Mucus glycoprotein biosynthesis in the human gall bladder: inhibition by aspirin

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Abstract

Aspirin, which inhibits mucin secretion in the gastrointestinal tract prevents gall stone formation in animals and may reduce gall stone recurrence in man. This study examines the effect of aspirin on mucin synthesis in human gall bladder explants. Two hundred explants were cultured with ^3H -glucosamine (74 kBq/ml) for 24 hours at 37°C. Mucin and other glycoproteins were isolated by papain digestion (72 hours) and exhaustive dialysis (144 hours) to remove non-incorporated radioactivity and digested protein. ^3H -glucosamine was readily incorporated into glycoprotein. Pooled gall bladder explants were fractionated on a CsCl density gradient and by gel filtration on Sepharose 2B and 4B to confirm that >90% radioactivity was incorporated into mucin. Acetylsalicylic acid (230–666 µg/ml) significantly reduced total ^3H -glucosamine incorporation (43–89%), $p < 0.01$ (unpaired t test). Diclofenac (125–1250 µg/ml), similarly reduced incorporation by 45–97% $p < 0.001$ (unpaired t test). Inhibition of mucin glycoprotein biosynthesis was irreversible with both drugs. Analysis of pooled samples on Sepharose 4B showed abolition of radioactive incorporation into mucin but no effect on incorporation into low molecular weight glycoprotein material (10% of total incorporation). This study provides a method for measuring human gall bladder mucin synthesis and shows its irreversible inhibition by acetylsalicylic acid and diclofenac at concentrations compatible with a therapeutic dose.

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Mucin has been identified in cholesterol and pigment gall stones using histochemical stains.^{1,2} In vitro studies have shown that mucus glycoprotein interacts with bilirubin³ and there is mounting evidence that it is involved in the nucleation of cholesterol.^{4–6} Early work in man⁷ revealed a higher concentration of hexosamine (a major constituent of mucin) in bile from gall stone patients as compared with controls. Both the cholesterol fed rabbit⁸ and cholesterol fed prairie dog⁹ secrete an increased amount of mucin before gall stone formation. This is accompanied by increased mucin biosynthesis in the prairie dog.

In the prairie dog mucin biosynthesis is stimulated by arachidonic acid and inhibited by aspirin.^{10,11} In man there is some early information to suggest that aspirin may reduce the risk of gall stone formation during weight loss¹² and decrease the risk of stone recurrence after successful dissolution therapy.¹³ The action of aspirin may be partially mediated through

inhibition of eicosanoid metabolism, as in the prairie dog or through the drugs anti-inflammatory properties.¹⁴ There is, however, little information available about the action of aspirin on the human gall bladder. We have therefore studied mucin biosynthesis in human gall bladder explants in 24 hour organ culture.

Methods

MATERIALS

D-[6- ^3H]-glucosamine hydrochloride (specific activity 1110 GBq/mmol) was obtained from New England Nuclear (Boston, USA) and Amersham UK Ltd, Weymouth MB 752/1 was supplied by Flow Labs, Rickmansworth, UK. Acetylsalicylic acid, prostaglandin F2 α , prostaglandin E2, ascorbic acid, and hydrocortisone were from Sigma (UK) and diclofenac from Ciba Geigy (UK). Sepharose 2B and 4B were from Pharmacia, Milton Keynes, UK.

ORGAN CULTURE

Gall bladders were collected fresh from patients undergoing routine open cholecystectomy. Upon removal gall bladders were opened, rinsed and immersed in ice cold isotonic saline 4°C. The fundus and neck of the gall bladder were taken for histology. The mucosa from the body of the gall bladder was then peeled from the underlying muscle and peritoneum and 20 to 50 small explants of mucosa weighing 1–5 mg were prepared using two scalpels to dissect a 80–100 mg piece of tissue. This was placed on a 3.2 cm, 8 µm pore size, ashless filter (Whatman, Maidstone, UK). A single filter was placed in a 5 cm Petri dish (Sterilin, Hounslow, UK) with 1 ml organ culture medium. The maximum time from collecting a gall bladder to placing in the organ culture incubator was one hour.

Organ culture medium consisted of Waymouth MB 752/1 medium to which was added 10% fetal calf serum (1 ml/10 ml), ascorbic acid (300 µg/ml), ferrous sulphate (0.45 µg/ml), hydrocortisone (3 µg/ml) and a mixture of penicillin, streptomycin, and mycostatin (100 IU/ml). To this solution was added ^3H -glucosamine at a concentration of 74 KBq/ml.¹⁵

Culture was carried out for 24 hours in 5% CO₂, 95% O₂ (humidified) at 37°C (Heraeus, Hanau, W Germany). Gall bladder viability was assessed at 0, 6, 12, and 24 hours using histological staining with haematoxylin and eosin and periodic acid/Schiff.¹⁶ Histological sections were examined for evidence of cell necrosis and depletion of intracellular mucin stores.

At the end of the incubation explants were rinsed and then homogenised in 0.1 M KH₂PO₄/

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Na₂HPO₄, containing 0.005 M EDTA and 0.005 M L-cysteine HCl, using a rotating metal blade (Ultra Turrax, IKA Werk, W Germany). Specimens were then proteolytically digested for 72 hours at 60°C using papain (Sigma UK) at 0.185 mg/ml. After digestion samples were dialysed against distilled water over six days (three changes per day) to remove non-incorporated radioactivity and digested protein leaving the non-dialysable fragmented mucin.¹⁷

Acetylsalicylic acid was dissolved in 0.9% NaCl to make a stock solution of 2 mg/ml (pH 7.5). Stock was added to tissue culture plates to give concentrations of 10 to 666 µg/ml. Diclofenac sodium (15 mg/ml) was added to organ culture plates to give a final concentration of between 125 µg/ml and 1250 µg/ml. Prostaglandin F₂α and prostaglandin E₂ were added to organ culture plates at concentrations from 1 to 50 µg/ml.

CsCl DENSITY GRADIENT FRACTIONATION AND SEPHAROSE 2B AND 4B CHROMATOGRAPHY

The size and distribution of radiolabelled macromolecules in pooled gall bladder mucosal explants was characterised by caesium chloride density centrifugation and gel filtration on 150 × 1.5 cm sepharose 4B and 2B columns. Each assay was repeated at least three times to establish reproducibility.

Samples studied on sepharose 2B were first fractionated on a CsCl gradient.¹⁸ The starting density was 1.425 (±0.005) and the solution was centrifuged at 50 000 rpm (mean of 201 100 g) for 18 hours using a vertical rotor (Sorvall TV850, Dupont, Wilmington, USA). The gradient was then fractionated into nine 4 ml fractions and each was analysed for protein, glycoprotein and radioactivity as well as density.

All samples for chromatography were eluted upwards using 0.2 M NaCl/0.02% NaN₃, 5 ml fractions were collected and assayed for glycoprotein,¹⁹ protein, and radioactivity.

STATISTICAL ANALYSIS

Results from each organ culture plate were expressed as fmol ³H-glucosamine incorporated per gram wet weight. Wet weight was shown to correlate in a linear fashion with total tissue protein and total tissue DNA. Data were compared visually using histograms and statistically using the Francia Shapiro W' test to test for normality. Data were found to be normally

distributed and the unpaired *t* test was used to compare the different groups.

Results

Incorporation of ³H-glucosamine by papain digested tissue explants was 3199 (SD 654) fmol/g wet wt over 24 hours and linear with time (Fig 1). Incorporation was linear in both control and aspirin treated cultures dishes. ³H-glucosamine incorporation was reduced to 855, 340, and 75 fmol/g wet wt by sodium azide (an inhibitor of aerobic metabolism) at concentrations of 20, 100, and 500 µg/ml respectively. Histological examination of cultured explants revealed no evidence of cell death or depletion of intracellular mucin.

Evidence that over 90% of the radioactivity in papain digested explants was incorporated into gall bladder mucin was obtained as follows. Fractionation of homogenised but undigested explants by CsCl yielded a single radioactive peak banding at a density between 1.48 and 1.55, coincident with standard undigested human biliary mucin. Fractionation of papain digested explants by CsCl yielded a single radioactive peak banding at a density between 1.49 and 1.55, coincident with standard papain digested human biliary mucin. Gel filtration on sepharose 2B of the radioactive peaks after CsCl fractionation gave a single excluded peak for undigested explants and a single included peak for papain digested explants. These peaks were coincident with those for undigested and papain digested human biliary mucin respectively (Fig 2).

The addition of acetyl salicylic acid to the culture medium caused dose dependant inhibition of ³H-glucosamine incorporation (Fig 3). At a concentration of 230 µg/ml significant inhibition of mucus biosynthesis was achieved (*p* < 0.04, 95% CI 95, 620). A second non-steroidal anti-inflammatory agent, diclofenac also significantly inhibited mucin biosynthesis. ³H-glucosamine incorporation was reduced from 1811 (SD 542) fmol/g wet wt in controls to 814 (SD 333) fmol/g wet wt in cultures to which 250 µg/ml of diclofenac had been added (*p* = 0.05, 95% CI -24, 2017). This inhibition of mucin biosynthesis as measured by ³H-glucosamine incorporation was irreversible after three hours incubation in acetyl salicylic acid at a dose of 300 µg/ml.

Analysis of pooled explant cultures on Sepharose 4B confirmed again that >90% of radioactivity was incorporated into mucus glycoprotein. The remainder of radioactivity was found in a small molecular weight PAS positive material (Fig 4) in the included volume of the column. Further analysis of pooled explants cultured in acetyl salicylic acid (Fig 4) at a dose of 300 µg/ml showed inhibition of incorporation into the mucin peak while incorporation of ³H-glucosamine into the minor low molecular weight glycoprotein peak was unaffected. Control experiments in which sodium azide was added to culture plates showed inhibition of incorporation into the mucin peak and the low molecular weight glycoprotein peak.

Addition of prostaglandin F₂α to the explant cultures at a dose of 10 µg/ml increased ³H-

Figure 1: Incorporation of ³H-glucosamine into human gall bladder mucosal explants. Explants were cultured for 24 hours, homogenised, digested with papain for 72 hours and dialysed for 144 hours to remove non-incorporated radioactivity and low-molecular weight glycoprotein.

Figure 2: Glycoprotein and ^3H -glucosamine content of undigested and papain digested mucosal explants. Mucosal explants were homogenised after 24 hours in tissue culture and either immediately fractionated on caesium chloride or fractionated after papain digestion. Mucus glycoprotein fractions were then separated on Sepharose 2B. Undigested material was excluded on Sepharose 2B. After papain digestion glycoprotein from mucosal explants was included in the column.

glucosamine incorporation from 1453 (SD 197) fmol/g wet wt to 2314 (SD 696) fmol/g wet wt ($p < 0.1$, 95% CI 290, 2013) while at a dose of 50 $\mu\text{g/ml}$ biosynthesis as measured by ^3H -glucosamine incorporation, was reduced to 707 (SD 48) ($p = 0.007$, 95% CI 424, 1070). The addition of prostaglandin E2 at doses between 1 and 25 $\mu\text{g/ml}$ had no influence on mucin biosynthesis.

Discussion

This series of experiments establishes a method for measuring ^3H -glucosamine incorporation into mucin by human gall bladder mucosal explants. A previously documented organ

explant culture method was used.^{15,16} Cell viability was confirmed histologically and by the linear incorporation of ^3H -glucosamine into mucin over the full time course of the experiment. Mucin biosynthesis is a very energy dependant process²⁰ and therefore itself provides a sensitive biochemical assay of cell viability under the conditions used. This is documented in this system by the total inhibition of ^3H -glucosamine incorporation into mucin in the presence of azide.

Radioactive incorporation into mucin was measured after first removing protein by papain digestion and dialysis.¹⁵ Protein digestion also removes part of the mucin protein core to leave the large molecular sized non-dialysable, degraded, glycosylated, mucin fragments containing the radioactively labelled carbohydrate.

This study also shows, for the first time, the selective inhibition of mucin biosynthesis by acetyl salicylic acid in human gall bladder mucosal explants. This finding is consistent with previous work, which showed that aspirin inhibits glycoprotein biosynthesis in the human gastric mucosa²¹ and the prairie dog gall bladder mucosa.¹⁰ Possible mechanisms for inhibition of mucin biosynthesis by aspirin include a reduction in prostaglandin levels or depletion of cellular energy.

The *in vitro* action of aspirin at a dose of 230 $\mu\text{g/ml}$ is compatible with a role for the drug *in vivo*. The inhibition of mucus glycoprotein biosynthesis by acetyl salicylic acid may be one reason for its action in the prevention of gall stones.^{12,13} Other anti-inflammatory properties of the drug may also prove important in gall stone prevention.¹⁴ Mucin is found at the centre of cholesterol gall stones^{1,2,6} and accelerates nucleation in model bile.^{22,23} Mucin also accelerates cholesterol nucleation in the gall bladder bile of the prairie dog⁹ and has been shown to increase nucleation in human gall bladder bile.²⁴ Furthermore, both synthesis and secretion of mucin are increased before stone formation in the cholesterol fed prairie dog model.⁹

In spite of this large amount of data linking mucin with gall stone formation it has been difficult to differentiate between gall stone patients and normal controls on the basis of total gall bladder bile mucin concentrations.²⁵ This may be because studies have not been able to examine patients during the critical phase of gall stone initiation and any transient differences in gall bladder mucin synthesis (as showed in the prairie dog model) will have long passed in patients with mature gall stones. Several studies^{14,26,27} have identified glycoproteins of between 60 000 Daltons and 130 000 Daltons which appear to act as potent nucleating agents in human gall bladder bile and appear in greater concentrations in the bile of gall stone patients but their role in the pathogenesis of gall stones is far from clear. Mucin, however, has been more closely linked with gall stone formation in that increased synthesis and secretion occur before gall stone formation in the animal model. Mucin itself causes nucleation of cholesterol in both model and native gall bladder bile and is found at the centre of human cholesterol gall stones.^{3,5,6}

This study has shown selective inhibition of

Figure 3: Inhibition of mucus synthesis by differing doses of acetyl salicylic acid. Each data point is the mean of six separate experiments with standard errors shown in each case. * $p = 0.02$, 95% CI 95, 620. ** $p < 0.0001$, 95% CI 410, 649. *** $p < 0.0001$, 95% CI 646, 851.

Figure 4: Glycoprotein and ^3H -glucosamine content in human gall bladder mucosal explants. Explants were homogenised after 24 hours in tissue culture, then digested in papain for 72 hours and dialysed for 144 hours. Pooled samples were then run on Sepharose 4B. ^3H -glucosamine was incorporated in parallel with glycoprotein, >90% in mucus glycoprotein and <10% in low molecular weight glycoprotein. ^3H -glucosamine incorporation into explants cultured without acetyl salicylic acid are shown by the solid line, those cultured with acetyl salicylic acid at a dose of 300 $\mu\text{g/ml}$ by the broken line. Acetyl salicylic acid selectively inhibits incorporation of ^3H -glucosamine into the mucin glycoprotein peak.

mucin biosynthesis in explanted human gall bladder mucosa. The concentrations of aspirin needed to inhibit mucin synthesis may well be compatible with an *in vivo* role for the drug. These observations provide one explanation for the observations that aspirin may prevent new gall stone formation and gall stone recurrence after successful oral dissolution therapy. Further *in vivo* studies in man are warranted.

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