Characterisation of MUC2 and MUC5AC mucins from a colorectal adenoma-mucinous carcinoma sequence: in vitro evidence for the neotransformation

MUC2 ve MUC5AC müsinlerinin bir kolorektal adenoma-müsinöz karsinoma sekansından karakterize edilmesi: neotransformasyon için in vitro bir bulgu

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Abstract

In this study we characterised colonic MUC2 and MUC5AC mucins from an in vitro adenoma-mucinous carcinoma sequence and tried to find out carcinoma-associated alterations occurring during the colorectal tumourogenesis. Using anti-MUC2 and MUC5AC polypeptide antibodies we detected a significant increase in the intracellular and secreted mucin molecules from adenoma to mucinous carcinoma stage of the colorectal cancer. Using the approach that we established previuosly approach to characterise mature mucin molecules and their precursor forms (1), we compared the different forms of MUC2 and MUC5AC between the benign (PC/AA8) and malign (PC/AA94) colonic cells. In the comparison, beside the increased levels of expression and secretion of the mucins, we detected some aberrant glycosylated MUC2 molecules in mucinous carcinoma cell line. Agarose gel electrophoretic analysis of the low-density fractions indicated that these molecules are more charged than precursors, however, they are smaller and/or less glycosylated than mature MUC2 molecules. We concluded that these aberrant glycosylated and/or novel MUC2 molecules detected in the mucinous carcinoma cell line may be used as a potential marker for the diagnosis and following of colorectal carcinoma.

Özet

Bu çalışmamızda MUC2 ve MUC5AC müsinlerini in vitro bir adenoma-müsinöz karsinoma sekansından karakterize edip, kolorektal tümorogenezis esnasında vukubulan karsinoma ile alakalı değişiklikleri ortaya çıkarmaya çalıştık. MUC2 ve MUC5AC antibadilerini kullanarak kolorektal kanserin adenoma devresinden müsinöz karsinoma devresine kadar intrasellüler ve sekrete edilen müsin moleküllerinde anlamlı bir artış tespit ettik. Daha önce müsin moleküllerini ve onların öncüllerini karakterize etmek için tayin ettiğimiz bir yaklaşımı kullanarak (1), MUC2 ve MUC5AC'nın farklı formlarını benign (PC/AA8) ve malign (PC/AA94) kolonik hücreler arasında mukayese ettik. Bu mukayesede müsinlerin artmış ekspresyon ve sekresyonları yanında müsinöz karsinoma hücrelerinde bazı aberrant glikolizlenmiş MUC2 molekülleri tespit ettik. Düşük dansiteli fraksiyonların agaroz jel elektrophoretik analizleri bu moleküllerin prekürsörlerden daha fazla yüklü, fakat matür MUC2 moleküllerinden daha küçük ve az glikolizlendiğini gösterdi. Müsinöz karsinomada tespit edilen bu aberrant glikolizlenmiş yeni MUC2 moleküllerinin kolorektal karsinomanın teşhis ve takip edilmesinde potansiyel bir belirleyici olarak kullanılabileceği sonucuna vardık.

INTRODUCTION

Colorectal cancer is one of the most common forms of malignant diseases. At least 50% of the Western population develops a colorectal tumour by the age of 70, and in about 1 in 10 of these individuals, progression to malignancy ensues. The most common malignant tumour of the colorectum is

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adenocarcinoma and the second is mucinous carcinoma (2-5). Although both of these secrete variable amounts of mucin which is the major secreted product of the colon, mucinous carcinoma is defined on the basis of the amount of the mucus component in the tumour mass. Many studies have shown that patients with mucinous carcinomas have a poorer prognosis than those with adenocarcinomas (2-5), however, it is still not clear whether the excessive mucin production makes the prognosis of the carcinoma worse and if so by which mechanisms.

Mucins are high-M_r and heavily O-glycosylated macromolecules found on the luminal side of the epithelial surfaces; for instance in the mouth, respiratory tract, gastrointestinal tract, urogenital tract and corneal surface. They are synthesised, stored and secreted from cells in the epithelial surface layer and glands from the underlying sub-mucosa. Mucins have been identified in both cell surface attached and fully released forms. A sub-family of the latter mucins, termed gel-forming mucins, are oligomeric, high M_r thread-like structures assembled from a variable number of subunits (2-3 x 10^6 Da) via the agency of disulphide bonds. These glycoproteins are the major macromolecular components of mucus and to date the MUC2, MUC5AC and MUC5B mucins have been demonstrated to be members of this family (1, 6-12).

The early passages of the intestinal PC/AA cell line makes copious quantities of mucins which are both stored within and secreted by the cells (13-15). We have previously identified the major mucin synthesised by this cell line as the product of the MUC2 gene (1,8,9,16). Furthermore we described an approach that allows the study of the total population of MUC2 molecules. In the present study we examined the expression and secretion of MUC2 and MUC5AC using anti-MUC2 and MUC5AC antibodies in a colonic adenoma-mucinous carcinoma sequence.

MATERIALS AND METHODS

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). Stock solution of guanidinium chloride 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 of 3-4 cm diameter that exhibited only mild dysplasia. The cells were continuously passaged in vitro at 37°C in 5% CO_2 in air and 3T3 feeder cells on 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-tumorogenic in nude mice, have an ultrastructural characteristics of colonic cells (17). If this cell line is passaged continuously it can be converted to its malignant phenotype PC/AA94 mucinous carcinoma cell line spontenously (17-20). The cell layers were solubilised with 6M GuHCl containing proteinase inhibitors and the media were mixed with an equal volume of 6M GuHCl.

Antibodies

The following antisera were used in this study; a 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, 22). The anti-MUC2 (LUM2-3) antiserum was raised against the hapten-linked synthetic peptide, NGLQPVRVEDPDGC present in the non-TR of the molecule towards the C-terminus (7). When using LUM2-3 for probing slot- or Western blots of unreduced mucins the molecules are reduced on the membrane (as described below) 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. MUC5AC (LUM5-1) antisera was raised against the keyhole limpet haemocyanin linked synthetic peptide, RNQDQQGPFKMC, present in a low glycosylation, C-terminal domain and in two domains flanking a tandem repeat region of MUC5AC (7). This antibody works on both whole and reduced MUC5AC molecules.

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 treatment 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.

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. After centrifuge tubes were emptied from the top and analysed by slot- and Western-blotting after agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed in 1% (w/v) agarose gels as described previously (1). 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.

RESULTS

A preliminary comparative electrophoretic study was undertaken between the cell layers of benign adenoma (PC/AA8) and its malignant phenotype (PC/AA94) cells. A significant increase was detected in the relative levels of MUC2 mucins of the mucinous carcinoma cell line compared to adenoma cell line lines (Fig. 1a,b). An increase was also detected in the relative levels of MUC5AC mucins of the mucinous carcinoma cell line compared to adenoma cell line lines, however, it was not valuable compared to the increase of the MUC2 mucin (Fig. 1a,b). The relative amounts of MUC2 and MUC5AC shown in Figure 1b were obtained by measuring the intensity

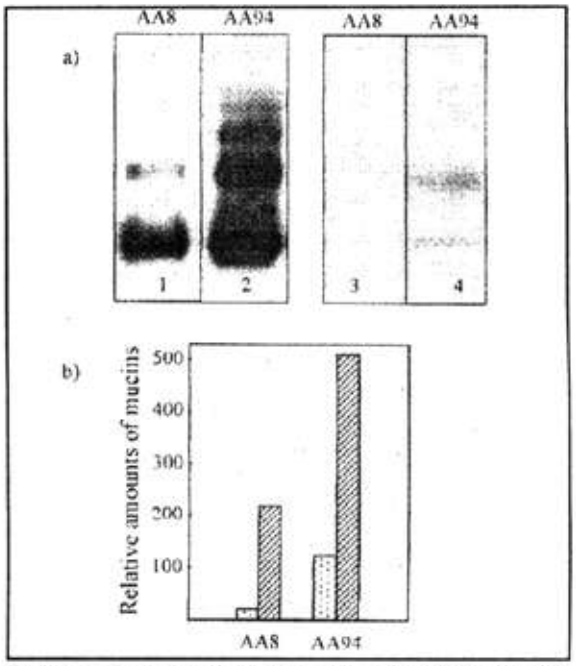

Figure 1. Comparison of the benign adenoma cell line PC/AA8 and its mucinous carcinomatous phenotype PC/AA94 by DS-Agarose gel electrophoresis

Reduced (R) layel extracts (a) were electrophoresed in a 1% agarose gel with 40 mM Trisacetate/1mM EDTA, pH 8.0, containing 0.1%SDS and blotted onto nitrocellulose membrane prior to detection with LUM2-3 (lanes 1-2) and LUM5-1 (lanes 3-4) antibodies. (b) Relative amounts of the reduced cell layer MUC2 and MUC5AC mucins of the AA8 and AA94 cell lines were obtained by measuring the densities of the LUM5-1 (□) and LUM2-3 (▨) stained bands.

of the LUM2-3 and LUM5-1 stained reduced mucin subunit bands.

The cell layer extract and corresponding medium of the mucinous carcinoma cell line PC/AA94 were subjected to isopycnic density gradient centrifugation in CsCl/4M GuHCl followed by agarose gel electrophoresis like we did for the PC/AA8 benign adenoma cell line previously (1).

The distribution of glycoconjugates in the density gradient was investigated after slot blotting using the LUM2-3 and LUM5-1 antibodies (Fig. 2a,b). Mature MUC2 and MUC5AC molecules were identified at densities 1.36-1.50 g/ml similar to those found in the PC/AA8 cell line (1). As we detected in the first comparison above a significant increase in the expression and secretion of MUC2 mucins was found after density gradient centrifugation. For further investigation of these extremely increased MUC2 molecules we previously used a monoclonal MUC2 antibody which was established as a specific probe

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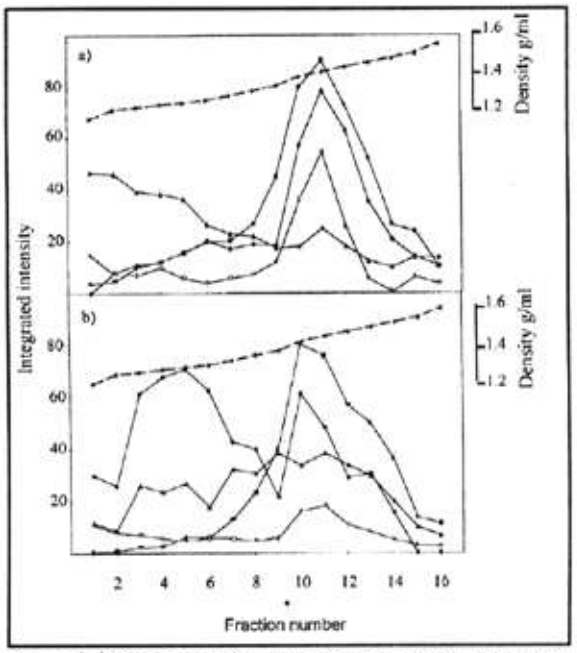

Figure 2. Density gradient centrifugation on the PC/AA94 cell layer(a) extract and medium (b).
The cell layer was solubilised in 6 M GuHCl containing proteinase inhibitors and the corresponding medium was collected and mixed with the same buffer. The molarity of GuHCl was brought to 4 M and thereafter subjected to isopycnic density gradient centrifugation in CsCl/4 M GuHCl, in a Beckman Ti70.1 rotor at 40.000 rpm at a denstiy of 1.40 g/ml for 68h at 15°C. After centrifugation the tubes were emptied from the top and fractions were analysed for PAS (filled diamonds), and reactivity with the MUC5AC antibody LUM5-1 (open circles) and two MUC2 antibodies LUM2-3 (filled squares) and 4F1 (filled triangles). The density of each fraction (dashed line) was determined by weighing.

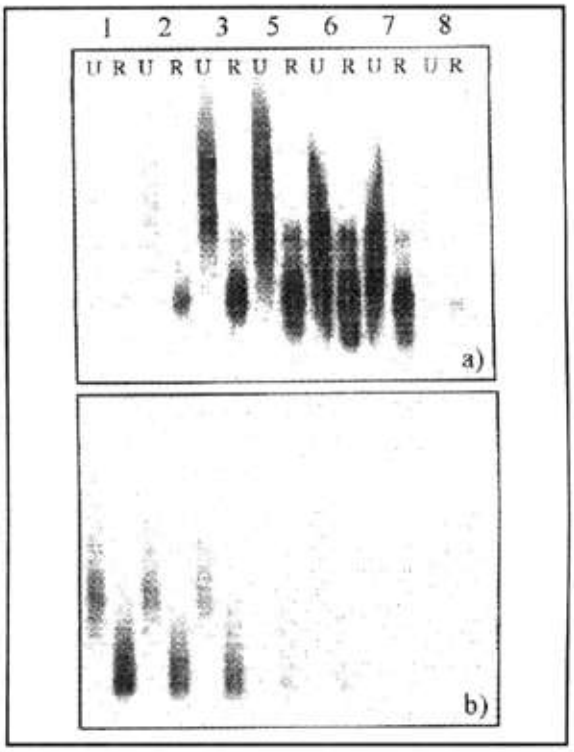

Figure 3. Comparison of the first fractions (1-8) of density gradients of the medium (a) and cell layer (b) of the mucinous carcinoma cell line PC/AA94 on SDS-agarose gel electrophoresis
The first 8 fractions of density gradients in the unreduced (U) and reduced (R) forms were electrophoresed in 40mM Tris-acetate/1mM EDTA, pH 8.0, containing 0.1% Agarose gel and blotted onto nitrocellulose membrane prior to detection with the 4F1 antibody.

for the MUC2 precursors (1). In particular, at the top of the density gradient of the cell layer, where we expect to find mucin precursors, a putative amount of 4F1-reactive molescules were detected .As predicted in the case of PC/AA8 cell line these molecules were not observed in the medium of the PC/AA94. However, in the fractions 3-6 of the density gradient of the PC/AA94 medium a significant amounts of 4F1 reactivity were found as compared with similar fractions of PC/AA8 (1). To analyse these ënovel' molecules Western blotting after agarose gel electophoresis was performed (Fig. 3a). These molecules were not found in the similar fractions of the cell layer either (Fig. 3b), and they had lower density than mature MUC2 molecules but higher than precursors.

DISCUSSION

Some recent studies have been focused on mucins as tumour markers (2-5, 16, 22-29). It is known that these glycoproteins have some TACA, for example sialyl-Le^a (CA 19-9), GalNAc-O-Ser/Thr (Tn antigen), Sialyl-Tn antigen, Galβ1, 3GαlNAca-O-Ser/Thr (Thomsen-Friedenreich antigen, also known as T antigen) and Sialyl-T antigen (23-25). Little information is available about carcinoma associated alterations on the mucins having a high immunogenicity. Therefore, if alterations of the mucin peptide and/or carbohydrate epitopes could be associated with tumour development, using them as specific tumour markers it may be possible to detect colorectal cancers very early, even before neotransformation in the benign adenoma stage, and to prevent the development of carcinoma.

It has been shown that over-expression or ectopic expression of MUC2 is the common property of mucinous carcinomas of the colon, pancreas, breast, and ovary (5). However, no specific mucin molecules have been characterised until today. This is the first study to investigate the total population of mucin molecules, from protein precursors through to fully mature mucins, produced by mucin-secreting aden-

oma and mucinous carcinoma cells at the protein/ glycoprotein level. Previously, we described an approach that allows the study of the total population of intracellular MUC2 molecules from a colonic benign adenoma cell line PC/AA using two MUC2-specific antibody probes (1). It has been established that this cell line can be converted to mucinous carcinoma cell lines in vitro (17-20). Using the approach developed for the PC/AA8 cell line we followed the MUC2 and MUC5AC mucin molecules, which are known as two predominant intestinal mucins, through the adenoma-mucinous carcinoma sequence to investigate possible carcinoma-related alterations at the protein/ glycoprotein level during the malignant transformation. Our initial comparative study using SDS-agarose gel electrophoresis indicated that there is a remarkable increase in both precursor and mature forms of MUC2and MUC5AC from benign adenoma to mucinous carcinoma during the colorectal carcinogenesis. These are consistent with those frequently observed in in vivo carcinogenesis (30).

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 (1). The results of the experiments on the mucinous carcinoma cell line PC/AA94 suggested that like the PC/AA8 benign adenoma cell line the PC/AA94 is making, storing and secreting MUC2 in particular. However, electrophoretic analysis of the fractions taken from density gradient showed the presence of some novel MUC2 species in the medium of mucinous carcinoma cell line. These molecules were not fully glycosylated, since they had lower density than mature MUC2 molecules and were more glycosylated than precursor forms since they had higher density than unglycosylated and/or partially glycosylated MUC2 precursors. Moreover, they have not been found in the cell layer, it may be assumed that after being synthesised they are secreted to the medium directly.

The assumption that altered glycosylation of MUC2 molecules is associated with the colorectal carcinogenesis is supported by others who have shown that MUC2 mucin molecules have modified glycosylation in colorectal adenocarcinoma as compared to the normal colon (31-36). 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 (22,31-33). One mechanism by which this could arise is by the down regulation or relation of key glycosyltransferases leading to a different population of oligosaccharides. There is no data available for mucins from the mucinous carcinoma cell lines or for surgical cases of mucinous carcinoma. The relationship of alteration in mucin apoprotein alteration and glycosylation patterns during colorectal cancer has not been accessed and is a further mechanism giving rise to abnormal carbohydrate patterns in cancer mucins (33-36).

In conclusion, the expression of colonic MUC2 and MUC5AC mucins increased from adenoma to mucinous carcinoma. These are consistent with those frequently observed in in vivo carcinogenesis (30). We have shown here the presence of some new MUC2 molecules in a mucinous carcinoma cell line at the protein level. The identification of unusual partially glycosylated forms of the major colonic mucin MUC2 is novel and unexpected. Implication of defective processes in the post translational modification/ processing of MUC2 opens a new field in the cancer mucin biology.

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