown to activate MUC4 mucin expression²¹ were 436 437 studied but had no effect on MUC1 (data not shown). 438

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Fig 1. Effect of bile acids and their conjugates on expression of *MUC1* mRNA and of MUC1 apomucin in OE33 cancer cells. **A**, Cells were treated with indicated bile acids for 24 hours, as described in Material and Methods. *MUC1* and *18S* PCR products (7.5 μl and 4 μl, respectively) were separated on a 1.5% agarose gel containing ethidium bromide after electrophoresis in 1X Tris-borate-EDTA buffer. Immunohistochemistry was performed as described in Material and Methods. **B-C**, Untreated cells. **B**, Alcian blue staining; **C**, MUC1 immunostaining; **D-I**, MUC1 immunostaining after treatment with TC (**D**), TDC (**E**), TCDC (**F**), GC (**G**), GNa (**H**) and DC (**I**). Original magnification ×200.

474 Effect of bile acids on MUC1 apomucin expres-475 sion in OE33 cancer cells. Having shown that 476 MUC1 mRNA expression was upregulated by bile 477 acids, we examined whether that regulation had 478 an impact on the expression of MUC1 apomucin 479 [T2] (Figs 1, B-I, and Table II). Untreated OE33 cells 480 showed a relatively low degree of differentiation, 481 because no vacuoles and a few grains of secretions 482 were detected (Fig 1, B). When immunostained 483 [Q11] with anti-MUC1 antibody, 1% to 5% of the cells 484 were labeled positively (Fig 1, C). The staining was seen in the membrane and cytoplasm (Fig 1, 485 486 C, arrow). The labeling became very intense and 487 up to 90% of the cells were stained positively for MUC1 when they were treated with TC (Fig 1, 488 489 D), TDC (Fig 1, E), TCDC (Fig 1, F), GC (Fig 1, 490 G), GNa (Fig 1, H), and DC (Fig 1, I).

491 Bile acid treatment under the same conditions492 did not induce any change in the morphology of

the cells (compare Figs 1, *B* and *C* [untreated cells] with Fig 1, D-I [bile acid-treated cells]) nor did it induce cell death, because trypan blue exclusion measurements were similar in untreated and bile acid-treated cells (0% to 1% dead cells). Absence of effect on apoptosis was also observed by studying caspase-3 expression at the mRNA and protein levels. No induction of caspase-3 mRNA (Fig 2, A) [F2-4/C] or cleaved caspase-3 protein level (Figs 2, B-D and Table II) was observed after bile acid treatment. Finally, under our experimental conditions, bile acids did not alter cell proliferation because the number of cells immunostained for the proliferation marker Ki67 was similar in untreated cells (Fig 2, E) and bile acid-treated cells (Fig 2, F and Table II).

Effect of bile acids on the expression of MUC1 mRNA in adenocarcinomatous esophageal mucosa. MUC1 regulation by bile acids was also

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	Control	TC	TDC	TCDC	GC	GNa	DC
MUC1	1-5	89	85	91	90	82	92
Trypan blue	0-1	0-1	0-1	0-1	0-1	0-1	0-1
Caspase-3	<1	<1	<1	<1	<1	<1	<1
Ki67	90	85	93	94	86	85	85

Table II. Effect of bile acids and their conjugates on MUC1 apomucin expression, cell death (trypan blue),

Cells were treated with the indicated bile acids for 24 hours and processed for immunostaining with anti-MUC1, anticleaved caspase-3, and anti-Ki67 antibodies (Material and Methods). Percentage of positive cells was calculated by 2 independent pathologists.

557 558 studied in an ex vivo organ culture model. To this 559 aim, surgical specimens of adenocarcinomatous esophageal mucosa from 9 patients were cultured 560 561 and exposed to bile acids. Expression of MUC1 was assessed by RT-PCR and immunochemistry. The 562 563 means ratio of MUC1 mRNA expression over 18S 564 **[F3]** from the 9 samples is shown in Fig 3, A. A 2-fold 565 induction of MUC1 mRNA level was observed in [F4] explants treated with TDC (Fig 4, *B*, *lane 3*), 566 567 TCDC (lane 4), GC (lane 5), and GNa (lane 6). 568 When treated with TC (lane 2) and DC (lane 7) a 569 4- to 4.5-fold induction was obtained. The increase 570 of MUC1 mRNA could not be correlated to an in-571 crease of MUC1 apomucin expression by immuno-572 chemistry because of an already high level of MUC1 expression in the control samples (90% to 573 574 100% of cells were MUC1-positive [data not 575 shown]). These results, however, indicate that 576 bile acids are able to activate MUC1 expression at the mRNA level in explants and that TC and DC 577 are the most potent activators in OE33-cultured 578 579 cancer cells and in mucosal explants.

580 Identification of bile acid-responsive regions in 581 the MUC1 promoter. To identify bile acid-responsive 582 regions responsible for MUC1 transcriptional upregu-583 lation, 1.47 kb of the MUC1 promoter was cloned into promoterless pGL3 basic vector (Fig 4, A). The 3 con-584 585 structs used in this study were active in OE33 cells (Fig 4. B). The fragment that covers the first 233 nucleo-586 587 tides of the promoter was already very active (15-fold 588 activation). The activity increased to 40-fold activation 589 when the intermediate -616/+33 deletion mutant was 590 transfected. With the longest fragment (-1472/+33), 591 the activity went up to 50-fold, indicating that all the 592 elements necessary for maximal activity of MUC1 593 promoter were present in the 1.47 kb region.

594 To identify bile acid-responsive regions within 595 the MUC1 promoter, transfected cells were treated 596 with bile acids for 24 hours (Fig 1) before measure-597 ment of luciferase activity. Promoter activity was 598 then compared between bile acid-treated cells 599 and untreated cells. As shown in Fig 4, C, a 2- to 2.5-fold activation of the -616/-234 promoter re-600 gion (gray bars) indicated that TC- and GC- responsive elements were present in that region of the promoter. For TDC and DC, similar activations (2-fold) of -1472/+33 and -616/+33 regions were observed, indicating that responsive elements for these 2 bile acids are present in the -1472/-234 region of the promoter. In contrast, responsive elements for TCDC and GNa were present within the more distal region -1472/-762 (2.5- and 2.2-fold activation [black bars]).

PI3K signaling pathway mediates upregulation 621 of MUC1 by bile acids in OE33 cells. Having 622 shown that bile acids regulate MUC1 expression 623 at the transcriptional level in OE33 cells, we 624 worked to identify the signaling pathway(s) re-625 sponsible for that regulation. To this aim, OE33 626 cells were pretreated for 30 minutes with specific 627 pharmacologic inhibitors of PI3K, MAPK, PKC, 628 and PKA, which are known to be activated by bile 629 acids,^{21,30-33} before adding bile acids to the cells 630 for another 24 hours. Inhibition of MAPK 631 (PD98059, U0126), PKC (GF109203X), or PKA 632 633 (KT5720) did not alter the level of MUC1 mRNA in the cells, indicating that these pathways were 634 635 not involved (data not shown). We also used Western blotting analysis to determine whether the 636 other 2 MAPK pathways (p38 MAPK and SAPK/ 637 [NK) were activated by bile acids. No variation in [Q12] 638 the level of phosphorylation of these 2 kinases, 639 however, was visualized, indicating that these path-640 ways are not triggered by bile acids under these 641 conditions (data not shown). In contrast, inhibi-642 643 tion of PI3K pathway with wortmannin (w) before cell treatment with TC, TDC, TCDC, and DC in-644 duced a substantial decrease of MUC1 mRNA level, 645 which then returned to the basal level (Fig 5, A, [F5-4/C]) lanes 3, 5, 7, and 13 vs lanes 2, 4, 6, and 12). Inhibi- [Q13] 646 tion of PI3K signaling pathway before cell treat-647 ment with GC and GNa had no effect on MUC1 648 mRNA level (compare lanes 9 and 11 to lanes 8 649 650 and 10).

The direct implication of the PI3K signaling 651 pathway in bile acid regulation of MUC1 endoge-652 653 nous expression was then assessed by performing knockdown assays with specific siRNA for PI3K 654

Surgery Volume ■, Number ■



6/9Fig 2. Effect of bile acids and their conjugates on apoptosis and proliferation in OE33 cancer cells. A, Level of caspase-3680mRNA by RT-PCR in untreated cells (*lane 1*) and bile acid-treated cells (*lanes 2-7*) as described in Material and Methods.681*Caspase-3* and 18S PCR products (15 μ l and 4 μ l, respectively) were analyzed on a 1.5% agarose gel. Cleaved caspase-3682immunostaining in untreated cells (B) and after treatment by TC (C) and TCDC (D). Original magnification $\times 200$.683Ki67 immunostaining in untreated cells (E) and after treatment by TDC (F). Original magnification $\times 200$.

(Fig 5, *B*). The results showed that knockdown of PI3K significantly (P < .05) inhibited TC-, TDC-, TCDC-, and DC-mediated increase of MUC1 mRNA level (73% ± 11%, 52% ± 19%, 44% ± 7%, and $84\% \pm 2\%$ inhibition, respectively). This indi-cates that PI3K mediates MUC1 endogenous ex-pression in response to these bile acids. Treatment of cells with GC and GNa enhanced the basal expression of MUC1 mRNA, but knock-down of PI3K did not significantly alter MUC1 mRNA level (P > .05, data not shown).

Immunostaining of OE33 cells with a specific anti-MUC1 antibody confirmed the involvement of the PI3K pathway in bile acid-mediated upregula-tion of MUC1 apomucin expression (Figs 5, C-H). The labeling decreased from 82% in TC-treated cells (Fig 5, C) to 43% in wortmannin-pretreated cells (Fig 5, F) and from 62% in TDC-treated cells **F6** (Fig 6, D) to 21% in wortmannin–retreated cells (Fig 5, G). The same conclusion was drawn regard-ing DC-mediated upregulation of MUC1 apomu-cin expression because 63% of DC-treated cells were positive for MUC1 (Fig 5, E), whereas only 29% remained labeled when cells were pretreated with wortmannin (Fig 5, H).

We then confirmed the involvement of the PI3K signaling pathway in mediating bile acid upregulation of *MUC1* at the promoter level (Fig 6). OE33 cells were transfected with responsive constructs to bile acids then pretreated with wortmannin for 30 minutes before adding the bile acids for another 24 hours. The results indicated that 75% to 80% of the transactivation of the -616/+33 region of the *MUC1* promoter by TC and TDC (Fig 6, gray bars) was lost when cells were pretreated with wortmannin. The same decrease was observed on the -1472/+33 region of the promoter when cells were pretreated with wortmannin before incubation with TDC, TCDC, and DC bile acids (Fig 6, *black bars*).

DISCUSSION

Bile reflux has been identified as a predominant and sufficient carcinogen to induce esophageal adenocarcinoma on Barrett's esophagus.⁷⁻⁹ The MUC1 mucin pattern of expression is altered profoundly during the esophageal carcinogenetic \sequence, but no link between MUC1 overexpression during early stages of Barrett's esophagus and the presence of bile acids in the reflux has been established to date. In the present study, we found



Fig 3. Effect of bile acids and their conjugates on expression of *MUC1* mRNA and of MUC1 apomucin in human explants of adenocarcinomatous esophageal mucosa by RT-PCR. Tissues were treated with indicated bile acids for 30 minutes, as described in Material and Methods. **A**, Diagram shows MUC1/18S ratio calculated from compilation of the results obtained from 9 different surgical specimens. **B**, RT-PCR profile for 1 patient. *MUC1* and *18S* PCR products (7.5 μ l and 4 μ l, respectively) were analyzed on a 1.5% agarose gel.

that bile acids are strong inducers of MUC1 mucin expression in human esophageal adenocarcinoma cells and in human tumoral tissues. We showed that the regulation occurs at the transcriptional level and is mediated by phosphatidyl-inositol 3 kinase. We also showed that, among the bile acids tested, DC and TC are the strongest inducers.

Activation of PI3K by bile acids is well-documented³⁰⁻³³ and may be one of the mechanisms of bile acid-induced carcinogenesis. We found that PI3K inhibitor wortmannin and PI3K siRNA efficiently blocked MUC1 induction by bile acids, indicating that PI3K is involved in the bile acid-de-pendent induction of MUC1. Pharmacologic inhi-bition of MAPK, PKC, and PKA did not alter the level of MUC1 mRNA in the cells, indicating that these pathways are not involved in this upregula-tion. Moreover, at this time, we have not been able to identify the signaling pathway that could mediate GC and GNa effects on the mRNA expres-sion of MUC1, suggesting indirect regulation for these bile acids. This result, and previous results in which we demonstrated that PI3K also was in-volved in the bile acid-mediated upregulation of the membrane mucin MUC4,²¹ suggest strongly that PI3K is a major and common signaling

pathway used to mediate bile acids effects on membrane-bound mucins. Bile acids could induce the transcription of another mucin gene that encodes the secreted mucin MUC2²²; in that case, however, the mechanism implied activation of an AP-1 complex. From these results and ours, we hypothesize that during esophageal carcinogenesis associated with reflux and the presence of bile acids in esophageal lumen, mucin expression is activated via different mechanisms: PI3K activation for membrane mucins and AP-1 activation for secreted mucins. This hypothesis suggests that the esophageal mucosa is able to respond to a chronic aggression by using specific intracellular mechanisms to drive either the secretion of MUC2, which participates in mucus formation and epithelium defense/integrity, or expression at the cell surface of MUC1 and/or MUC4 membrane mucins. $[\mathbf{Q}\mathbf{14}]$

We showed in this work that MUC1 activation by bile acids in esophageal cancer cells was not accom-panied by increased cell proliferation or cell apo-ptosis. Moreover, MUC1 is known to activate PI3K/ Akt and Bcl- x_L antiapoptotic pathways,³⁴ a pathway (PI3K) also activated by bile acids to induce cell sur-vival.³² Having shown that PI3K activates MUC1 ex-pression, our work suggests that esophageal cancer

Surgery Volume ■, Number ■

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Fig 4. Characterization of the promoter activity of *MUC1* in OE33 cancer cells by transient transfection and effect of bile acids on the transcriptional activity of *MUC1* promoter. A, Schematic representation of 3 promoter constructs covering 1.47 kb of the *MUC1* promoter. B, Luciferase diagram showing activity of 3 deletion mutants. Ref., Cells transfected with the empty vector pGL3 basic. C, Diagram representing the ratio of luciferase activity in bile-acid treated cells versus untreated cells on 3 pGL3-*MUC1* promoter constructs. Cells were treated with bile acids for 24 hours. Ref., cells transfected without bile acid treatment (this value was arbitrarily set to 1). Mean ± SD values were calculated from the values obtained in triplicate from 3 separate experiments.

919 cells, when submitted to bile acid aggression, may 920 use an autocrine system to overexpress MUC1 mu-921 cin at their surface via PI3K (a pathway implicated 922 in the regulation of genes contributing to tumorigenesis³⁵) and thereby promote cancer progression. 923 Our conclusions are in agreement with recent 924 925 data^{11,14-16} that demonstrated that overexpression 926 of MUC1 (a transmembrane protein with a long ex-927 tracellular glycosylated domain, which plays the role 928 of sensor of the cellular environment) favors tumor 929 progression and metastasis.

Transcription factors implicated in *MUC1* upregulation by bile acids after the activation of PI3K are unknown. Activation of gene transcription by PI3K may be mediated by several families of transcription factors. Among those that have been identified are the following: activating transcription factor-1/cAMP-responsive element-binding protein (ATF/CREB),³⁶ nuclear factor- κ B (NF- κ B),³⁷⁻³⁹ and factors of the hepatocyte nuclear factor family (HNF).⁴⁰ Putative binding sites for these factors are present in the *MUC1* promoter, and



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1110**Fig 6.** Inhibition of PI3K signaling pathway impairs *MUC1* promoter activation by bile acids in OE33 cells. pGL3-*MUC1*1111promoter constructs (-616/+33 and -1472/+33) were transfected in OE33 cells that were then pretreated for 30 minutes1112with wortmannin (W) before adding bile acid for another 24 hours. Means \pm SD were calculated from the values ob-1113tained in triplicate from 3 separate experiments.

1116 preliminary cotransfection studies indicated that 1117 ATF/CREB and HNF-4 α transcription factors 1118 were able to transactivate the MUC1 promoter 1119 (Piessen G and Van Seuningen I, unpublished 1120 [Q15] data). Cdx-2 transcription factor was also hypothe-1121 sized to be a good candidate, because it regulates mucin expression²⁶, was shown recently to be activated by bile acids,⁴¹ and is able to transactivate 1122 1123 human MUC1 promoter (Piessen G and Van Seu-1124 1125 [Q16] ningen I, unpublished data).

Bile acids, amphophilic derivatives of choles-1126 1127 terol, have been shown to enhance cellular trans-1128 formation in vitro and are known promoters of gastrointestinal neoplasia in vivo.42 Moreover, con-1129 1130 jugated bile acids exacerbate esophageal mucosa injury either in combination with acid or alone, 1131 both in vitro or in animals^{8,43,44} and humans.⁴⁵ 1132 1133 Wide variations in biologic activity have been re-1134 ported for individual bile acid fractions, and minor changes in the structure of bile acids dramatically 1135 alter their biologic effects.³² Moreover, the concen-1136 trations of the individual bile acids present in bile 1137 reflux are modified as a result of the process of 1138 reflux itself.⁴⁶ All these data make bile acids impor-1139 tant molecules to consider in esophageal carcino-1140 1141 genesis associated with bile reflux; identifying 1142 their target genes, therefore, will help in a better 1143 control their biologic effects on epithelial cells. 1144 We showed in the current study that the unconju-1145 gated bile acid DC and, in a lower proportion, 1146 the taurine conjugate bile acid TC are the major 1147 bile acids responsible for MUC1 upregulation. Consequently, these bile acids may be considered 1148 1149 as the most important components of the bile in

mediating MUC1 upregulation and promoting its role in tumor progression in esophageal cancer associated with bile acid refluxate. From this work and others, DC appears as the major bile acid to activate mucin gene transcription, because it is able to activate MUC1, MUC4,²¹ and MUC2.²² We thus hypothesize that, in esophageal carcinoma associated with bile reflux, mucin activation is under the control of DC.

Previous studies have shown the following: (1) 1180 secondary bile acids in patients with Barrett's 1181 esophagus may contribute to the metaplastic 1182 change, and (2) a significant proportion of the 1183 1184 bile acids in patients with extensive mucosal injury were composed of the dehydroxylated taurodeox-1185 ycholic acid (TDC) and the unconjugated deoxy-1186 cholic acid (DC).⁴⁶ DC has also been under 1187 considerable interest because of excessive free 1188 bile acid deconjugation in the small intestine, lead-1189 ing to extensive damage with loss of villi through 1190 inhibition of the main active transport systems 1191 for glucose, amino acids, and sodium in the jeju-1192 num.⁴⁷ Finally, DC has been shown to do the fol-1193 lowing: (1) increase colon tumor incidence in 1194 N-methyl-N-nitrosourea-treated rats,48 indicating 1195 that DC is a tumor promoter rather than a com-plete carcinogen, 48,49 (2) cause apoptosis in meta-1196 1197 plastic esophageal cells expressing wild-type p53⁷ 1198 and in colonic cells, especially in goblet cells,⁵⁰ 1199 and (3) cause extensive cell death in rat colonic 1200 mucosa, which is followed by a period of increased 1201 cell proliferation.⁵¹ In contrast, we showed that un-1202 der our experimental conditions, neither cell pro-1203 liferation nor apoptosis were altered by bile acid 1204

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treatment, suggesting that the molecular mecha-nisms induced by bile acids are tissue-specific anddifferent in colonic and esophageal cells.

1208 Of the different components of the refluxate, 1209 bile acids-either alone or in combination with acid-are probably the most important.⁵² The pH 1210 of the refluxate has been shown to modify the 1211 effect of bile acids on epithelial cells.42 One expla-1212 1213 nation is that pH variation can modify the biologic characteristics of bile salts, thus facilitating pene-1214 tration of certain bile salts in epithelial cells.⁴⁶ 1215 1216 Other elements of the refluxate include gastric 1217 pepsin, cholesterol, and pancreatic juice (trypsin, 1218 lipase). Pancreatic juice has been suggested to be 1219 to be most potent component of the duodenal 1220 reflux in the promotion of esophageal carcinogen-1221 esis in a rat model with the use of dimethylnitrosomorpholine as a carcinogen.⁵³ In one study, ⁵³ 1222 1223 biliary reflux exerted a co-carcinogenic effect 1224 when combined with pancreatic secretions. The 1225 clinical relevance of these findings, however, needs 1226 further evaluation.

1227 In conclusion we have shown that treatment of 1228 human esophageal adenocarcinomatous cells and 1229 tissues with bile acids leads to MUC1 upregulation 1230 via a PI3K-mediated molecular transcriptional 1231 mechanism. The biologic consequences of the 1232 induction of MUC1 expression by bile acids in 1233 esophageal cancer cells are still unknown, but our 1234 data favor a role in tumor progression and metas-1235 tasis. Further studies are needed to determine 1236 whether the induction of MUC1 by bile acids 1237 may increase the invasive and metastatic potential 1238 of esophageal cancer cells. The development of a 1239 rat model of esophageal carcinogenesis in which 1240 esophageal mucosa is exposed to bile or acid 1241 reflux or both will be very informative regarding 1242 the following: (1) deciphering the precise molec-1243 ular mechanisms activated by bile acids to induce 1244 MUC1 expression, (2) evaluating the conse-1245 quences of MUC1 overexpression during the 1246 carcinogenetic sequence on tumor cell behavior 1247 and biologic properties, and (3) evaluating PI3K 1248 signaling pathway as a therapeutic target in esoph-1249 [Q17] ageal carcinogenesis.⁵⁴⁻⁵⁶ This rat model will also be useful in demonstrating the pivotal role of DC 1250 1251 as the main inducer of the expression of both 1252 membrane and secreted mucins. 1253

1253This paper is dedicated to Dr Jean-Pierre Aubert,
Director of our Laboratory, who died in September
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and scientists of the Research Unit to stimulate transla-
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