Inhibition of human gall bladder mucus synthesis in patients undergoing cholecystectomy

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Abstract

Hypersecretion of gall bladder mucus is associated with gall stone formation in animal models.¹ Aspirin inhibits both mucus synthesis and secretion, prevents gall stone formation in animals² and reduces gall stone recurrence in man after dissolution therapy.³ Mucus biosynthesis in human gall bladder mucosal explants is inhibited by aspirin in vitro.⁴ We have studied the effects of aspirin in vivo. Fifty five patients with functioning gall bladder and stones have been randomised, 27 to group 1 (aspirin EC 300 mg once daily for seven days before cholecystectomy) and 28 to group 2 (controls). Gall bladder bile composition was analysed and mucus synthesis rates measured using ³H-glucosamine incorporation into mucosal explants cultured for 24 hours.⁴ Patient age, sex, and gall bladder histology were similar in both groups. There were no differences in stone composition, gall bladder bile calcium concentration, cholesterol saturation and cholesterol nucleation time. The mean ³H-glucosamine incorporation in aspirin treated patients was 1347 fmol/g wet weight as compared with 2008 fmol/g wet weight in controls (95% confidence interval 222-1100, $p < 0.005$, unpaired t test). This reduction in biosynthesis was associated with gall bladder bile mucus concentrations of 7.6 mg/ml in patients and 7.1 mg/ml in controls (ns). Treatment with aspirin led to a significant reduction in mucus biosynthesis by the gall bladder mucosa. This action is consistent with a role for aspirin in the prevention of gall stones.

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The advent of laparoscopic cholecystectomy is making a dramatic impact on the management of symptomatic patients with gall bladder stones. Despite this, many patients are still being treated with alternative approaches to cholecystectomy (both open abdominal and laparoscopic) in which the stones are removed or dissolved, but the gall bladder is left in situ. These include oral bile acid treatment,⁵⁻⁷ contact gall stone dissolution with solvents such as methylterbutylether,⁸ extracorporeal shock wave lithotripsy (ESWL) +/- adjuvant oral bile acids⁹⁻¹¹ and percutaneous cholecystolithotomy.^{12,13} A major disadvantage of all these approaches is that approximately 50% of the patients develop recurrent stones.¹⁴⁻¹⁸ As yet, there is no reliable way of preventing this recurrence but studies of the pathogenesis of recurrent stones are important as they may provide clues about primary gall stone formation.

At least three factors contribute to the

development of both primary and recurrent cholesterol stones: (i) supersaturated bile in which the amount of cholesterol present exceeds the limits of thermodynamic equilibrium,^{19,20} (ii) stasis associated with gall bladder motor dysfunction^{21,22} and (iii) abnormal nucleation of cholesterol crystals from the supersaturated bile because of an excess of promoters or a deficiency of inhibitors of crystallisation, or both.²³ Several lines of evidence suggest that excess mucus glycoprotein synthesis by the gall bladder mucosa, and secretion into the gall bladder lumen may play an important role in the nucleation and trapping or immobilising of cholesterol crystals.^{24,25} First, in prairie dogs fed a lithogenic diet, increased mucus glycoprotein synthesis and secretion by the gall bladder mucosa antedate the appearance of crystals and stones.¹ Aspirin treatment, however, prevents this excess mucus glycoprotein production and the development of gall stones in this animal model.² Second, during weight reduction, obese patients are at risk of developing microcrystals, microstones and gall stones but Broomfield and colleagues²⁶ showed that aspirin reduced this risk. Third, in a retrospective survey of patients in the British-Belgian post dissolution trial, Hood *et al*³ found that regular non-steroidal anti inflammatory drug (NSAID) ingestion prevented gall stone recurrence.

The aim of the present investigation, therefore, was to extend these preliminary observations by studying, prospectively, the effect of preoperative oral enteric coated aspirin treatment on the composition of gall bladder bile and on the rate of mucus glycoprotein synthesis by explanted gall bladder mucosa from gall stone patients scheduled for elective open cholecystectomy.

Methods

PATIENTS

Patients awaiting elective open cholecystectomy for gall stones were recruited from three hospitals: the Royal Victoria Infirmary and the Freeman Hospital in Newcastle upon Tyne and Ashington Hospital in Northumberland. Patients with a non-functioning gall bladder, previous peptic ulceration, or hypersensitivity to aspirin were excluded from the trial. In addition, patients already taking long term non-steroidal antiinflammatory agents were excluded.

Seventy three patients were randomised into two groups, 37 to group 1 (aspirin treated) and 36 to group 2 (untreated controls). Patients in group 1 were given 300 mg of enteric coated aspirin (aspirin EC) once daily for seven days before

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cholecystectomy and a further single 600 mg dose two hours before surgery. Controls received no NSAIDs during the week before surgery.

The choice of aspirin dose was based on the known safety and efficacy of 300 mg aspirin EC/day while the decision to treat the patients for seven days before cholecystectomy was made to improve compliance and facilitate administration of the trial.

ETHICAL CONSIDERATIONS

Informed consent was obtained from each patient and ethical approval for the study was granted by both the Newcastle and Northumberland Ethical Committees.

LABORATORY METHODS

MUCUS GLYCOPROTEIN BIOSYNTHESIS IN MUCOSAL EXPLANTS

Mucosal explants of freshly excised gall bladder were grown for 24 hours in tissue culture.²⁷ Glucosamine hydrochloride, D-[6-³H(N)]-, with a specific activity of 1110·0 GBq/mmol, was used to label macromolecules. ³H-glucosamine was used at a concentration of 74 KBq/ml in the tissue culture medium. Total ³H-glucosamine incorporation into mucin was measured after removal of protein and low molecular weight radioactive material by papain digestion (72 hours) and exhaustive dialysis against distilled water (144 hours). Papain digestion is an effective method for isolation of mucus glycoprotein. After digestion, dialysis removes protein and non-glycosylated regions of protein core, leaving behind non-dialysable, mucus glycoprotein.²⁸

Glucosamine incorporation into the glycoprotein component of mucin was confirmed by fractionation on a caesium chloride density gradient²⁹ and by gel filtration on sepharose 4B. All radioactive mucin fractionated in a peak coincident with purified, papain digested human gall bladder mucin. Gall bladder mucosa, killed in liquid nitrogen was used as a control to check for non-specific interactions between the tissue and ³H-glucosamine.

MUCUS GLYCOPROTEIN CONTENT OF GALL BLADDER BILE

Quantitation of mucus glycoprotein was undertaken using a modified 'Slotblot' technique.³⁰ After blotting samples of bile onto nitrocellulose membranes, mucus glycoprotein was visualised using the periodic acid/Schiff reaction.³¹ Colour yield was then measured using a scanning densitometer (Shimadzu, Japan). Results were compared with those obtained by measuring glycoprotein after purification of the mucin by the traditional method of fractionation on a caesium chloride density gradient and showed close correlation.

CHOLESTEROL

The cholesterol concentration in bile was measured using the enzymatic method first described by Roda³² and modified by Bolton.³³

Briefly, this involved diluting samples of gall bladder bile obtained by needle puncture at laparotomy 1 in 10 with isopropanol and incubating the diluted sample with three enzymes. Sequential treatment of bile with catalase, cholesterol esterase and cholesterol oxidase (Sigma, UK) produces cholestenone and hydrogen peroxidase. The hydrogen peroxide is then used as an oxidising agent in a colorimetric assay.

The cholesterol content of gall stones was measured by crushing the dried stones with a pestle and mortar, extracting the cholesterol in isopropanol and quantifying it as described above.

PHOSPHOLIPID

Phospholipid was assayed using an enzymatic method first described by Takayama³⁴ and modified by Qureshi *et al.*³⁵ Dual enzymatic digestion of bile with phospholipase and choline oxidase (Wako Chemical Company, Eastleigh, UK) leads to the release of hydrogen peroxide which is used as the oxidant in a colorimetric assay.

BILE ACIDS

The total bile acid content of gall bladder bile was measured using an enzymatic method in which 3- α -hydroxy-steroid dehydrogenase (Sigma UK) is used to oxidise bile acids allowing reduction of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide hydride (personal communication).

CHOLESTEROL SATURATION

The cholesterol saturation was determined by calculating the actual percentage of cholesterol in bile and dividing it by the theoretical maximal cholesterol solubility.³⁶

CALCIUM

The total calcium of gall bladder bile was measured using atomic absorption spectrophotometry.³⁷

CHOLESTEROL NUCLEATION TIME

Nucleation time was measured in the so called isotropic 'micellar' phase obtained by centrifugation of bile for one hour at 200 000 g. After centrifugation samples were examined for the presence of cholesterol crystals then incubated at

TABLE I Ten patients in the aspirin treated group were withdrawn from the study as compared with eight in the control group. Reasons for withdrawal are indicated together with the numbers of patients in each category

Patients withdrawn from trial		Group - 2 Control	
Group - 1 Aspirin			
7 Women -	3 mucocoele 2 fibrosis 1 adenocarcinoma 1 aspirin withdrawn	3 Women -	2 fibrosis 1 oedema 2 mucocoele
3 Men -	3 aspirin withdrawn	5 Men -	1 no bile 1 oedema 1 infected

TABLE II Age, sex, symptom duration and histology in the two groups. The severity of cholecystitis was graded independently by a pathologist

Patient age, sex, symptoms and histology		
Group – 1 Aspirin		Group – 2 Control
n	27	28
Age (yr)	54.4 (range 21–84)	52.0 (range 23–74)
F: M	21: 6	26: 2
Symptoms (yr)	2.7 (SD 2.7)	2.9 (SD 2.5)
Histology	normal – 3 mild chronic cholecystitis – 14 moderate chronic cholecystitis – 7	normal – 2 mild chronic cholecystitis – 13 moderate chronic cholecystitis – 5

37°C under nitrogen. Further aliquots of this isotropic phase were removed daily and examined with polarizing light microscopy (Leitz, Wetzlar) to look for cholesterol crystals.³⁸

STATISTICAL ANALYSIS

Data were compared visually using histograms and statistically using the Francia Shapiro W’ test to test for normality. Where data were found to be normally distributed, the unpaired *t* test was used to compare the two patient groups. For those data which were not normally distributed, log transformations was utilised to obtain a normal distribution and the unpaired *t* test was then used to compare the two groups.

Results

Of 73 patients randomised, 18 were withdrawn from the study (Table I). Patients who had fibrotic oedematous or acutely infected gall bladders were withdrawn from the study because it was not possible to conduct explant culture. Of the 55 patients who were suitable for further study, 27 were randomised to the aspirin treated group and 28 to the control group. The male to female ratio, mean age and mean duration of symptoms were similar in the two groups (Table II). Gall bladder histology was also comparable in both groups.

MUCUS GYLCOPROTEIN SYNTHESIS BY GALL BLADDER MUCOSA

Gall bladder mucus biosynthesis, as measured by ³H-glucosamine incorporation, was significantly less in aspirin treated patients than in the untreated controls. Gall bladder explants from controls incorporated a mean of 2008 fmol/g wet weight (SD 892) of ³H-glucosamine as compared with 1347 fmol/g wet weight (SD 722) in aspirin treated patients (*p*<0.005, 95% confidence interval for the difference 222 to 1100, unpaired *t* test), (Figure). Incorporation of ³H-glucosamine

into mucosal explants linear over 24 hours. Controls containing liquid nitrogen killed tissue incorporated a maximum of 3 fmol/g wet weight of ³H-glucosamine.

GALL BLADDER BILE COMPOSITION

Both gall bladder bile mucus concentration and cholesterol nucleation time were similar in the two groups (Table III). Cholesterol saturation, calcium concentration and stone composition were also similar in the two groups (Table III).

SIDE EFFECTS AND COMPLICATIONS

There was no postoperative morbidity in the control group. Two patients in the aspirin treated group had complications. One had evidence of a prolonged clotting time postoperatively and her haemoglobin fell from 12.5 g/dl to 10 g/dl on the first postoperative day. No transfusion was needed and the patient showed no clinical signs of blood loss. The second had a small melaena after two days of aspirin treatment and he was therefore withdrawn from the study.

Discussion

This study shows that low dose aspirin significantly inhibits mucus synthesis in the human gall bladder *in vivo*. Our earlier work¹ has shown that aspirin will inhibit human gall bladder mucus glycoprotein biosynthesis *in vitro* and this present study shows that this inhibition occurs *in vivo*. This is not, however, accompanied by significant changes in soluble mucin content in gall bladder bile or gall bladder bile composition. This may be as a result of short term treatment (seven days), or because of the low dose of aspirin chosen for this study.

What is clear is that the mucus biosynthetic machinery of the gall bladder mucosa has been reduced by short term aspirin treatment. Over a longer period of time this would be expected to establish a decreased level of mucus production by the gall bladder and thus diminish its potential as a nucleating agent. Mucus has been shown to be a nucleating agent *in vitro*^{39, 40} and *in vivo* increased mucus glycoprotein synthesis and secretion antedates gall stone formation in the rabbit⁴¹ and the prairie dog.¹ Aspirin inhibits mucus synthesis and secretion and prevents gall stones in the prairie dog.² Our results here show that in man aspirin has similar actions on gall bladder mucus glycoprotein biosynthesis.

Stone recurrence is a major problem with all forms of gall stone treatment which leave the gall bladder *in situ*. Ruppin *et al*¹⁶ followed 54 patients successfully treated with oral bile acids for a mean of 23 months and found that 25 (46%) suffered recurrent stones. O'Donnell *et al*¹⁷ found a similar recurrence rate in their study of 40 patients, 50% of whom had a recurrence within five years. In a longer term study, Villanova *et al*¹⁴ found a 61% gall stone recurrence rate at 11 years in 86 patients. Gall stone recurrence has also been studied in patients after percutaneous cholecystolithotomy and shock wave lithotripsy although the length of follow up is much shorter than in post dissolution studies. Eight months

TABLE III Gall bladder bile composition and gall stone composition in the two groups

Gall bladder bile and gall stone composition	Aspirin n=27	Control n=28	t test
Mucus glycoprotein (mg/ml)	7.6 (SD 7.7)	7.1 (SD 5.4)	ns
Nucleation time (days)	6 (SD 2.4)	5.2 (SD 1.9)	ns
Cholesterol (mmol/l)	15.3 (SD 7.9)	13.5 (SD 8)	ns
Phospholipids (mmol/l)	36.1 (SD 19.1)	33.2 (SD 17.1)	ns
Bile acids (mmol/l)	103 (SD 50.1)	102 (SD 49.2)	ns
Saturation (% cholesterol)	145 (SD 39)	160 (SD 82)	ns
Total calcium (mmol)	6.9 (SD 3.3)	5.4 (SD 2.9)	ns
Stone composition (% cholesterol)	66 (SD 34)	75 (30)	ns

Figure: Mucus glycoprotein biosynthesis as measured by ^3H -glucosamine incorporation over 24 hours and treatment with papain for 72 hours followed by exhaustive dialysis (144 hours). Each patient is represented by a dot and the mean of each group by a horizontal line. Mean incorporation in aspirin treated patients with 1347 fmol/g wet weight (SD 722) as compared with 2008 (SD 892) in controls; $p < 0.005$, unpaired t test, 95% confidence interval for the difference 222–1100.

follow up in 53 patients who underwent successful percutaneous removal of their gall stones revealed recurrent stones in five¹⁸ whilst the same duration of follow up in 49 patients who underwent shock wave lithotripsy also showed five to have recurrent stones.¹⁹

It has been suggested that recurrent stones are amenable to repeat dissolution therapy⁴² but experience with this approach is limited. One possible way of preventing gall stone recurrence is the use of long term, low dose aspirin. There is extensive evidence that aspirin prevents gall stone formation in the prairie dog through its inhibition of mucus synthesis and secretion.² Broomfield *et al*²⁶ studied the effects of aspirin (1200 mg/day) and ursodeoxycholic acid (1300 mg/day) on duodenal bile composition and on microcrystal, microstone and gall stone formation in 53 patients dieting to lose weight. Patients taking aspirin or ursodeoxycholic acid had significantly lower bile glycoprotein concentrations when compared with the placebo group and both had a reduced incidence of gall stone, microstone and cholesterol crystal formation. Hood *et al*³ found that gall stone recurrence after dissolution was significantly less in patients taking long term non-steroidal anti inflammatory agents, a result consistent with the work of Broomfield.

Our study has shown inhibition of gall bladder mucin biosynthesis using aspirin at a dose of 300 mg/day. At this dose we have failed to show significant prolongation of cholesterol nucleation time. Longterm treatment with aspirin at a dose of 300 mg/day has been shown to be safe in asymptomatic subjects who took part in The Physicians Health Study⁴³ and the UK TIA study.⁴⁴ If future studies show prolongation of cholesterol nucleation time as a consequence of longterm treatment with aspirin at a dose between 300 mg/day and 1200 mg/day, there might be a

role for the drug in the prevention of gall stones among high risk groups.

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