Turk J Med Sci
30 (2000) 359-365
© TÜBİTAK

Nurten AKSOY[1]
Anthony P. CORFIELD[3]
Christos PARASKEVA[4]
David J. THORNTON[2]
John K. SHEEHAN[2]

Alterations of MUC2 Mucin in Colorectal Adenocarcinoma

Received: July 27, 1999

[1]Department of Biochemistry, Faculty of Medicine, Harran Unversity, Şanlıurfa 63300, Turkey,
[2]Wellcome Trust Centre for Cell-Matrix Research, Division of Biochemistry, 2.205 School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK;
[3]University of Bristol, Department of Medicine, Bristol Royal Infirmary, Bristol, BS2 8HW, UK;
[4]CRC Colorectal Tumour Biology Group, Department of Pathology and Microbiology, University of Bristol, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK.

Abstract: MUC2 is a well-identified intestinal mucin, present predominantly in the small intestine and colon. It is known that pathological alterations in the expression and composition of this mucin occur in several serious diseases related to epithelial surfaces, e.g., carcinoma and colitis ulcerosa. In this study, our aim was to find out whether there are any specific alterations in the expression and secretion of MUC2 in an adenoma-adenocarcinoma sequence that has been established as an in vitro model (1,2). The adenoma-carcinoma sequence represents the first example of the malignant progression of human colonic adenoma cells in vitro (2,3). Using a novel approach that we established previously (4) — the combination of density gradient centrifugation and agarose gel electrophoresis— we have characterised and compared the cell layer and medium MUC2 mucins among the colonic cell lines representing different stages of colorectal carcinogenesis. The results indicate that the expression and secretion of MUC2 mucin decrease from adenoma to adenocarcinoma. This is consistent with frequently observations in in vivo carcinogenesis (5) and strongly suggests that intestinal MUC2 mucins may be a useful marker for malignant transformation.

Key Words: Mucins, MUC2, PC/AA cell line, carcinogenesis, tumour markers.

Introduction

An analysis of the pre-cancer stages is essential in understanding the complex initiation and promotional events involved in the development of carcinoma. It may also provide an opportunity to detect malignancy before early premalignant changes and alter the natural history of carcinogenesis through earlier diagnosis. Unfortunately, it is not always possible to detect pre-cancerous stages of cancers in the human body. The development of colorectal cancer is an excellent example of the multistep nature of carcinogenesis. In colorectal carcinogenesis a clear premalignant stage, adenomas (benign epithelial tumours, sometimes called polyps) have been recognised, and most colorectal cancers are thought to originate from premalignant adenomas in what is often called the adenoma-carcinoma sequence (5-7).

Colorectal mucosa, like the other epithelial surfaces, is covered by a visco-elastic gel (mucus) whose biological functions include lubrication, maintenance of tissue hydration, and cytoprotection against proteases, pH extremes, chemical irritants and biological agents. The molecules responsible for the rheological properties of mucus are mucin glycoproteins produced from cells in the epithelial surface layer and glands from the underlying sub-mucosa. These extremely large macromolecules are characterised by a very high molecular mass containing >70% of their dry weight as carbohydrate in the form of O-linked oligosaccharides linked to serine and threonine residues in the mucin peptide.

So far a family of mucin genes (the MUC genes) coding for at least 9 distinct members has been reported (8-12). These mucin genes are located on several chromosomes (9,10,13). Interestingly, there is a cluster on 11p15.5 in the sequence telomere HRAS-MUC6-MUC2-MUC5AC-MUC5B-IGF2 centromere (14). MUC1, MUC2, MUC3 and MUC4 are commonly expressed in the colon, with MUC2 representing the major gel-forming secretory component (15-20).

Previously, we described an approach that allows the study of the total population of MUC2 molecules, both those inside the cell and those secreted into the culture medium from a colonic cell line PC/AA, using two MUC2-

specific antibody probes (4). In the present work, employing the same procedure for several colonic cell lines representing different stages of colorectal adenoma-carcinoma sequence, we followed the expression and secretion of MUC2 during the conversion from adenoma to malignant transformants.

EXPERIMENTAL

Materials

Guanidinium chloride (GuHCl), goat anti-rabbit IgG horseradish peroxidase conjugate, goat anti-mouse IgM horseradish peroxidase and alkaline phosphatase conjugate were purchased from the Sigma Chemical Co (Poole, Dorset UK). Tween 20 and CsCl were from BDH Ltd (Dagenham, Essex. UK). A stock solution of GuHCl was treated with charcoal before use. Agarose UltraPURE (electrophoresis grade) was from GIBCOBRL (Paisley, Scotland). The enhanced chemiluminescence (ECL) Western detection kit was from Amersham International Plc (Buckinghamshire, UK).

Methods

Cell culture and collection of mucins

The PC/AA adenoma cell line was derived from a single, large, colonic tubular adenoma 3-4 cm in diameter that exhibited only mild dysplasia. The cells were continuously passaged in vitro at 37°C in 5% CO2 in air and 3T3 feeder cells in collagen type IV-coated T25 flasks. The PC/AA cells at passage 8 used as a benign step for the adenoma-carcinoma sequences in this study are non-tumourogenic in nude mice, and have ultrastructural characteristics of colonic cells (1). Isolation of the clonogenic variant, designated AA/C1, and the malignant phenotype AA/C1/SB10 is schematically represented in Figure 1 (1-3).

The cell layers holding the same numbers of cells (2 x 10^7) were solubilised with 6M GuHCl containing 5mM EDTA, 10mM Benzamidine, 5mM N-ethylmaleimide, 0.1 mg/ml soy bean trypsin inhibitor, and 1mM PMSF proteinase inhibitors, and the media were mixed with an equal volume of 6M GuHCl.

Preparation of reduced mucins

Reduced mucins were prepared following dialysis of the whole mucins into GuHCl reduction buffer (6M GuHCl/0.1M Tris/5mM EDTA, pH 8.0) and then treated with 10mM DTT for 5h at 37°C. Iodoacetamide was added to a final concentration of 25mM and the mixture left in the dark overnight at room temperature. Alternatively mucins were reduced on nitrocellulose membranes after slot or Western blotting. In brief, the blotted membrane was washed in distilled water for a few minutes and incubated in urea or GuHCl reduction buffer containing 10mM DTT at room temperature for 15 minutes. After removing the DTT solution, the membrane was incubated in the same buffer containing 25mM iodoacetamide at room temperature for 10 minutes and then washed twice (5min) with distilled water.

Antibodies

Monoclonal antibody 4F1 (IgM ascites, 1:1000) was a kind gift from Dr. M. McGuckin (University of Queensland, Australia). This antibody was raised to a synthetic peptide corresponding to a single repeat of the MUC2 tandem repeat (TR) and recognises two different sites, TPTP and PTTT (21). These epitopes would be expected to be covered by glycans in the mature MUC2 mucins; thus, this may only be a suitable probe for the polypeptide. Polyclonal antiserum LUM2-3 was a kind gift from Dr. I. Carlstedt (University of Lund, Sweden). This antiserum was raised against a synthetic peptide NGLQPVRVEDPDGC in the non-TR of the molecule towards the C-terminus (22). When using LUM2-3 for probing slot or Western blots of unreduced mucins, the molecules are reduced on the membrane prior to incubation with the antiserum, because its activity is dramatically enhanced by reduction of disulphide bonds, indicating that these epitopes are buried within tertiary structural elements of the molecule. Therefore, this antiserum is an effective probe for the reduced MUC2 subunit regardless of its state of O-glycosylation.

Isopycnic density-gradient centrifugation

The cell layer extracts and media were centrifuged at a starting density of 1.40 g/ml in 4M GuHCl/CsCl using a Beckman Ti70.1 rotor at 40 000 rpm for 68h at 15°C. Centrifuge tubes (13 ml) were then emptied manually, from the top, into sixteen 800 µl fractions. Fractions were analysed by slot- and Western-blotting after agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed in 1% (w/v) agarose gels (4). After electrophoresis, molecules were transferred to nitrocellulose membrane by vacuum blotting in 0.6M sodium chloride/ 0.06M sodium citrate using a Pharmacia LKP VacuGene XL at a suction pressure of 40mbar (4000 Pa) for 2h prior to detection of mucins using antibodies.

Concentration of mucin containing samples

Dilute samples were concentrated by the following three methods:

a) They were dialysed into 6M urea containing 1-5% PEG (polyethylene glycol powder) until the required reduction in volume was reached.

b) They were sealed in a dialysis bag and buried under Aquacide II powder until the required reduction in volume was reached.

c) They were put into a small centricon concentrator and centrifuged at 3,000 g until the required reduction in volume was reached.

Results

It has previously been demonstrated that the benign colonic adenoma cell line PC/AA can be converted in vitro to adenocarcinoma (Figure 1) (1-3). The levels of MUC2 expression, at the protein level, in this adenoma-adenocarcinoma sequence were compared by Western blotting after agarose gel electrophoresis.

The cell layer extracts (Figs. 2a,b,c) and media (data not shown) that we selected from this sequence were subjected to agarose gel electrophoresis followed by Western blotting. In each experiment, the same numbers of cells (2×10^7) were analysed by using the same techniques under the same conditions. A dramatic decrease in the cell layer MUC2 mucins (precursor and mature forms) and the medium MUC2 mucins (mature forms) was detected from the benign adenoma to the premalign and malign stages of the adenoma-adenocarcinoma sequence.

The cell layer extracts and media were subjected to isopycnic density gradient centrifugation in CsCl/4M GuHCl as performed previously for the PC/AA8 cell line (4). In that study, the distribution of MUC2 glycoconjugates in the density gradient of the benign PC/AA cell layer and the corresponding medium was then assessed using the 4F1 and LUM2-3 antibodies by Western blotting after agarose gel electrophoresis. The mature MUC2 molecules extracted from both the cell layer and the medium were recovered in the density range 1.36-1.48 g/ml. However, in the low-density

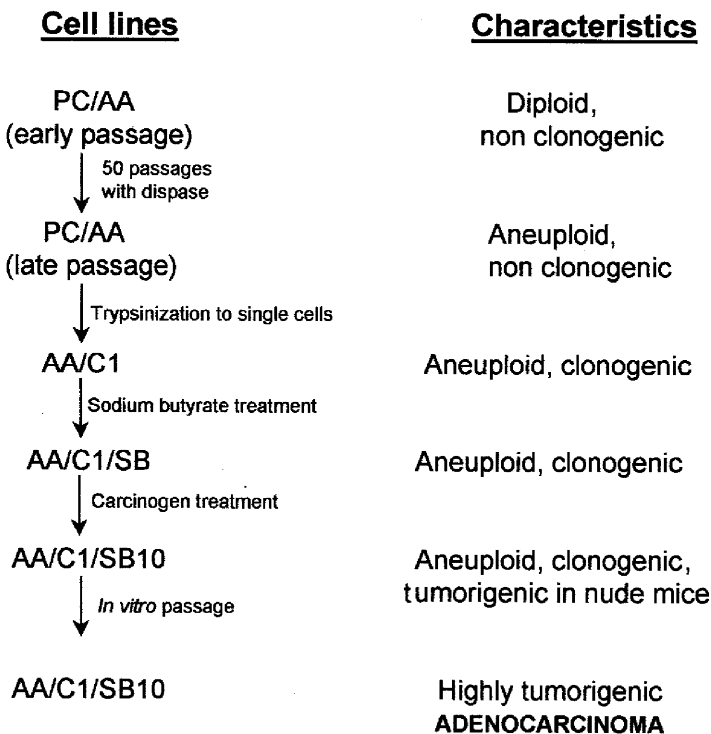

Figure 1. Schematic representation of the progression of the PC/AA8 adenoma derived cell line to the tumourogenic phenotype adenocarcinoma (AA/C1/SB10) in athymic mice (slightly adapted from Williams et. al., 1996)

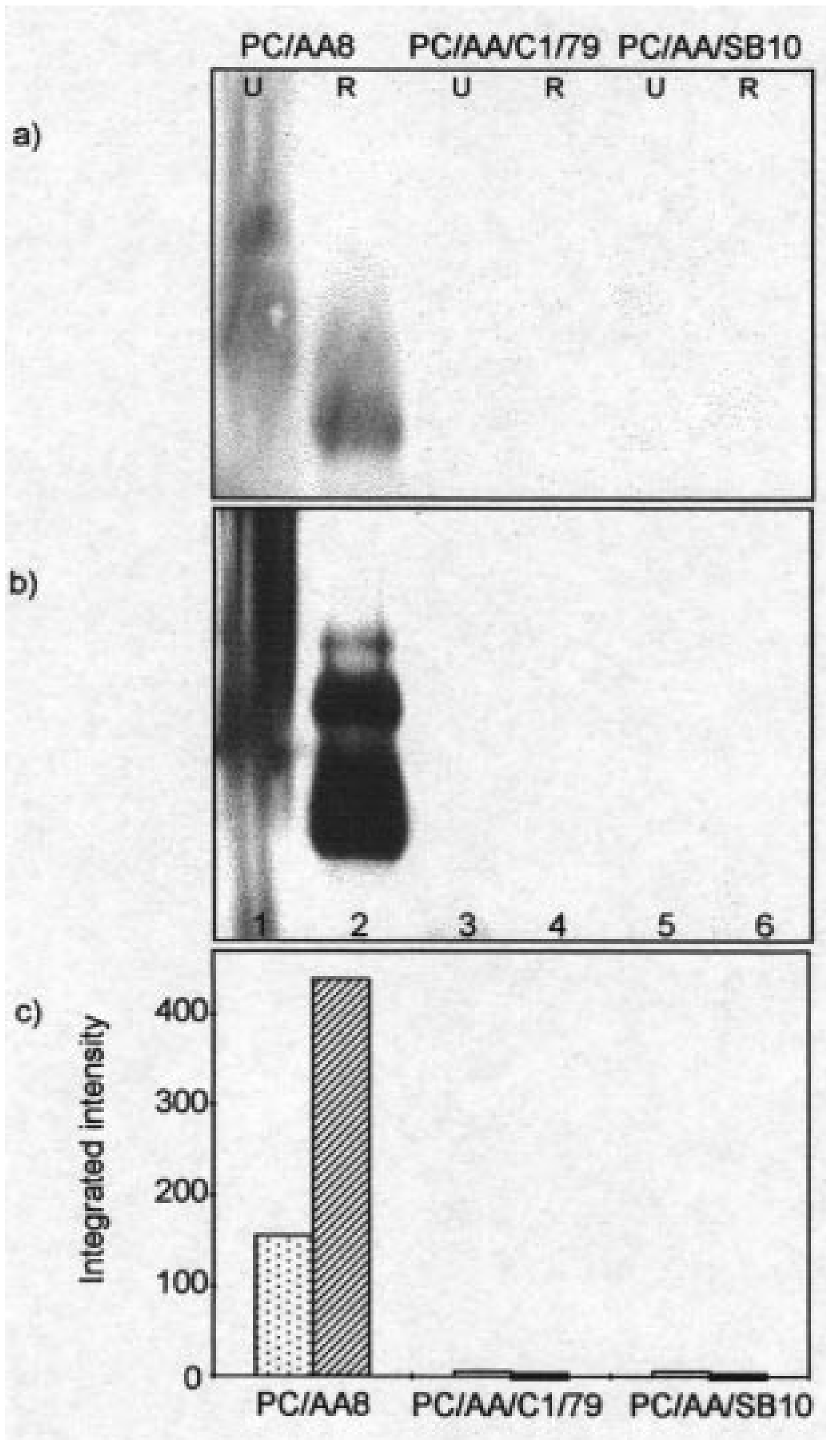

Figure 2. Comparison of the benign adenoma cell line (PC/AA8) and its premalign (PC/AA/C1/79) and adenocarcinomatous phenotypes (PC/AA/SB10) on SDS-agarose gel electrophoresis
Unreduced (U) and reduced (R) original cell layer extracts (a and b) were electrophoresed in a 1.0% agarose gel with 40mM Tris-acetate / 1mM EDTA, pH 8.0, containing 0.1% SDS and blotted onto nitrocellulose membrane prior to detection with two anti-MUC2 mucin antibodies; (a) 4F1 and (b) LUM2-3.
(c) Relative amounts of the reduced cell layer MUC2 mucins of the PC/AA8, PC/AA/C1/79 and PC/AA/SB10 cell lines: 4F1 (dotted) and LUM2-3 (hatched).

fractions of the cell layer extract we found unglycosylated and/or partially glycosylated MUC2 precursors, which were not detected in the medium (4). In this previous work, we demonstrated that the 4F1 and LUM2-3 antibodies are quite useful probes for the precursor forms of MUC2 and the mature MUC2 molecules, respectively (4). We applied the same approach and the same antibodies to the cell layers and media from the

adenoma-adenocarcinoma sequence; however, the same blotting experiments did not visualise MUC2 in density gradients of the PC/AA/C1/79 and PC/AA/SB10 cell lines as insufficient MUC2 was present (data not shown). Therefore, we decided to pool and concentrate the top fractions (1-6) that were expected to contain precursors and/or partially glycosylated forms of MUC2 and the mid-density fractions (9-12) that were expected to contain mature MUC2 molecules with one of the methods described in methods. In spite of the different concentration techniques used for different cell lines, we had quite similar results with these three methods. The concentrated samples were then analysed by Western blotting (Figs. 3a,b,c,d). Although mature MUC2 molecules from both the cell layer (Figure 3a,b, lanes 3-4) and the medium (data not shown) were detected in the fractions 9-12 of the density gradient on the benign cell line PC/AA8 by the LUM2-3 only, no mature MUC2 molecules (neither cell layer nor medium) were found in the equivalent fractions of the density gradient of the premalign PC/AA/C1 cell line (Figure 3c, lanes 3-4) and adenocarcinoma PC/AA/SB10 cell line (Figure 3d, lanes 3-

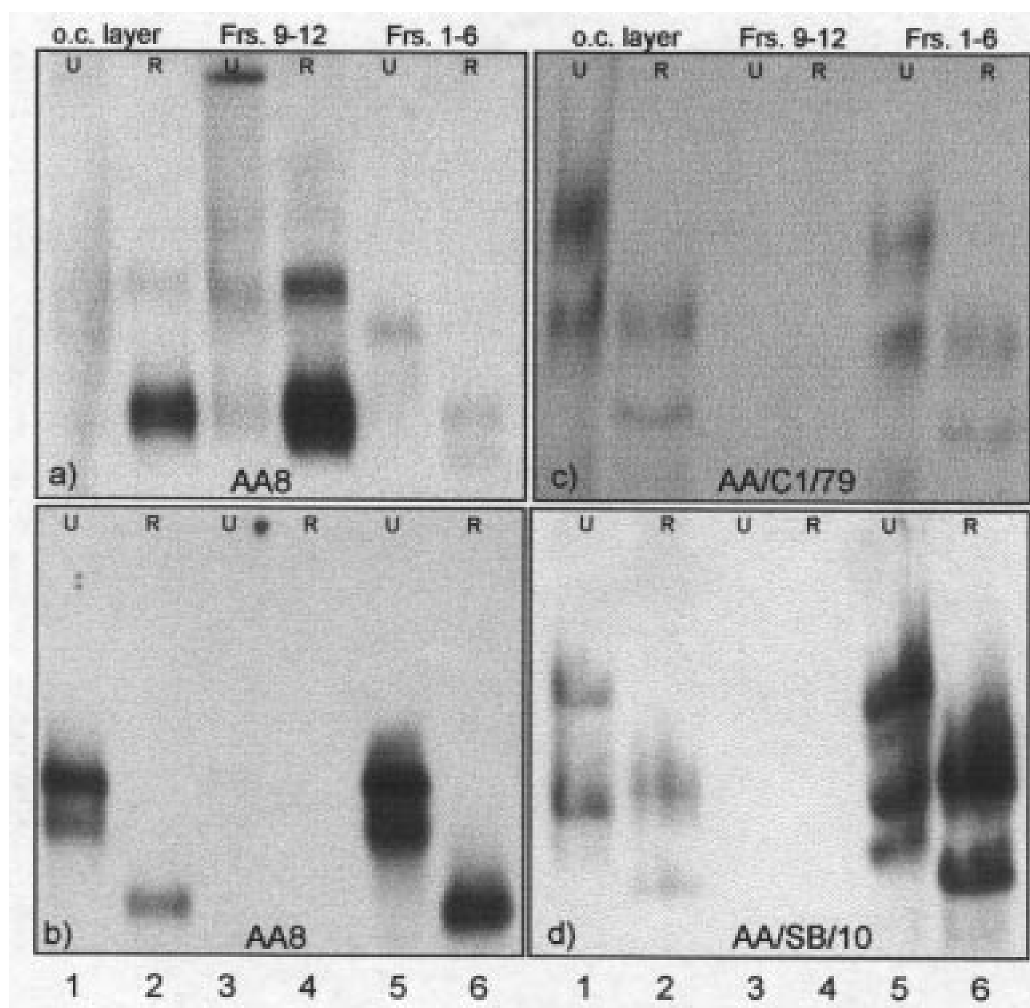

Figure 3. Comparison of the concentrated cell layers and fractions 1-6 and 9-12 from CsCl density gradient of PC/AA8, PC/AA/C1/79 and PC/AA/SB10 cell lines on SDS-agarose gel electrophoresis
Unreduced (U) and reduced (R) original cell layer extracts (o.c. layer), fractions 9-12 and fractions 1-6 taken from density gradients of the PC/AA8 (a, b), PC/AA/C1/79 (c), and PC/AA/SB10 (d) were concentrated (see the text) and subsequently electrophoresed in a 1.0% agarose gel with 40mM Tris-acetate / 1mM EDTA, pH 8.0, containing 0.1% SDS and blotted onto nitrocellulose membrane prior to detection with two anti-MUC2 mucin antibodies; LUM2-3 (a) and 4F1 (b, c, d).

4). Precursors and/or partially glycosylated MUC2 molecules with the 4F1 antibody were detected in the cell layers of benign adenoma cell line PC/AA8 (Figure 3b, lanes 5-6), premalign cell line PC/AA/C1 (Figure 3c, lanes 5-6) and adenocarcinoma cell line PC/AA/SB10 (Figure 3d, lanes 5-6). The molecules detected with 4F1 in the PC/AA/C1/79 and PC/AA/SB10 cell layers showed a double band. The slow migrating band was not found in the benign adenoma cell line. The origin of this band is unknown.

Discussion

Alterations of the MUC2 expression occurring during malignant transformation have been demonstrated in colorectal adenocarcinomas at the mRNA level in colonic tissues (4,6,7,16,19,20). It is not known, however, at which step of the transformation process these alterations become detectable (adenoma vs. carcinoma) and how they are related to the morphogenesis of colorectal cancer.

The early passages (8-20) of the intestinal adenoma PC/AA cell line make copious quantities of mucins which are both stored within and secreted by the cells (4,15,18,20). We have previously identified the major mucin synthesised by this cell line as the product of the MUC2 gene (4,18). It has been established that this cell line can be converted to the adenocarcinoma cell line in vitro (1-3). Using the approach developed for the identification of precursor and mature forms of MUC2 mucin from the early passages of the PC/AA cell line (4), we followed all of the biosynthetic intermediates of MUC2 through the adenoma-adenocarcinoma sequence to investigate possible carcinoma-related alterations at the protein/glycoprotein level during malignant transformation.

Initially, the levels of MUC2 expression, at the protein level, were compared by Western blotting after agarose gel electrophoresis using the two MUC2-specific antibodies 4F1 and LUM2-3, among the several colonic cell lines representing different stages of colorectal adenocarcinoma. The relative amounts of MUC2 indicated that the expression and secretion of the MUC2 significantly decreased from adenoma to adenocarcinoma. This result was in agreement with the previously established pattern of expression of MUC2 at the mRNA level in colonic tissues (6,23,24).

The distribution of unglycosylated and/or partially glycosylated MUC2 precursors and mature forms were investigated by the combination of density gradient centrifugation and Western blotting after agarose gel electrophoresis. This combined approach was previously established to define all biosynthetic intermediates of MUC2 using the PC/AA8 benign adenoma cell line (4). Comparison of the parental cell line PC/AA8 with the premalignant adenoma cell line PC/AA/C1/79 and adenocarcinoma cell line PC/AA/SB10 showed insignificant quantities of MUC2 gene products. These products represented precursors and/or partially glycosylated MUC2 molecules and were present only in the cell layers. They were detected with 4F1 and were not reactive with LUM2-3, indicating an abnormal post translational metabolism for MUC2. As they are not found as stored matured mucin, and are not secreted into the medium, we propose that these immature MUC2 molecules are being turned over within the cell and are not secreted into the medium due to defective processing.

Aberrant glycosylation may account in part for the abnormal pattern of MUC2 molecules found here. Others have described cancer associated mucins with fewer and shorter oligosaccharides for each glycosylation site (16,25,26). One mechanism by which this could arise is through the down regulation (abolishment) of key glycosyltransferases involved in the several maturation steps necessary to produce the normally posttranslationally modified MUC2 apoprotein. It has been shown that this is the case for cell lines including the malignant adenocarcinoma phenotype (PC/AA/SB10) (25). The loss of several glycotransyltransferase activities, including the core 3 and core 4 enzymes was identified by Corfield et al. in the same adenoma-adenocarcinoma sequence (16). This resulted in a switch to core 1 and 2 structures, giving rise to a cancer-related oligosaccharide pattern (16,25). The relationship of the alteration in mucin apoprotein and glycosylation patterns during colorectal cancer has not been assessed and is a further mechanism giving rise to abnormal carbohydrate patterns in cancer mucins (11,19).

This work demonstrates that using a cell culture model of the adenoma-carcinoma sequence shows the significantly altered expression and secretion of the major colonic mucin MUC2 in human colorectal adenoma cells during progression to cancer. The characterisation of differently glycosylated forms of the MUC2 is novel and unexpected. The implication of defective processes in the post translational modification/processing and secretion of MUC2 opens a new field in cancer mucin biology.

References

1. Paraskeva C, Buckle BG, Sheer D, Wigley CB. The isolation and characterisation of colorectal epithelial cell lines at different stages in malignant transformation from familial polyposis coli patients. Int. J. Cancer 34: 49-56, 1984.
2. Paraskeva C, Hague A, Rooney N, Williams AC, Harper SJ, Hanlon KA, Atkinson RJ, Corfield AP. A single human colonic adenoma cell line can be converted in vitro to both a colorectal adenocarcinoma and a mucinous carcinoma. Int. J. Cancer 51: 661-4, 1992.
3. Williams AC, Harper SJ, Paraskeva C. Neoplastic transformation of a human colonic epithelial cell line: in vitro evidence for the adenoma to carcinoma sequence. Cancer Res 50: 4724-30, 1990.
4. Aksoy N, Thornton DJ, Corfield AP, Paraskeva C, Sheehan JK. A study of the intracellular and secreted forms of the MUC2 mucin from the PC/AA intestinal cell line. Glycobiology. 9(7): 739-46, 1999.
5. Fearon ER, Vogelstein B. A genetic model for colorectal tumourigenesis. Cell. 61: 759-67, 1990.
6. Blank M, Klussmann E, Krüger-Krasagakes S, Schmitt-Gräff A, Stolte M, Bornhoeft G, Stein H, Xing P, McKenzie IFC, Verstijnen CPHJ, Riecken EO, Hanski C. Expression of MUC2-mucin in colorectal adenomas and carcinomas of different histological types. Int. J. Cancer. 59: 301-6, 1994.
7. Hanski C, Hanski ML, Zimmer T, Ogorek D, Devine P, Riecken EO. Characterisation of the major sialyl-Le^x -positive mucins present in colon, colon carcinoma, and sera of patients with colorectal cancer. Cancer Res. 55: 928-33, 1995.
8. Gum JR, Hicks JC, Toribara NV, Lamport DTA, Kim YS. Molecular cloning of human intestinal mucin cDNAs. Sequence analysis and evidence for genetic polymorphism. J. Biol. Chem. 264: 6480-87, 1989.
9. Gum JR. Mucin genes and the proteins they encode: Structure diversity and regulation. Am. J. Respir. Cell Mol. Biol. 7: 557-64, 1992
10. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EL, Wilson D. Molecular cloning and expression of human tumour-associated polymorphic epithelial mucin. J. Biol. Chem. 265(25): 15286-93, 1990.
11. Velcich A, Palumbo L, Selleri L, Evans G, Augenlicht L. Organisation and Regulatory aspects of the human intestinal mucin gene (MUC2) locus. J. Biol. Chem. 272(12): 7968-76, 1997.
12. Desseyn JL, Aubert JP, Van Seuningen I, Porchet N, Laine N. Genomic organisation of the 3' region of the human mucin gene MUC5B. J. Biol. Chem. 272: 16873-83, 1997.
13. Verma M, Davidson EA. Mucin genes: structure, expression and regulation. Glycoconjugate J. 11: 172-9, 1994.
14. Pigny P, Guyonet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, Collyn D'Hooge M, Laine A, Van-Seuningen I, Degand P, Gum JR, Swallow DM, Aupert JP, Porchet N. Human mucin genes assigned to 11p15.5: Identification and organisation of a cluster of genes. Genomics 38: 340-52, 1996.
15. Corfield AP, Clamp JR, Casey AD, Paraskeva C. Characterisation of a sialic-acid-rich mucus glycoprotein secreted by a premalignant human colorectal adenoma cell line. Int. J. Cancer. 46: 1059-65, 1990.
16. Corfield AP, Myerscough N, Gough M, Brouckhausen I, Schauer R, Paraskeva C. Glycosylation patterns of mucins in colonic disease. Biochem. Soc. Trans., 23: 840-5, 1995.
17. Tytgat KMAJ, Büller HA, Opdam FJM, Kim YS, Einerhand AWC, Dekker J. Biosynthesis of rat MUC2 in colon and its analogy with human MUC2. Gastroenterology. 107: 1352-63, 1994.
18. Sheehan JK, Thornton DJ, Howard M, Carlstedt I, Corfield AP, Paraskeva C. Biosynthesis of MUC2 mucin: evidence for a slow assembly of fully glycosylated units. Biochem. J. 315: 1055-60, 1996.
19. Ho SB, Ewing SL, Montgomery CK, Kim YS. Altered mucin core peptide immunoreactivity in the colon polyp-carcinoma sequence. Oncol. Res. 8(2): 53-61, 1996.
20. Aksoy N. Mucin may be used as tumour markers. The New J. Med. 14, 1(Supp.): 34-8, 1997
21. McGuckin MA, Devine PL, Ward BG. Early steps in the biosynthesis of MUC2 epithelial mucin in colon cancer cells. Biochem. Cell Biol. 74: 87-93, 1996.
22. Carlstedt I, Herrmann A, Hovenberg H, Lindell G, Nordman H, Wickstrom C, Davies JR. 'Soluable' and 'Insoluable' mucins-Identification of distinct populations. Biochem. Soc. Trans. 23: 845-51, 1995.
23. Chang S, Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS. Localisation of mucin (MUC2 and MUC3) messenger RNA and peptide expression in normal human intestine and colon cancer. Gastroenterology. 107: 28-36, 1994.
24. Weiss AA, Babyatsky MW, Ogata S, Chen A, Itzkowitz SH. Expression of MUC2 and MUC3 mRNA in normal, malignant and inflammatory intestinal tissues. J. Histochem. Cytochem. 44: 1161-6, 1996.
25. Vavasseur F, Dole K, Yang J, Matta KL, Myerscough N, Corfield AP, Paraskeva C, Brockhausen I. O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. Eur. J. Biochem. 222: 415-24, 1994.
26. Boland CR, Deshmukh GD. The carbohydrate composition of mucin in colonic cancer. Gastroenterology. 98: 1170-7, 1990.