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Heterogeneity in the protein cores of mucins isolated from human middle ear effusions: evidence for expression of different mucin gene products

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High molecular weight mucins were isolated and purified from human middle ear effusions of children with Otitis Media with Effusion (OME) classified into three groups, (1) thick and (2) thin from anatomically normal children and (3) effusions from cleft palate patients. Amino acid analyses of the purified mucins from the three pools were similar but not identical with characteristic contents of serine threonine and proline (32%, 28%, and 38% for pools (1) (2) and (3) respectively). Proteinase resistant glycopeptide fragments corresponding to the tandem repeat domains of cloned mucin genes showed marked differences both between the three mucin pools and with the composition of the tandem repeat sequences of the cloned mucin genes expressed in the airways. Studies on the antigenic identity of middle ear mucins found an epitope likely to be present on MUC5AC, but only accounting for a maximum of 15% by weight and no reactivity was found with antibodies to MUC2 or MUC1. A polyclonal antibody raised to thick effusion mucins reacted strongly with human salivary mucin suggesting the presence of MUC5B epitopes. These studies suggest that more than one mucin gene product is secreted by the human middle ear mucosa and that there may be further mucin genes expressed by the middle ear that have yet to be cloned.

***Keywords*: mucin, human middle ear, *MUC* genes, *MUC5AC*, *MUC5B*, *MUC2*, *MUC1*, effusion, otitis media**

Introduction

Otitis media with effusion (OME) is the commonest cause of childhood deafness in the developed world [1]. The disease is characterized by an increase in goblet cells and mucus glands and accumulation of viscous effusions in the middle ear cleft that cannot be cleared by the normal mucociliary transport mechanisms. Mucins are a major component of middle ear effusions and are responsible for the rheological properties of the effusions [2]. Effusions vary in viscosity from thin (serous) to mucoid (thick). Purified mucins from thick effusions have been extensively characterized and shown to be high molecular weight glycoprotein polymers maintained by disulphide bridges [3]. The disulphide linked subunits are broken down into smaller units by proteolysis. Preliminary studies on mucins purified from thin effusions and mucins from effusions from cleft palate patients have suggested that they may have a different chemical composition from each other and from thick effusion mucins [4, 5].

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Nine human mucin genes (*MUC*s *1, 2, 3, 4, 5AC, 5B, 6, 7* and *8*) have now been at least partially characterized [6, 7]. A cluster of mucin genes *MUC6, MUC2, MUC5AC* and *MUC5B* is found on chromosome 11p15.5 and it has been suggested that these genes are part of a multigene family whose members code for secreted mucins [8]. The complete amino acid sequences of *MUC1, 2* and *7* have been reported [9–11] and the sequence of the large central exon of *MUC5B* has recently been published [12]. Domains containing tandem repeats (VNTR) rich in potential O-glycosylation sites are characteristic of all of these sequences and probably correspond to the highly glycosylated (proteinase resistant) regions found in mucins. The composition and sequence of amino acids in the tandem repeats differ markedly between all the secreted mucins. Repeats vary in length between 8 and 169 amino acids, *MUC5AC* and *MUC6* respectively [6].

In situ hybridization studies have shown more than one gene to be expressed by the epithelial cells in the same tissues [13–15] making it likely that mucus secretions are a mixture of mucin gene products. There have, however, been relatively few attempts to relate the sequences of mucin genes to the analysis of mucins isolated from mucus secretions [16–18].

Polyclonal antibodies raised to synthetic peptides made from sequences of *MUC5AC*, in the tandem repeat region and in 'unique' regions flanking tandem repeat regions and from sequences present on the C-terminal side of the large tandem repeat region of *MUC2*, have been used to discriminate between populations of mucins in the airways [19–21]. *MUC5AC* was found to be a prominent mucin in respiratory secretions, however, *MUC2* could not be detected in the high density mucin rich fractions although some reactivity was present in low density fractions [21]. Recently the tracheobronchial mucin *MUC5B* has been identified as a major fraction of high molecular weight salivary mucin *MG1* [22].

There is as yet no information as to which mucins are expressed by the human middle ear, however, middle ear mucosa is a modified respiratory epithelium and eight of the nine mucin genes have been shown to be expressed in the airways [6, 7, 11, 14]. One study reported immunohistochemical staining of goblet cells and submucosal glands of rat middle ear mucosa by polyclonal antibody to human *MUC6*, however, in northern analysis mRNA from rat middle ear mucosa did not hybridize with specific human MUC6 cDNA probes [23].

As a first step in determining which mucin gene products are secreted by human middle ear mucosa we have isolated in as undegraded state as possible mucins present in the middle ear effusions of three clinical groups (1) thick (mucoid) and (2) thin (serous) effusions from anatomically normal children and (3) effusions from cleft palate patients with OME. The protein composition of these mucins has been analysed before and after exhaustive proteolytic digestion and compared with the composition of the tandem repeat sequences of cloned genes already published. We have also used antibodies to mucins to try and establish the antigenic identity of the middle ear mucins. The results presented here suggest that more than one mucin gene product is indeed secreted by human middle ear mucosa and that at least one of the genes expressed may be different to those so far characterized.

Methods

Mucin preparation

Middle ear effusions were collected from children undergoing myringotomy for OME and stored at $-20\,^{\circ}$C until used. Effusions were pooled and solubilized by homogenization for 30 s in 67 mM sodium phosphate buffer, pH 6.7, containing an extensive mixture of proteinase inhibitors [2]. Insoluble debris was removed by centrifugation, $8000 \times \mathbf{g}$, 1 h, 4 °C and soluble mucin was separated from other effusion components by equilibrium density gradient centrifugation in 60% (w/v) CsCl, 40 000 rpm, 24 h, 4 °C Sorvall vertical rotor. Glycoprotein rich fractions identified using a PAS assay [24] were pooled and purified further in a second CsCl gradient. Mucins were also prepared as above from pig gastric mucus gel scrapings and from human saliva for use as controls in the antisera experiments. Unstimulated human saliva was collected into proteinase inhibitors at 4 °C. Human colonic mucus gel scrappings were stirred for 24 h at 4 °C in 6 M GuHCl containing proteinase inhibitors, insoluble material was recovered by centrifugation ($8000 \times \mathbf{g}$, 1 h, 4 °C) and re-extracted twice with the same solvent. The residual insoluble material was solubilized by reduction with 10 mM dithiothreitol and mucin purified by equilibrium density gradient centrifugation in CsCl [25]. This mucin was also used as control in the antisera experiments. An extract of human ileum was prepared by homogenizing $\sim$2 g of tissue in guanidinum thiocyanate-phenol-chloroform buffer [26]. Protein was precipitated from the phenol phase by the addition of H_2O, resolubilized in 6 M GuHCl and blotted onto nitrocellulose membranes for use as a positive control in the experiments with NCL-MUC1 and NCL-MUC2 monoclonal antibodies (see below).

Mucin fragmentation

Purified mucins were proteolytically digested with papain (E.C. 3.4.22.1; 1:100 [w/w] papain/mucin), 48 h, 60 °C sodium phosphate buffer, pH 6.25, containing, 5 mM cysteine/HCl, 5 mM EDTA, Mucin glycopeptides were purified from the products of digestion and from papain by a further equilibrium density gradient centrifugation step in 60% (w/v) CsCl, 40 000 rpm, 24 h, 4 °C, Sorvall vertical rotor. The purity of the mucin glycopeptides was assessed by SDS PAGE using the Pharmacia 'Phast' system. Mucin samples (10 μg) were applied to 4–15% gradient gels and the gels stained with PAS, Coomassie blue and silver [27].

Deglycosylation

Purified mucins were deglycosylated with trifluoromethane sulphonic acid (TFMS) as described in Aubert *et al*., 1991 [28]. Alternatively purified mucins were immobilized on polyvinylidene difluoride (PVDF) membranes. Sialic acid was removed with neuraminidase and other *O*-glycosidically linked sugars with TFMS. The time of exposure to TFMS was reduced to 5 min, from 15 min in Thornton *et al.* [19] as in our hands this was adequate for exposure of VNTR epitopes and significant losses of mucin from the membrane occurred with longer exposure times.

Antisera and ELISA

The following antisera were used in this study. Monoclonal antibodies NCL-HGM-45M1, NCL-MUC1 and MUC2 were purchased from Novacastra Laboratories Ltd. Purified mucin from thick effusions of anatomically normal children was used to prepare a polyclonal antisera (TEPA1) in rabbits. NCL-HGM-45M1 recognizes a non-VNTR epitope in the protein core of human gastric mucin and NCL-MUC1 and NCL-MUC2 were raised from synthetic

peptides produced from the tandem repeat sequence of MUCs 1 and 2. The polyclonal antisera raised to mucin purified from thick effusions (TEPA1) probably recognizes a protein epitope in the non-VNTR region, the reasons are as follows. Papain digestion of thick effusion mucin, which removes the non/sparsely glycosylated regions abolishes antisera reactivity. These papain digestion studies demonstrate that the TEPA1 antisera is not recognizing the major carbohydrate containing domain, or any carbohydrate epitopes on that domain, which in our methodology is still firmly bound to the slot blot membrane via wheat germ agglutinin [24]. A synthetic peptide with the sequence NGLQPVRVEDPDGC present in the 'unique' region present on the C-terminal side of the large tandem repeat region of the *MUC2* gene was conjugated with BSA and used to prepare rabbit polyclonal antisera (LUM 2-3) in the University of Newcastle Upon Tyne Molecular Biology Unit.

Samples were blotted onto nitrocellulose sheets (0.45 μm) and incubated overnight at 4 °C in PBS containing 2% (w/v) BSA. NCL-HGM-45M1, NCL-MUC1 and NCL-MUC2 were used at dilutions of 1 : 50 and 1 : 100 respectively, the polyclonal antisera TEPA1 and LUM 2-3 were used without any preabsorption steps at a dilution of 1 : 200 and 1 : 1000 respectively. Antibody binding was measured with anti-mouse and anti-rabbit IgG secondary antibodies conjugated to peroxidase with H_2O_2 as substrate and 3′3′ diaminobenzidine for colour development. Staining of the slots was quantitated using a Shimadzu CS 930 dual wavelength scanning densitometer at 595 nm.

Analytical methods

Glycoprotein content was estimated after blotting the samples onto nitrocellulose membranes pretreated with wheat germ agglutinin [24].

Amino acid analyses were performed by the method of Carlton and Morgan [29]. Samples were hydrolysed for 24 h at 110 °C and derivitized with 9-fluoroenylmethylchloroformate prior to analysis by reversed phase HPLC.

Thiol content was measured by the method of Mantle *et al.* [30]. Purified mucin was reduced in 8 M urea, 20 mM Na_2EDTA and 0.33 M $NaBH_4$ pH 8.0 at 37 °C (90 min). Thiol groups were labelled with 4,4 dithiopyridine (0.15 M final concentration, 30 min, 20 °C) and detected at OD 324 nm. The total number of thiols was calculated using the molar absorption coefficient. Free thiols were determined by the above procedure but omitting the reduction step.

Results

Purification and physiochemical characteristics

The major mucin population from each of the three pools had similar buoyant densities in 3.5 M CsCl between 1.41 and 1.48 g ml^{-1}. After two CsCl gradients mucin from each of the pools was shown to be free of contaminating protein by SDS PAGE on 4–15% gradient gels using high loadings (10 μg) of mucin and staining with coomassie blue. Purified mucins from each of the three pools were predominantly excluded from Sepharose CL-2B (>70%) (results not shown). The thiol content of the mucins was measured by the method of Mantle *et al.* [30] and the results are summarized in Table 1. The majority of thiols ($\sim$20 nmol mg^{-1}) in mucin from 'thick' effusions were involved in disulphide bridges as were those in mucin from 'cleft palate' effusions ($\sim$50 nmol mg^{-1}) Mucin from 'thin' effusions contained less total thiols and less were involved in disulphide bridges ($\sim$5 nmol mg^{-1}). Undegraded mucins from all three pools remained at the point of application when electrophoresed on 4–15% SDS gradient gels and stained with PAS. Reduced mucins from each of the three pools migrated through the stacking gel to the interface of the gradient gel indicating that reduction of disulphide bridges causes a size change (results not shown).

Antigenic identity

Purified mucin from each of the three pools was probed with the monoclonal antibodies and polyclonal antiserum. NCL-HGM-45M1 (anti-human gastric mucin) recognized epitopes in all three of the middle ear mucin pools. The mucins reacted in a concentration dependent manner (Figure 1a), however, the 'thick' effusion mucin was approximately three times more reactive than 'thin' effusion mucin and 1.5X as reactive as mucin from 'cleft palate' effusions. Pig gastric mucin was used as a positive control and also reacted in a concentration dependent manner but much more strongly than the middle ear mucins. The levels of reactivity in comparison (by weight) to pig gastric mucin were 14.8 $\pm$ 3.6% (3) [x $\pm$ SEM (n)] for 'thick' mucin. Thin and 'cleft palate' mucin reactivity were 4.2 $\pm$ 0.42 (3) and 10.2 $\pm$ 1.3 (5) respectively NCL-HGM-45M1 did not react with purified human colonic mucin or with human salivary mucin.

The polyclonal antisera raised to mucin purified from 'thick' effusions reacted strongly with 'thick' mucin, cleft palate' mucin and human salivary mucin. If reactivity with 'thick' mucin was assigned as 100% reactivity, human salivary mucin and cleft palate effusion mucin had $\sim$160% and $\sim$86% reactivity on a weight for weight basis. A weak positive reaction was found with 'thin' mucin and human colonic mucin at the highest loading studied (400 ng) approximately 32% and 21% of 'thick' effusion mucin reactivity (Figure 1b).

LUM 2-3 reacted in a concentration dependent manner with purified human colonic reduced mucin subunit (Figure 1c), no reactivity was found with any of the pools of middle ear mucins.

Mucins were probed with NCL-MUC-1 and NCL-MUC-2 monoclonal antibodies after deglycosylation either

Table 1. Amino acid composition of mucins purified from three pools of human middle ear effusions: comparison with cloned gene sequences.

Amino acid	*Thick*	*Thin*	*Cleft palate*	*Thick*	*Thin*	*Cleft Palate*	MUC1	MUC2	MUC7	*Central exon of* MUC5B
	(μg mg^{-1} mean ± SEM)			*(%)*			*(%)*			
his	10.3 ± 2.3	14.4 ± 1.1	6.2 ± 3.9	5.6	8.2	4.3	2.7	1.5	3.4	1.9
arg	7.2 ± 0.8	9.5 ± 1.8	4.2 ± 2.7	4.0	5.4	2.9	2.1	1.7	3.1	2.9
ser	19.8 ± 1.9	18.0 ± 3.8	18.8 ± 4.5	10.9	10.3	13.1	22.4	4.7	12.3	13.6
thr	19.5 ± 2.0	13.6 ± 4.2	16.4 ± 4.2	10.7	7.7	11.4	12.2	34.9	17.1	28.4
asp	23.9 ± 1.7	12.6 ± 2.1	10.4 ± 3.2	13.0	7.2	7.2	7.3	4.6	5.6	2.1
glu	28.5 ± 1.9	24.7 ± 5.2	11.9 ± 3.3	15.7	14.1	8.3	5.2	6.1	7.3	4.8
gly	9.9 ± 1.4	13.2 ± 4.2	12.0 ± 3.1	5.5	7.6	8.3	5.2	5.5	0.8	6.3
ala	11.4 ± 1.8	10.5 ± 2.5	11.7 ± 1.5	6.3	6.0	8.1	7.8	2.3	11.8	8.8
pro	18.7 ± 3.9	17.4 ± 3.4	20.2 ± 4.7	10.3	9.9	14.0	9.2	15.8	21.0	11.2
val	5.1 ± 1.3	7.8 ± 3.3	1.5 ± 0.4	2.8	4.5	1.0	7.5	5.3	3.1	4.4
ile/leu/phe	16.1 ± 1.0	17.1 ± 4.3	16.1 ± 1.7	8.9	9.8	11.1	12.3	8.3	8.7	8.5
lys	4.5 ± 1.4	12.6 ± 3.0	Trace	2.5	7.2	Trace	1.9	2.1	3.9	1.3
Total thiol (nmol mg^{-1})	26.9 ± 7.2	14.1	61.0 ± 27.9	3.6	1.9	10.1	0.5 (cys)	4.1 (cys)	0.6 (cys)	2.0 (cys)
free thiol	6.6 ± 3.1	9.2	10.6				[9]	[10]	[11]	[12]
Protein (%)	18.1	17.5	14.4							

4, 4 and 3 effusion pools were analysed in duplicate from thick, thin and cleft palate respectively: square brackets contain reference source for data

in solution or after immobilization on PVDF membranes. An extract of human ileum and reduced human colonic mucin were used as controls. The human ileal extract reacted positively with NCL-MUC-1 and NCL-MUC-2 and the reduced human colonic mucin subunits reacted positively with NCL-MUC-2 after deglycosylation on PVDF membranes. No reactivity was found for any of the middle ear mucins deglycosylated in solution or on the membranes.

Amino acid composition of polymeric mucins

Table 1 shows the amino acid composition of polymeric mucins purified from the three effusion types. The protein content of polymeric mucins was 18.1%, 17.5% and 14.4% of the freeze dried weight for 'thick', 'thin' and 'cleft palate' effusion mucins respectively. The general amino acid composition of the three mucins was similar but some important differences were apparent. 'Thick' effusion mucin contained 32% serine threonine and proline (11% serine, 11% threonine, 10% proline) and 29% acidic amino acids (13% asp and 16% glu). 'Thin' effusion mucin contained less serine, threonine and proline (28%) the reduction being in threonine (8%) and also aspartic acid which was reduced to almost half of that in the 'thick' effusion mucin (*ie* 7% by weight), the acidic amino acids making up 21% of the protein core. Mucin from 'cleft palate' effusions contained a higher proportion of serine, threonine and proline (38%) and a relatively low proportion of acidics (16%). In each mucin pool there was at least as much serine as threonine present and the ratios of Ser : Thr were 1 : 1, 1.3 : 1 and 1.2 : 1 for 'thick', 'thin' and 'cleft palate' effusion mucins respectively.

Amino acid analysis of mucin glycopeptides resistant to proteolysis

Amino acid analysis of mucin glycopeptides produced by proteolytic digestion with papain are shown in Tables 2a and b. The protein contents of the glycopeptides were between 9 and 10% of the freeze dried weight. This represents losses of 45%, 45% and 37% from the original mucin forms isolated from 'thick', 'thin' and 'cleft palate' effusions respectively suggesting that the greater amount of protein found in 'thick' mucin and 'thin' mucin compared to 'cleft palate' mucin was accessible to proteolysis. There were also some differences in the composition of the remaining protein cores resistant to proteolysis.

In the proteinase resistant glycopeptides from 'thick' mucin, of the major amino acids no loss of threonine was observed and serine was largely conserved (74%), 58% of the proline was lost and about half of the glutamic acid. Approximately 50–70% of the other residues were lost,

except lysine where no loss was observed and isoleucine, leucine and phenylalanine of which ~60% was conserved. As a result of these changes on digestion serine, threonine and proline now make up 43% of the core protein.

In proteinase resistant glycopeptides from 'thin' mucin no loss of serine was observed, and the acid residues were largely conserved (~90%) however there were substantial losses of threonine and proline, 54% and 76% respectively.

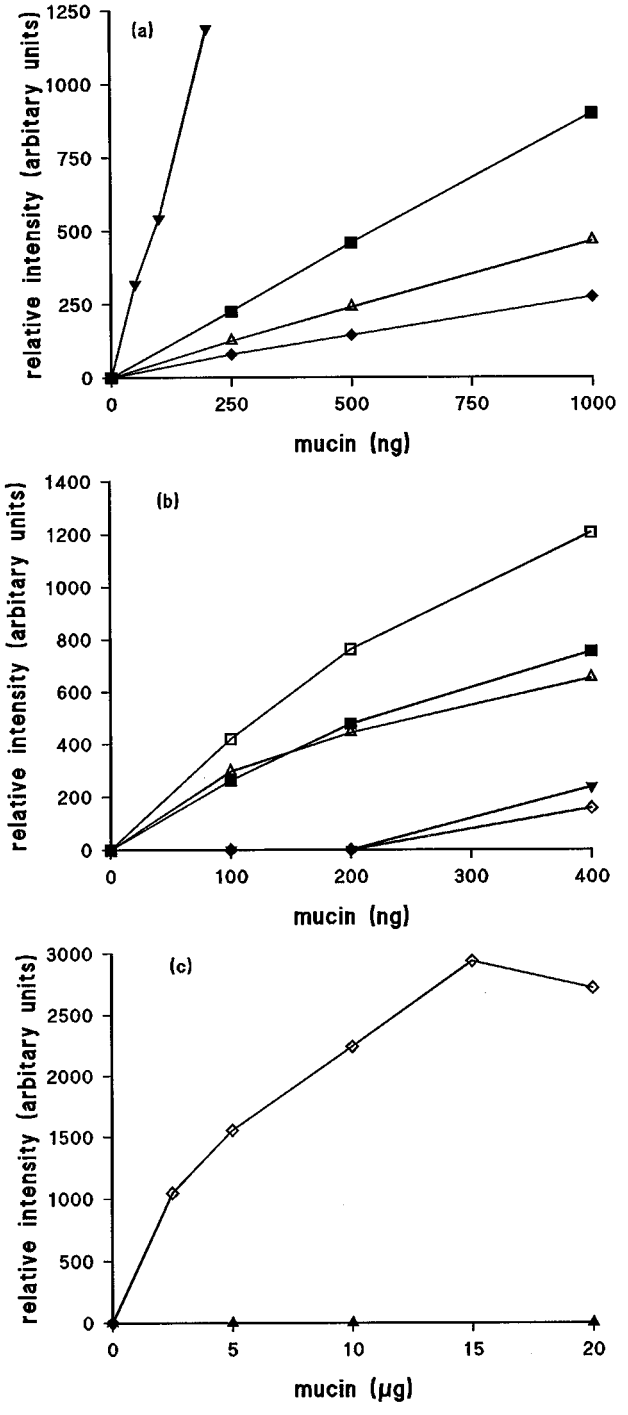

Table 2a. Amino acid composition of papain digested middle ear mucins.

Amino acid	*Thick*	*Thin*	*Cleft Palate*
	(µg mg^{-1} mean ± SEM)		
his	3.8 ± 0.4	3.4 ± 0.4	3.1 ± 0.6
arg	3.0 ± 0.23	1.9 ± 0.3	2.7 ± 0.4
ser	14.7 ± 0.9	18.7 ± 1.6	19.2 ± 2.2
thr	20.4 ± 2.8	6.2 ± 0.5	6.6 ± 1.3
asp	7.7 ± 2.2	11.3 ± 2.4	9.2 ± 1.5
glu	13.4 ± 2.7	21.6 ± 3.4	16.1 ± 1.5
gly	4.9 ± 0.5	9.0 ± 3.3	6.7 ± 1.8
ala	5.6 ± 1.1	5.9 ± 0.9	5.6 ± 0.7
pro	7.8 ± 1.7	4.1 ± 0.3	8.5 ± 1.3
val	2.2 ± 0.8	2.7 ± 0.5	2.9 ± 0.7
ile/leu/phe	10.7 ± 0.4	10.6 ± 4.2	8.5 ± 1.7
lys	6.3 ± 2.8	2.4 ± 0.2	2.3 ± 0.2
cys	0	ND	ND
protein	10.1%	9.8%	9.1%

ND, not determined; 3, 4 and 6 effusion pools were analysed in duplicate from thick, thin and cleft palate respectively.

Losses of the other residues varied between 32% (glycine) and 81% of lysine. Serine, threonine and proline now make up 30% of the protein core which is very similar to the percentage present in the undigested mucin.

Proteinase resistant mucin glycopeptides from 'cleft palate' effusions showed 60% loss of threonine compared to the undigested polymer and a 58% loss of proline, no detectable losses of serine and glutamic acid and small losses of aspartic acid (12%), the contribution of the latter acidic residues increasing from 16% to 28% of the protein core. Serine, threonine and proline contributed 38% of the core protein in the digested mucin, the same as that in the undegraded polymeric mucin. The major difference between the proteinase resistant mucin glycopeptides from 'thick', 'thin' and 'cleft palate' effusions

Figure 1. Studies on the antigenic identity of human middle ear mucins. (a) Reactivity of NCL-HGM-45M1 monoclonal antibody with mucins. Pig gastric mucin (50–200 ng) (▼); thick (■), cleft palate (△) and thin (◆) human middle ear mucins (250–1000 ng). Mucins (50–1000 ng) were blotted onto nitrocellulose membranes and tested for reactivity with NCL-HGM-45M1 (1 : 50 dilution) in ELISA. (b) Reactivity of mucins with TEPA1 polyclonal antisera; (□) human salivary mucin; (■) thick, (▼) thin and (△) cleft palate human middle ear mucins; (◇) human colonic mucin subunits. Mucins (100–400 ng) were blotted onto nitrocellulose membranes and tested for reactivity with TEPA1 (1 : 200 dilution) in ELISA. (c) reactivity of mucins with LUM 2-3 polyclonal antisera; (◇) human colonic mucin subunits; (▲) human middle ear mucins. Mucins (2.5–20 µg) were blotted onto nitrocellulose membranes and tested for reactivity with LUM 2-3 polyclonal antisera (1 : 1000 dilution) in ELISA. In all cases staining of the slots was quantitated using a Shimadzu CS 930 dual wavelength scanning densitometer at 595 nm.

Table 2b. Comparison of amino acid composition of papain digested middle ear mucins with that of tandem repeat of known mucin genes.

Amino acid	*Thick*	*Thin*	*Cleft Palate*	MUC1	MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6	MUC7	MUC8
	(%)			*(% in VNTR)*								
His	3.8	3.5	3.4	5	0	6	0	0	0	8	0	0
Arg	3.0	1.9	3.0	5	0	0	0	0	0	1	0	7
Ser	14.6	19.1	21.0	10	0	29	19	25	3	18	17	14
Thr	20.2	6.3	7.2	15	61	41	25	50	45	30	22	14
Asp	7.6	11.6	10.1	5	0	0	6	0	0	1	0	0
Glu	13.3	22.1	17.6	0	4	0	0	0	0	3	4	14
Gly	4.9	9.2	7.3	10	4	0	0	0	7	4	0	14
Ala	5.5	6.0	6.1	20	0	0	13	13	14	6	22	0
Pro	7.7	4.2	9.3	25	22	5	13	13	10	15	35	21
Val	2.2	2.8	3.2	5	4	0	6	0	3	3	0	0
Ile/Leu/Phe	10.6	10.8	9.3	0	4	12	0	0	7	8	0	7
Lys	6.2	2.5	2.5	0	0	0	6	0	0	1	0	0
Cys	0	ND	ND	0	0	0	0	0	0	0	0	7
				[9]	[10]	[6]	[6]	[6]	[6]	[6]	[11]	[7]

ND, not determined; square brackets contain reference source for data.

was therefore the content of threonine. In digested mucin from 'thick' effusions the ratio of serine to threonine was 0.8 : 1.0, whereas for mucin glycopeptides from 'thin' and 'cleft palate' effusions this ratio was 3 : 1. In addition the relative levels of acidics increased in the 'thin' and 'cleft palate' pools but decreased in the 'thick' pool on digestion. Proline was extensively lost from all the mucin pool types.

Discussion

This study describes the isolation and characterization of mucins from human middle ear effusions. Comparisons have been made between the structural and chemical analyses of these mucins with those predicted for the gene products of the nine identified mucin genes and attempts have been made to establish the antigenic identity of middle ear mucins. It is not known which of the human mucin genes cloned so far are expressed by the middle ear mucosa, however, middle ear mucosa is a modified respiratory epithelium and eight of the nine mucin genes cloned so far (excluding *MUC6*) have been shown by *in situ* hybridization and Northern blotting to be expressed in the airways [6, 7, 11, 14]. The products of any of these genes could therefore be candidates for expression and secretion by the middle ear. It is important to attempt to identify which mucins are actually present in mucous secretions as anomalies do exist between mRNA expression in particular tissues and mature mucin gene products identified in secretions, *eg MUC2* has been shown to be expressed in the airways based on mRNA detection [31] but could not be detected in tracheal mucus secretions of tracheal mucosa using antibodies to non-VNTR regions of the protein core [21]. The amino acid composition of mucins isolated from respiratory secretions were also different to that predicted for *MUC2* from the cDNA sequence [20].

Effusions were classified into 3 groups, 'thick' and 'thin' from anatomically normal children and effusions from 'cleft palate' patients. Previous studies have suggested differences between the mucins present in effusions divided into these three groups [4, 5]. Whole effusions were solubilized by mild homogenization, conditions which have been previously shown to solubilize $\sim$95% of the PAS positive material in middle ear effusions [3] and a cocktail of proteinase inhibitors was included to effectively inhibit *in vitro* degradation of the mucins. Mucins from each of the three pools had similar buoyant densities in the range 1.41–1.48 g ml^{-1} and these are characteristic of other mucins purified under these conditions [17]. The purified mucins were shown to be free of contaminating protein by applying high loadings to SDS polyacrylamide gels. Mucins from 'thick' and 'cleft palate' effusions contained 32 and 38% serine, threonine and proline respectively which is comparable with mucins isolated from other secretions *eg* human cervical mucins [32]. Mucin from thin effusions had a lower content of these amino acids (28%). The overall amino acid compositions of the mucins from the three pools differed from the predicted compositions of any of the three fully sequenced mucin genes *MUC1* [9], *MUC2* [10] or MUC7 [11] and from the large central exon of *MUC5B* (Table 1) and mucins from 'thick' and 'thin' effusions are especially rich in acid amino acids (29% and 21% respectively). All of the protein compositions predicted by the cDNA sequences contained less acidic residues (particularly

glutamic acid) than the middle ear mucins (Table 1). *MUC2* and the large central exon of *MUC5B* contain particularly large amounts of threonine (Table 1) and *MUC7* has very high contents of alanine and proline.

The middle ear mucins from all three pools were largely excluded from Sepharose CL-2B and therefore contained high M_r, weight mucins ($>2\times10^6$). Mucins from 'thick' and 'cleft palate' effusions had a high content of thiols (Table 1) the majority of which were reducible with a concomitant reduction in molecular size. Thin effusion mucin contained less disulphide bridged thiols than the others which would suggest it is less polymerized. These polymeric characteristics of middle ear mucins would apparently exclude *MUC1* and *MUC7*) (monomeric, relatively low M_r mucins) from being major mucin components of middle ear effusions.

It is the consensus of opinion in the mucin field that the tandem repeat sequences of these genes correspond to the heavily glycosylated domains of mucins, indeed glycopeptide units from human colonic mucus have been found to have a similar composition to *MUC2* [18]. It is therefore important to compare the predicted size and composition of these gene products with the composition of the mucin glycopeptides from middle ear effusions isolated in this study. It is possible, however, that proteolysis may produce fragments containing sequences outside of the VNTR region of mucin genes. This is to some extent supported by Thornton *et al.* [33] who give an amino acid analysis of a proteolytically derived glycosylated region of putative MUC5B which differs from that of the VNTR region [33]. Threonine and serine make up a major proportion of amino acids in the tandem repeat sequences of cloned mucin *genes* (between 39 and 75% for *MUCs 2–7*) (Table 2b) [6]. The proportion of threonine and serine in digested mucin from thick effusions (35%) approaches these levels and the ratio of serine to threonine is similar to that found in *MUC4* (*ie* 1 : 1.4 and 1 : 1.3 for the 'thick' effusion mucin and *MUC4* respectively), however the rest of the composition is different (Table 2b). This probably reflects the presence of other mucin gene products in 'thick' effusions. In all of the other mucin genes so far found to be expressed in the airways the ratio of threonine to serine is at least 2 : 1.

The tandem repeat sequence of *MUC8* contains a smaller proportion of threonine and serine (28%) (as does *MUC1*, 25%) and this is comparable to the total amounts of these residues found in digested mucin from thin (25%) and cleft palate (27%) effusions. The proportions of serine and threonine in these digested mucins (3 : 1 serine : threonine) is however different to that predicted by the consensus sequence of the tandem repeat of *MUC8* which contains equal amounts of serine and threonine (7). There are also other differences in particular the proportion of proline found in these digested mucins (4% for thin and 8% for cleft palate) is considerably lower than that found in *MUC8* (21%). Interestingly the tandem repeat sequence of *MUC8* also contains quite a high proportion of glu (14%) which approaches the levels found in digested mucin from thin and cleft palate mucins (22% and 18% respectively), it does not however contain any asp, found as 11% and 9% of thin and cleft palate digested mucins respectively.

MUC5AC, has been identified in respiratory secretions using polyclonal antibodies to synthetic peptides prepared from cloned gene sequences and tryptic peptides of *MUC5* [19–21] and shown to account for approximately 60% of the mucins in normal secretions [20]. The structure of *MUC5AC* and *MUC5B* [12] predicted from the gene sequence cloned up until now (*ie* tandem repeat domains interupted by non-tandemly repeated cys-rich domains), is compatible with that previously described for middle ear mucins isolated from 'thick' effusions [3]. It was therefore particularly interesting to investigate further the possibility that *MUC5AC* and/or *5B* was a component of middle ear effusion mucins.

The monoclonal antibody NCL-HGM-45M1 reacts with an epitope of human gastric mucin destroyed by reduction with mercaptoethanol, partially lost on trypsin proteolysis but stable to periodate oxidation [34, 35] *ie* located in a region of the protein core rich in cysteine and outside the VNTR region. The antibody stains the surface gastric epithelium of normal human gastrointestinal tract and precancerous and cancerous colonic mucosa but not normal colon [36]. Concurrently Northern blotting and *in situ* hybridization have shown *MUC5AC* to be strongly expressed in normal stomach mucosa [37], and in rectosigmoid villous adenomas while normal adult rectal mucosa is negative [37]. The *MUC5AC* gene product has also been identified as a prominent mucin in human gastric mucosal scrapings reacting strongly with an antibody raised to a synthetic peptide prepared from a non-VNTR sequence [37]. *MUC6* is also strongly expressed in the stomach but appears to be associated with the gastric glands and not the mucosal surface [38]. NCL-HGM-45M1 is therefore highly likely to react with a non-VNTR region of *MUC5AC* although reactivity with the *MUC6* gene product or other novel mucins cannot be completely ruled out.

Mucins from all three pools reacted positively with the anti-human gastric monoclonal NCL-HGM-45M1, however, 'thick' effusion mucin was 3 fold more positive than 'thin' mucin and 1.5-fold more positive than the 'cleft palate' effusion mucin, emphasizing the differences between the pools. Despite being recognized by the antibody the levels of reactivity of middle ear mucins were only about 15%, for 'thick' mucin, 5% for 'thin' mucin and 10% for 'cleft palate' mucin, of the levels of reactivity found for the positive control (pig gastric mucin). Cysteine rich domains of pig gastric mucin apoprotein have 60% homology with *MUC5AC* [39]. This suggests that *MUC5AC* is a component of middle ear mucin secretions but probably not a major one and confirms the conclusions of the amino acid analyses.

The polyclonal antibody TEPA1 raised to mucin from thick effusion mucins reacted strongly with high molecular weight mucin from human saliva which has recently been identified as the product of *MUC5B* [22]. This suggests that epitopes present on *MUC5B* are present in mucins from thick effusions and it will be interesting to investigate this further with antibodies raised to synthetic peptides designed from sequences of *MUC5B*. A plausible alternative explanation is that TEPA1 is reacting with a novel mucin, not *MUC5AC* or *MUC5B* in thick middle ear effusions and saliva. Mucins from 'cleft palate' effusions reacted at a similar level to 'thick' effusion mucins, however, mucins from thin effusions were much less reactive (approximately 30% by weight), again highlighting the difference between mucins from 'thick' and 'thin' effusions.

None of the mucins isolated from middle ear effusions reacted with LUM2-3 polyclonal antisera or with NCL-MUC2 or NCL-MUC1 suggesting that *MUC2* and *MUC1* are not present in significant amounts. Less than 5% of the PAS positive material was left unextracted by homogenization, however the presence of insoluble *MUC2* in this residue cannot be ruled out.

These studies suggest that human middle ear mucosa secretes more than one mucin, that *MUC5AC* forms a minor component and that *MUC5B* may be present in middle ear effusions. This could reflect different stimuli in the multifactorial aetiology of OME or different stages of the proliferative cellular processes taking place in the disease in the clinical groups studied.

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