Virchows Arch (1999) 435:479–485

ORIGINAL ARTICLE

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Mucins and mucin-associated carbohydrate antigens expression in gastric carcinoma cell lines

Received: 19 April 1999 / Accepted: 23 June 1999

Abstract Mucins are high-molecular-mass glycoproteins with high carbohydrate content and marked heterogeneity both in the apoprotein and in the oligosaccharide side chains. Mucin genes are expressed in a regulated manner, namely in the human stomach. The first aim of the present study was to characterise the expression of mucins and mucin-associated carbohydrate antigens in seven gastric carcinoma cell lines, and to compare their expression profiles with those of normal gastric tissues and human gastric carcinomas. Secondly, we aimed to see whether or not there is an association between the expression of mucins and mucin-associated carbohydrate antigens. Our results show that mucin expression in gastric carcinoma cell lines: (a) follows in part the mucin expression profile of normal gastric mucosa and gastric carcinomas with wide expression of MUC1 and MUC5AC; (b) parallels the aberrant pattern of mucin expression observed in human gastric carcinomas with occasional expression of MUC2, MUC3, MUC4 and MUC5B; (c) does not include, at least in our series, the expression of MUC6 mucin; and (d) follows in part the differentiation pattern of the carcinomas from which the cell lines originated, keeping S-Tn expression in cell lines derived from glandular carcinomas. Our results further demonstrate that there is no apparent relationship between the mucin core proteins and the simple mucin-type or Lewis carbohydrate antigens.

Key words Mucins · MUC1 · MUC5AC · MUC6 · Gastric carcinoma cell lines

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Introduction

Mucins are glycoproteins characterised by high molecular mass, high carbohydrate content (50–80% of their mass) and marked heterogeneity involving both the apoprotein and the oligosaccharide side chains [18]. To date nine human mucin genes have been partially or totally sequenced and characterised [1, 4, 14, 19, 22–24, 33, 34, 38, 43, 46].

There are consistent data indicating that mucin genes are expressed in a regulated cell- and tissue-specific manner. The stomach provides a good example of such differential expression of mucin genes. MUC1 is detected in mucous cells of the surface epithelium and neck region of the gastric antrum, as well as in pyloric glands and in oxynthic glands of the body region [5, 20, 27, 28, 35, 40]. MUC5AC is highly expressed in foveolar epithelium of both body and antrum [2, 6, 13, 28, 39], whereas MUC6 protein expression is limited to mucous neck cells of the body and pyloric glands of the antrum [6, 13, 28]. Finally, the expression of MUC2 in normal stomach has been reported either as absent [28, 51] or as very weak [2, 10, 15, 16, 27, 41].

Glycosylation is thought to be essential for the biological functions of mucins, and it has been postulated that the cell-type-specific mucin expression in gastric cells may be related to the type of glycosylation. De Bolós et al. [13] demonstrated an association between the expression of MUC5AC and the expression of Lewis^b, Lewis^a, and sialyl-Lewis^a (type 1 chains), and be-

tween the expression of MUC6 and that of Lewis^y (type 2 chains).

During gastric carcinogenesis, there are often expression of underglycosylated forms of MUC1 mucin, decreased expression of MUC5AC and MUC6, and expression of mucins not found in the normal gastric mucosa, including MUC2 [3, 28, 39, 40, 42]. It has been shown that aberrant glycosylation occurs in cancer development in the stomach as it does elsewhere [25], leading to the accumulation of simple mucin-type carbohydrates such as Tn, Sialyl-Tn and T (Thomsen-Friedenreich) antigens [26, 31, 45].

The first aim of the present study was to characterise the expression of mucins and mucin-associated carbohydrate antigens in a panel of seven gastric carcinoma cell lines, and to compare the expression profiles in the cell lines with those previously described in normal gastric tissues and human gastric carcinomas taking into consideration that three of the cell lines (GP202, GP220 and KATOIII) were developed from isolated cell carcinomas and the remaining four cell lines (MKN45, St23132, St2957 and St3051) originated from glandular-type gastric carcinomas. Secondly, we aimed to see whether or not there is an association between the expression of mucins and mucin-associated carbohydrate antigens, in order to evaluate whether, as in normal conditions, the glycosylation is related to the mucin proteins expressed.

Materials and methods

Materials

Culture materials and standard RNA marker were obtained from Life Technologies. Nylon membranes Hybond-N$^+$ and [α-^{32}P]dCTP were purchased from Amersham. Mucin probes were obtained from different sources: MUC1 [19], MUC2 [21], MUC3 [22], MUC4 [38], MUC5AC [24], MUC5B [14], MUC6 [46], MUC7 [47], and the actin probe and 1st-strand cDNA synthesis kit were obtained from Clontech (Clontech, Ozyme, France). The smart ladder and 100-bp ladder used as standards for reverse transcription–polymerase chain reaction (RT-PCR) were purchased from Eurogentec. The random-primer labelling kit was obtained from Boehringer Mannheim. Ampli Taq Gold was purchased from Perkin Elmer. The primers were synthesised by MWG-BIOTECH. Anti-MUC1 monoclonal antibodies HMFG-1, HMFG-2 and SM3 were a generous gift from Dr. Joyce Taylor-Papadimitriou and Dr. Joy Burchell (Imperial Cancer Research Fund, London). Monoclonal antibodies PMH1 [41], CLH2 [39] and CLH5 (unpublished) were produced by our group. Anti-Le^b monoclonal antibody BG.6 was purchased from Signet Laboratories. All the other monoclonal antibodies to the carbohydrate structures were a kind gift from Prof. Henrik Clausen (Faculty of Health Sciences, Copenhagen). Rabbit normal nonimmune serum, biotinylated rabbit anti-mouse secondary antibody, and avidin-biotin-peroxidase complex were obtained from Dako.

Cell culture

Seven gastric cancer cell lines were analysed. GP202 and GP220 cell lines were recently established in our laboratory [17], while MKN45 and KATOIII are commercially available cell lines (American Type Cell Collection) and the St23132, St2957, St3051 cell lines were a kind gift from Dr. Peter Vollmers [49]. All cells lines were grown in RPMI 1640 with Glutamax, supplemented with 10% inactivated (30 min, 56°C) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 U/ml nystatin. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Table 1 Monoclonal antibodies used for immunohistochemistry and respective dilutions

Antigen	Antibody	Dilution
MUC1	HMFG-1	– [a]
	HMFG-2	– [a]
	SM3	– [a]
MUC2	PMH1	– [a]
MUC5AC	CLH2	– [a]
MUC6	CLH5	– [a]
Tn	5F4	1:15
S-Tn	3F1	1:8
T	3C9	1:10
Le^a	CA3F4	1:5
S-Le^a	CA19.9	1:5
Le^b	BG.6	1:60
Le^x	SH1	1:5
S-Le^x	FH6	1:5
Le^y	AH6	1:5

[a] Hybridoma supernatant

Northern blotting

Large RNAs were extracted from the different cell lines (~10^8 cells) at confluence using an improved method for the isolation and transfer of large RNAs as previously described [12]. All the probes used to *MUC1*, *MUC2*, *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC7* and actin (positive control) were labelled with [α-^{32}P]dCTP using a commercial random-primer labelling kit according to the manufacturer's protocol.

Signals obtained were scored as: – (no expression) or + (expression).

Reverse transcription–polymerase chain reaction

cDNAs were prepared using the 1st-strand cDNA synthesis kit. PCRs for mucin genes were carried out in 50 µl of final solution (5 µl Perkin Elmer buffer, 5 µl $MgCl_2$ 25 mM, 5 µl dNTPs, 10 pmol of each primer, 2 U Taq polymerase, and 5 µl cDNA). Cycling conditions were as follows: (1) denaturation at 96°C, 30 s for one cycle; (2) denaturation at 96°C, 30 s, annealing at 60°C, 30 s, and extension at 72°C, 60 s for 30 cycles; (3) extension at 72°C, 15 min. The primers were chosen in the 3' part of the different mucin genes (unpublished data). GAPDH mRNAs were co-amplified in each experiment with each primer set. The same PCR conditions were performed to study all mucin genes except for *MUC5AC*. The conditions for the amplification of *MUC5AC* were: (1) denaturation at 94°C, 1 min, annealing at 58°C, 30 s, extension at 72°C, 30 s for 35 cycles.

FUT1 and FUT2 fucosyltransferases transcripts were amplified by PCR using primers described by Kelly et al. [29, 30] in the following conditions: (1) denaturation at 94°C, 1.5 min, and annealing/extension at 72°C, 2.5 min for 40 cycles; (2) extension at 72°C for 10 min. For FUT3 mRNA detection primers published by Cameron et al. [7] were used with the following conditions: (1) denaturation at 94°C, 1.5 min, annealing at 70°C, 1.5 min, and extension at 72°C, 2 min for 40 cycles; (2) extension at 72°C for 10 min.

Signals obtained were scored as: – (negative) or + (positive).

Table 2 Comparison between the results for mucin expression by Northern blotting (*N*), reverse transcription–polymerase chain reaction (*RT*) and immunohistochemistry (*I*). Results are expressed as positive (+) or negative (–)

	MUC1			MUC2			MUC3		MUC4		MUC5AC			MUC5B		MUC6			MUC7	
Cell line	N	RT	I	N	RT	I	N	RT	N	RT	N	RT	I	N	RT	N	RT	I	N	RT
GP202[a]	+	+	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
GP220[a]	+	+	+	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–
MKN45[b]	+	+	+	–	–	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–
KATOIII[a]	+	+	+	+	+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	–
St23132[b]	–	+	+	–	–	–	+	+	–	–	+	+	+	–	+	–	–	–	–	–
St2957[b]	–	+	+	–	+	+	–	–	–	–	+	+	+	–	+	–	–	–	–	–
St3051[b]	–	+	+	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–

[a] Cell lines developed from isolated-cell-type carcinomas
[b] Cell lines developed from glandular-type gastric carcinomas

Immunohistochemistry

Sections from formalin-fixed paraffin-embedded cytospins were used for immunostaining. The avidin-biotin-peroxidase complex (ABC) method was applied. The paraffin sections were dewaxed and then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min, to block endogenous peroxidase. The sections were incubated for 20 min with normal nonimmune serum to eliminate nonspecific staining. Excess normal serum was removed and replaced by specific primary monoclonal antibodies in the appropriate dilution (Table 1). Sections were incubated overnight (18–22 h) at 4°C. After the slides had been washed, sections were incubated with biotin-labelled secondary antibody diluted 1:200 in PBS with 5% BSA for 30 min and with ABC for 60 min. Sections were stained for 5 min with 0.05% 3'3 diaminobenzidinetetrahydrochloride (DAB) freshly prepared in 0.05 M tris/hydroxymethylaminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2, counterstained with haematoxylin, dehydrated and mounted.

A semi-quantitative approach was used to score the staining: + (<25%), ++ (25–50%), +++ (50–75%) and ++++ (>75%).

Results

Northern blotting

The results of the Northern blotting study of the different mucins are summarised in Table 2. *MUC1* mRNA was detected in GP202, GP220, MKN45 and KATOIII. Low levels of *MUC2* mRNA were only detected in KATOIII. GP220 expresses *MUC3* mRNA. Low levels of MUC3 and MUC4 were detected in St23132 and KATOIII, respectively. *MUC5AC* mRNA was detected in St23132, St2957 and St3051. *MUC5B*, *MUC6* and *MUC7* mRNAs were not detected in any of the cell lines.

Reverse transcription–polymerase chain reaction

Table 2 summarises the RT-PCR results for the different mucin genes. All cell lines expressed *MUC1*. *MUC2* expression was detected in KATOIII and St2957, and *MUC3* expression was detected in GP220 and St23132. *MUC5AC* was detected in six cell lines and not in GP202 (Fig. 1). *MUC5B* was detected in four cell lines: MKN45, St23132, St2957 and St3051. None of the cell lines expressed *MUC4*, *MUC6* or *MUC7* as far was detected by RT-PCR.

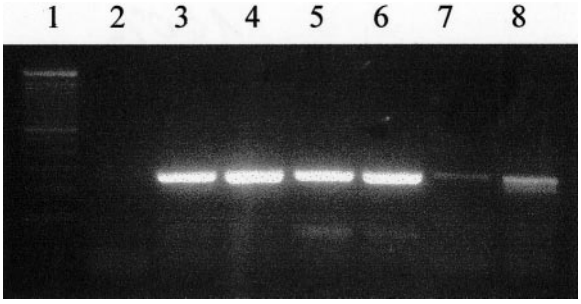

Fig. 1 RT-PCR analysis of *MUC5AC* (257-bp product) in the seven gastric carcinoma cell lines. *Lanes 1–8* correspond to: 100-bp ladder, GP202, GP220, St2957, St3051, St23132, MKN45 and KATOIII cell lines

All cell lines express FUT1 and FUT2, whereas FUT3 is expressed in GP220, KATOIII, St23132, St2957 and St3051 and is not detected in GP202 and MKN45.

Immunohistochemistry

The results obtained in the immunohistochemical study of mucin core proteins are summarised in Table 2. All cell lines expressed MUC1 and MUC5AC at different levels, except for GP220 that did not express MUC5AC. Expression of MUC1 was detected in all cell lines using three different monoclonal antibodies: HMFG-1 MAb stained more cells than HMFG-2, and HMFG-2 stained more cells than SM3. Four cell lines expressed MUC2: GP202 and St2957 at low levels and MKN45 and KATO III at high levels. None of the cell lines expressed MUC6.

The expression of mucin-associated carbohydrate antigens is summarised in Table 3. All the cell lines expressed high levels of type 1 and type 2 chain Lewis antigens. In most cell lines, the staining for the sialylated epitopes, S-Le^a and S-Le^x, was lower than that for the respective non-sialylated antigens (Le^a and Le^x, respectively). Exceptions are MKN45 and St2957, which show similar levels of Le^a and S-Le^a, KATOIII, which shows higher levels of S-Le^x than Le^x, and St23132, which shows similar levels of Le^x and S-Le^x. The expression

Table 3 Immunohistochemical expression of mucin-associated carbohydrate antigens in the seven gastric carcinoma cell lines

Cell line	Simple mucin-type carbohydrate antigens			Lewis antigens					
				Type1 chain			Type 2 chain		
	Tn	S-Tn	T	Le^a	S-Le^a	Le^b	Le^x	S-Le^x	Le^y
	5F4	3F1	3C9	CA3F4	CA19.9	BG.6	SH1	FH6	AH6
GP202[a]	+	–	+	++	–	++++	++++	+	++++
GP220[a]	–	–	–	++++	–	+++	++++	–	++++
MKN45[b]	+	+	–	+	+	+	+++	++	–
KATOIII[a]	–	–	–	++++	++	+	++	++++	++
St23132[b]	+	+	–	++++	+++	++++	++	++	+++
St2957[b]	+	+	–	++	++	++	++	+	+++
St3051[b]	–	+	–	+++	++	++	+++	++	++++

[a] Cell lines developed from isolated-cell-type carcinomas
[b] Cell lines developed from glandular-type gastric carcinomas

of simple mucin-type antigens is low or absent in every cell line. Expression of Tn antigen was observed in four cell lines: GP202, MKN45, St23132 and St2957. The sialylated form, S-Tn, was observed in MKN45, St23132, St2957, St3051. T antigen was only detected in GP202.

Discussion

To the best of our knowledge this is the first comprehensive study on mucin expression in a panel of gastric carcinoma cell lines. Mucin expression was evaluated at mRNA and protein levels using different methodologies.

Our data show that the seven cell lines express MUC1 and that most of them express MUC5AC. Our results thus fit in with the in vivo data and suggest that gastric type differentiation, evaluated by MUC1 and MUC5AC expression, is maintained in all gastric carcinoma cell lines.

The absence of *MUC1* mRNA detectable by Northern blotting in three of the gastric carcinoma cell lines, despite their positivity on RT-PCR and immunohistochemistry, may indicate the presence of very low amounts of *MUC1* mRNA (under the detection threshold for Northern blotting).

A similar interpretation is tenable for the understanding of MUC5AC protein expression in MKN45 and KATOIII without detectable amounts of *MUC5AC* mRNA by Northern blotting. In GP220, *MUC5AC* expression was detected by RT-PCR alone, suggesting that very low levels of gene expression are responsible for undetectable levels of *MUC5AC* mRNA by Northern blotting and of MUC5AC protein by immunohistochemistry. Finally, we cannot rule out the possibility that the immunohistochemical detection of MUC5AC in GP202, in which *MUC5AC* transcripts are not detected by Northern blotting and RT-PCR, may represent a false-positive result of immunohistochemistry despite the high specificity of the antibody we have used [39].

MUC6 mRNA and MUC6 protein were absent in every cell line of the present study. Expression of MUC6 protein is limited to mucopeptic cells of the normal stomach [6, 13, 28], and in a series of 104 gastric carcinoma cases only 29.8% showed MUC6 protein expression (C.A. Reis et al., unpublished data). The absence of expression of MUC6 probably reflects the low frequency of mucopeptic differentiation in gastric carcinoma cell lines as well as in gastric carcinomas in vivo [44].

MUC2 protein was expressed in GP202 and MKN45, but *MUC2* mRNA was not detected by Northern blotting or by RT-PCR. One cannot exclude the possibility that some cross-reactivity of monoclonal antibody PMH1 may be responsible for these discrepant results, although it seems unlikely since the detection of MUC2 protein was confirmed by another anti-MUC2 monoclonal antibody (data not shown). The present study, showing MUC2 expression in four cell lines, demonstrates that both in vivo and in vitro, MUC2 expression reflects a nonuniversal aberrant differentiation pattern of gastric carcinoma cells.

Studies on the intestinal mucin MUC3 in human stomach are scarce, but low levels of *MUC3* mRNA have been demonstrated in normal gastric tissues [2, 48]. Expression of *MUC4* in normal stomach has also been reported in the literature [2, 48] and the expression of *MUC3* and *MUC4* mRNA was previously demonstrated in gastric carcinomas [28]. In accordance with the in vivo expression of MUC3 and MUC4 in gastric tissues, consistent results were obtained in the present study regarding Northern blotting and RT-PCR expression of MUC3 in GP220 and St23132 and of MUC4 in KATOIII by Northern blotting.

To the best of our knowledge expression of MUC5B has not been reported in the normal stomach. Four cell lines expressed *MUC5B* mRNA as detected by RT-PCR. The absence of signal in Northern blotting may be due to low levels of expression of *MUC5B* mRNA. In vitro expression of MUC5B in cell lines may indicate an aberrant differentiation program of these gastric carcinoma cell lines in culture.

Expression of the low-molecular-weight salivary mucin, MUC7, has not been reported to date in normal gastric tissues and was not detected in any of the cell lines examined in this study.

Expression of mucins by the seven cell lines was not correlated with the characteristics of the carcinomas from which they derived. This contrasts with previous results from human tumours, which showed preferential expression of MUC5AC in isolated cell-type gastric carcinomas [39] and preferential expression of MUC2 in glandular type gastric carcinomas [3].

All cell lines expressed FUT1 and FUT2 transcripts in accordance with the detection of type 2 and type 1 chain Lewis antigens, respectively. Two of the cell lines analysed lack the expression of the FUT3 enzyme despite the high levels of type 1 Lewis antigens, suggesting that some other α-3,4-fucosyltransferase gene must be expressed in these cells.

The most striking changes of glycosylation occurring in gastric carcinomas are the increased and/or de novo expression of Le^a antigen and the decreased or absent expression of type 2 Lewis antigens in comparison with normal gastric mucosa [36]. The gastric carcinoma cell lines we have studied do not apparently reproduce the results described for gastric carcinomas, since Le^a, S-Le^a and Le^b and all type 2 Lewis antigens (Le^x, S-Le^x and Le^y) are widely expressed in most cell lines. The relationship between mucin expression and Lewis antigens expression reported for the human stomach [13], namely the association between expression of MUC5AC and type 1 Lewis structures, and between MUC6 and type 2 Lewis structures, was not found in the cell lines. In fact, type 1 Lewis antigens are expressed in all gastric carcinoma cell lines and type 2 Lewis antigens are also expressed in all cell lines despite the absence of MUC6 expression. These findings suggest that different carrier mucins are used for building type 1 and type 2 Lewis chain antigens in gastric carcinoma cell lines.

The expression of simple mucin-type antigens is low or absent in the gastric carcinoma cell lines. Expression of Tn and S-Tn antigens was observed in four cell lines, and T antigen was only detected in GP202. It has been reported that Tn and S-Tn antigens are expressed in the majority of gastric and colon carcinomas [8, 9, 11, 26, 31, 32, 50], whereas T antigen is detected in a low proportion of gastric carcinoma cases [11]. S-Tn expression is associated with the glandular type of gastric carcinoma [9, 32], suggesting that mucin glycosylation may also be related to cell differentiation. The gastric carcinoma cell lines parallel the in vivo model in that the four cell lines expressing S-Tn are those derived from glandular-type gastric carcinomas. The expression of T antigen by a single gastric carcinoma cell line is in keeping with the low levels of expression of T antigen in gastric carcinomas [11].

Similarly to the absence of an association between mucin expression and Lewis-type antigens expression, our data also demonstrate the absence of any relationship between mucin expression and simple mucin-type carbohydrate antigen expression, in keeping with the results reported by Ogata et al. [37] in colon cancer cell lines.

Summing up, our results show that the profile of mucin expression in gastric carcinoma cell lines: (a) follows the mucin expression profile of normal gastric mucosa and gastric carcinomas in part, with wide expression of MUC1 and MUC5AC; (b) parallels the aberrant pattern of mucin expression observed in human gastric carcinomas, with occasional expression of MUC2, MUC3, MUC4 and MUC5B; (c) does not include, at least in our series, the expression of MUC6 gastric mucin; (d) follows in part the differentiation pattern of the carcinomas from which the cell lines have originated, keeping S-Tn expression in the cell lines derived from glandular type carcinomas. Our results further demonstrate that, in contrast to normal gastric tissues, there is no apparent relationship between the mucin core proteins and the simple mucin-type or Lewis carbohydrate antigens that are expressed in each cell line.

Acknowledgements The authors thank Mrs. D. Leitão and M.P. Delescaut for technical assistance. This work was supported by the Fundação para a Ciência e a Tecnologia, PRAXIS XXI (project no PECS/P/SAU/255/95) and by BIOMED 2-PL963222.

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