Detection of a mucin marker for the adenoma-carcinoma sequence in human colonic mucosa by monoclonal antibody AM-3

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Abstract

The monoclonal antibody AM-3 was raised against mucins extracted from human colorectal carcinomas. It reacted strongly with sections of paraffin wax embedded colorectal carcinoma. In colonic adenoma tissue the percentage of cells expressing the epitope detected by AM-3 correlated with the degree of dysplasia. In contrast to immunohistochemical staining, which did not show the presence of the antigen in histologically normal mucosa, the more sensitive enzyme linked immunosorbent assay (ELISA) and immunoblot assays showed that it was weakly expressed in this tissue. AM-3 reacted with variable frequency with several normal and malignant human tissues, indicating that the detected epitope is not restricted to colonic tissue. In colonic carcinomas it is present on a sialomucin of apparent relative molecular mass of more than 440 000. These data suggest that the antigen detectable with AM-3 may be useful in the assessment of premalignant changes in colonic adenomas.

The generally accepted concept of the adenoma-carcinoma sequence is that most colorectal carcinomas arise from adenomas. Most adenomas, however, never become malignant. The criteria for assessing the malignant potential of adenomas are size, the presence of a villous component, and the grade of dysplasia.¹ The most selective histopathological marker of increased cancer risk is severe dysplasia.² Because the grading of dysplasia is subjective and imprecise,³ biochemical and immunological methods have also been used. They include histochemical staining,⁴⁻⁶ lectin binding,⁷⁻⁸ and immunohistochemistry.⁹⁻¹⁰ The potential of these methods lies in the detection of early molecular changes in adenomas which may be indicative of malignant transformation. Among the molecules affected by this process are the colonic mucins. During the course of malignant transformation of colonic tissue they may undergo structural modifications, including the increased synthesis of sialomucins instead of sulphomucins, the reappearance of blood group antigens, changed accessibility of sugar moieties to lectins, as well as modifications of the peptide core. Several antigens present on mucins

associated with human colon carcinoma have recently been identified by monoclonal antibodies. Monoclonal antibody 19-9 detects the ganglioside CA 19-9 in about 60% of colonic carcinomas¹¹ and a mucin in the serum of patients with tumours.¹² The antibody B 72-3 detects the mucin TAG-72 in more than 80% of colonic carcinomas,¹³ while the mucin recognised by the antibody 1D₃ is present in 51% of colonic carcinomas.¹⁴ The anti-mucin antibody SPan-1 stains 62% of colonic carcinomas.¹⁵ None the less these antibodies were not used for assessing the early changes in the malignant process. In this study we attempted to develop an antibody which could detect early mucin changes in colonic adenomas. The monoclonal antibody AM-3 described here detects a sialomucin whose expression correlates with the grade of dysplasia of colorectal adenomas. AM-3 was extensively tested on human malignant and normal tissue sections. Furthermore, the colonic carcinoma mucin carrying the recognised epitope was partially characterised.

Methods

Two month old Balb/c mice of both sexes were used for the preparation of immunised spleen cells and the production of ascites.

MUCIN EXTRACTION

Fresh, non-neoplastic and malignant tissue from colorectal carcinomas used for mucin extraction was obtained at the time of surgery. The tumour was dissected and necrotic tissue and fat removed. Colon segments 10 cm or more from the tumour were regarded as normal. Tissue was washed with saline, dried with paper towel, and weighed. Tissue (2 g) was minced with scissors and suspended in 6 ml of extraction buffer (10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 1 mM MgCl₂, 30 mM NaCl, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl-fluoride, 0.02% NaN₃, 0.01% DNase).¹⁶ Homogenisation was carried out on ice with an Ultraturrax T25 (Janke & Kunkel, Staufen, West Germany) at the highest setting (24 000 rpm) four times for 30 seconds each with 30 second intervals. The homogenate was centrifuged for 30 minutes at 100 000 × *g* and the supernatant was decanted. The extraction procedure was repeated with the pellet and both supernatants (15 ml) were pooled. The protein concentration in the supernatant was determined according to the method of Lowry¹⁷ and the concentration of hexoses was

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determined with anthrone reagent.¹⁸ All chemicals were obtained from Sigma (Deisenhofen, West Germany). The pooled supernatants were fractionated on Sepharose 6B and the mucins, which were eluted in the void volume, were pooled and used for immunisation.

GENERATION AND SELECTION OF MONOCLONAL ANTIBODIES

Eight week old pristane primed Balb/c mice of both sexes were immunised by seven injections with a mucin fraction (50–100 µg hexoses) obtained from three human colorectal carcinomas. The fusion of spleen cells with NS-1 cells was carried out as described by Köhler and Milstein¹⁹ with 50% polyethylene glycol 1450 (Eastman, Kodak, Rochester, New York, USA) as the fusion inducing agent. The selection of clones was carried out in an ELISA on microtitre plates coated with pooled mucins extracted from normal colonic mucosa or from colonic carcinomas. For detection of bound antibodies, β galactosidase conjugated rabbit anti-mouse IgG (Amersham-Buchler, Braunschweig, West Germany) was used. Hybridomas, which secreted antibodies differentiating between mucins extracted from non-malignant colonic tissue and those from colorectal carcinoma tissue were cloned twice by limiting dilution.

DETERMINATION OF IMMUNOGLOBULIN ISOTYPE
The immunoglobulin isotype of monoclonal antibodies in the cell supernatants was determined by the immunoblot technique with the Amersham Isotyping Kit RPN 29 (Amersham-Buchler, Braunschweig, West Germany).

ELECTROPHORESIS

The electrophoretic separation of mucins was carried out in a discontinuous sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) (3–10% gradient). Molecular weight markers SDS-6H (Sigma) and murine laminin (Collaborative Research Inc, Bedford, Massachusetts, USA) were used for determination of molecular weights in the gel. Mucins were applied in sample buffer as described by Laemmli²⁰ and were boiled for five minutes before being applied.

IMMUNOBLOTTING

Blotting of the separated mucins on to nylon membrane (Millipore, Eschborn, West Germany) was carried out in a dry blot apparatus (Biometra, Göttingen, West Germany) at 1 mA/cm² for four hours. The detection was carried out according to the standard protocol of Towbin *et al*²¹ with β galactosidase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG). Slot-blotting was performed in a BioRad blotting chamber (BioRad, München, West Germany), with β galactosidase and BCIG used for detection.

EVALUATION AFTER APAAP STAINING

Biopsy specimens and resected tissue were either frozen in liquid nitrogen or fixed immediately after surgery in 4% formaldehyde

and embedded in paraffin wax. Cryostat sections or dewaxed 5 µm thick tissue sections were sequentially incubated for 30 minutes with the hybridoma supernatant, rabbit anti-mouse immunoglobulin, mouse anti-alkaline-phosphatase-alkaline-phosphatase (APAAP) complex (Dianova, Hamburg, West Germany), and naphthol AS-biphosphate (Sigma, München, West Germany) as the substrate. A preimmune mouse serum served as a negative control. For serial staining with AM-3 the cell supernatants from several flasks were pooled to a volume of about 500 ml and used for all specimens without further dilution. A deep red staining was scored as 3, red staining was scored as 2, and a pink staining as 1. Staining which covered less than 0.5% of the whole area investigated was scored as negative. The percentage of stained tumour area was also estimated.

Dysplasia was graded according to the following criteria: (i) change in the structure of cells, particularly the cytoplasm : nucleus ratio; (ii) changes in cell polarity; (iii) structural disorganisation of the tissue. It was classified according to Morson²² into mild (grade I), moderate (grade II), and severe (grade III). After staining, the percentage of cells displaying each degree of dysplasia and the fraction of positive cells in each of the dysplastic areas were assessed. All histological and immunohistochemical determinations were carried out by at least two independent investigators. The median values of the results, which never differed by more than 10%, were used for plotting. The commercial anti-CEA and anti-CA-19-9 antibodies were obtained from Histocis-Isotopendiagnostik (Dreieich, West Germany) and the anti-MAM-6 antibody from Biochrom (Berlin).

Results

PRESELECTION OF MONOCLONAL ANTIBODIES IN THE ELISA

The cell supernatants were tested on pooled mucins extracted from non-neoplastic colonic tissue and from colorectal carcinoma tissue, and the signal ratio of tumour mucin to normal mucin was determined. Finally, the monoclonal antibody AM-3 was selected, which strongly reacted with native mucins extracted from colorectal carcinomas and only weakly with mucins from non-neoplastic colonic tissue. The ratio of both signals depended on the mucin used for coating and varied between 20 and 100. Antibody AM-3 was characterised by immunoblotting with a commercial isotyping kit. AM-3 belongs to the IgM subclass with the κ light chain.

PARTIAL CHARACTERISATION OF THE EPITOPE DETECTED WITH AM-3 IN COLORECTAL CARCINOMAS

To investigate the nature of the epitope detected with AM-3 in colorectal carcinomas the mucins were fractionated on Sepharose 6B and further analysed by ELISA, electrophoresis, and immunoblotting. AM-3 reacted more strongly with fractionated mucins from colorectal carcinomas than with the corres-

Figure 1 Mucins extracted from histologically normal colonic tissue (1) or from colorectal carcinoma (2) of the same patient. To each lane mucins purified on Sepharose 6B (3 µg hexoses) were applied, analysed on SDS-PAGE (3–10% polyacrylamide gradient) blotted on to nylon membrane, and stained with AM-3. The broad band with molecular weight of more than 440 000 represents mucins detectable in colorectal carcinomas; mucins from histologically normal colonic tissue exhibit a very weak staining.

Table 1 Distribution of epitope detected with AM-3 in human malignant tissues

Tumour localisation	No of cases positive/tested	Intensity of staining	Percentage positive area
Colon and rectum	19/19	3	90-100
Colon*	1/1	1	10
Small intestine†	5/5	2-3	90-100
Oesophagus	5/6	1-2	5-25
Stomach	6/8	1-2	10-90
Breast	7/9	1-2	5-50
Pancreas	3/5	2-3	40-90
Liver	3/4	1-2	5-80
Kidney	3/4	1-2	80-90
Bladder	4/5	1-2	20-85
Lung/bronchus	4/10	1-2	25-100
Ovary	2/4	2	90-100
Prostate	2/7	1	1-5

*mucinous carcinoma
†ampullary carcinoma.

Table 2 Distribution of epitope detected by AM-3 in human non-malignant tissues

Tissue	No of cases positive/tested	Intensity of staining	Percentage of positive cells
Colon and rectum	0/10		
Small intestine	0/3		
Oesophagus			
Squamous epithelium	5/5	2	100
Submucosae	0/5		
Stomach	0/6		
Breast			
Secretory material	5/5	2	100
Lobules	0/5		
Ductules	5/5	2	100
Pancreas	4/4	1	1
Liver			
Hepatocyte plasma membrane	4/4	3	100
Hepatocyte cytoplasm	4/4	1	100
Kidney			
Tubules	4/4	3	100
Glomeruli	0/4		
Lung			
Alveoli	0/4		
Mucus secreting bronchus cells	4/4	1	30
Prostate	3/3	1	1
Endometrium			
Epithelial cells and secretory material	3/4	2	50
Placenta	0/1		
Thyroid gland	0/1		
Testis	0/1		
Muscle	0/1		
Bone marrow	0/1		

ponding fractions from histologically normal colonic tissue (not shown), thus confirming the integral measurements obtained in the ELISA used for hybridoma selection. The major peak eluting in the void volume of the column contains components with relative molecular mass of greater than 10⁶. After SDS-PAGE and immunoblotting the material from the peak yielded a broad band with the apparent relative molecular mass of more than 440 000 (fig 1). The same result was obtained after mercaptoethanol in the sample buffer was omitted. The epitope detected with AM-3 was completely destroyed by neuraminidase treatment but was not sensitive to mercaptoethanol and SDS treatment nor to paraffin wax embedding. These data indicate that the sialomucins carrying the epitope eluted in the void volume of Sepharose 6B column in the form of non-covalently bound aggregates which dissociated during treatment with SDS. The denaturation and the reduction of disulphide bridges (if any) influenced neither the antigenicity of the epitope nor the apparent molecular mass of the colon carcinoma mucin. A very weak signal with the same characteristics was also obtained with mucins extracted from non-neoplastic colorectal mucosa (fig 1).

Figure 2 Intense staining of colon carcinoma tissue but not adjacent non-malignant tissue with AM-3.

Figure 3 Staining of lymph node metastases of colon carcinoma depicted in figure 4. Note intense staining of secretory material.

EXPRESSION OF THE EPIOTOPE DETECTED WITH AM-3 IN NORMAL AND MALIGNANT HUMAN TISSUES

The antibody reacted strongly with cryostat sections as well as with parallel paraffin wax embedded samples of colorectal carcinomas but not with the adjacent non-malignant tissue. The results of APAAP staining of malignant

Table 3 Analysis of colorectal adenomas according to proportion of tissue exhibiting each grade of dysplasia and percentage of stained cells in each area

Specimen number	Growth* pattern	Grade I	Positive	Grade II	Positive	Grade III	Positive
1	TV	20	0	80	70		
2	TV	90	0	10	50		
3	TV	10	0	90	35		
4	TV	40	10	60	50		
5	TV	40	10	40	20	20	80
6	T	100	5				
7	T			100	17.5		
8	TV	20	0	80	35		
9	TV	15	0	85	20		
10	TV	45	0	55	65		
11	T	30	0	65	10	5	90
12	TV	7.5	0	70	10	22.5	80
13	TV	10	0	90	7.5		
14	TV	15	0	80	20	5	90
15	V	12.5	0	82.5	10	5	80
16	TV	30	0	70	40		
17	TV	50	10	50	15		
18	TV	15	0	57.5	10	27.5	90
19	V			100	30		
20	TV	20	0	80	17.5		

*T: tubular; V: villous; TV: tubulovillous.

and non-malignant paraffin wax embedded human tissues are shown in tables 1 and 2.

Nineteen out of 20 colorectal carcinomas (well and poorly differentiated) as well as six corresponding lymph node metastases stained strongly (intensity 3, 90–100% of tumour area) (figs 2 and 3). One carcinoma of the colon (mucinous type) stained only weakly (intensity 1, 10% of tumour area) and its lymph node metastases did not stain at all, and five out of five tumours located in the small intestine and diagnosed as ampullary carcinomas were also strongly positive (intensity 2–3, 90–100% of tumour area). The staining included secretory material as well as membranes and the cytoplasm of malignant cells.

Six out of eight stomach carcinomas were positive, with 10–90% of tumour area stained. Three out of five pancreatic carcinomas

Figure 4 Expression of epitope detected with the monoclonal antibody AM-3 in adenoma areas exhibiting different grade of dysplasia.

exhibited strong expression of the antigen (intensity 2–3, 40–90% of tumour area).

Oesophageal carcinoma and liver carcinoma displayed the antigen in lower amounts (intensity 1–2) and in smaller and variable fractions of stained tumour cells (5–80%). A positive reaction was observed in four out of 10 bronchial carcinomas (intensity 1–2, 25–100% of tumour area), in seven out of nine breast carcinomas (intensity 1–2, 5–50% of tumour area), and in four out of five bladder carcinomas (intensity 1–2, 20–85% of tumour area).

The epitope detected by AM-3 was also present in several non-malignant epithelial tissues (table 2). It could be consistently detected on the plasma membrane of adult hepatocytes, in the squamous epithelium of the oesophagus, in the tubules of the kidney and in the ductules of the mammary gland. Furthermore, about 30% of the bronchial epithelial cells and 50% of the endometrial epithelium focally expressed the epitope. One per cent of normal pancreatic and prostate cells also weakly expressed the epitope. The antigen was not detectable in normal stomach, small and large bowel tissues, thyroid gland, testis, peripheral nerves, muscle and bone marrow.

Figure 5 Staining of adenomatous tissue exhibiting dysplasia grade I (a): sporadic staining of cells and secretory material; grade II (b): single cells, apical membranes, and secretory material are stained; grade III (c): predominantly secretory material is stained.

EXPRESSION OF THE ANTIGEN DETECTED WITH AM-3 IN COLORECTAL ADENOMAS

Twenty colorectal adenomas with areas exhibiting different grade of dysplasia were evaluated (table 3). The staining was very rare in the areas displaying mild dysplasia (grade I), while the areas with moderate (grade II) and severe (grade III) dysplasia were always positive. Quantitative estimation of the stained portion of each area showed that 10% or less of mildly dysplastic cells were stained. Areas of moderate dysplasia showed positive staining in 10–70% of cells, while adenoma tissue exhibiting severe dysplasia expressed the antigen in 80–90% of cells (fig 4). A positive reaction corresponding to score 3 was detectable in the secretory material, on the apical part of the epithelial layer, and frequently in the cytoplasm (fig 5).

Discussion

The purpose of this study was to identify early mucin changes associated with the malignant transformation of colonic tissue. As it is known that tumour-associated antigens may be lost in culture,²³ mucins isolated from freshly resected human colorectal carcinomas, instead of cultured cells, were used for immunising mice. This direct approach yielded the monoclonal antibody AM-3 which discerned mucins derived from normal and malignant colon tissue in an ELISA. When tested on paraffin wax embedded tissue, AM-3 recognised 100% of colorectal carcinomas, regardless of the degree of differentiation. It also detected six out of seven lymph node metastases from colorectal carcinomas. The epitope was also expressed in a number of other carcinomas as well as in the plasma membrane of normal hepatocytes, in the tubules of the kidney, and in the squamous epithelium of the oesophagus.

This distribution, as well as direct comparison with parallel tissue sections (data not shown) ruled out the crossreactivity of AM-3 with 19-9 and anti-CEA antibodies. The antigen detectable with AM-3 was also present in the ductules of the mammary gland, on the mucinous bronchial epithelial cells, and in small amounts on about 1% of cells in pancreatic and prostate tissue. Whether this crossreactivity is due to the presence of a common mucin molecule or only to a widespread epitope on different glycoproteins is currently under investigation. Of particular interest was the positive correlation of the antigen expression with the grade of dysplasia of colorectal adenomatous tissue.

Immunoblotting experiments indicate that in colorectal carcinomas AM-3 detects a denaturation- and mercaptoethanol-insensitive epitope present on a sialomucin with a relative molecular mass of more than 440 000. As the epitope in normal colonic mucosa exhibited the same properties, it is concluded that the adenoma-carcinoma sequence is accompanied by the gradual increase in expression of the detected antigen. The expression of the well characterised T antigen in adenomatous polyps is also related to polyp size, histological type, and grade of dysplasia.²⁴ The

T antigen, however, can be detected with peanut agglutinin as well as with monoclonal antibody in the transitional mucosa²⁴ which was negative in the present study (fig 2). Moreover, a direct comparison with serial sections showed a different tissue distribution of peanut agglutinin binding antigens and the antigen detectable with AM-3 (not shown). Griffioen *et al* found that the production of mucins in the large intestine during the adenoma-carcinoma sequence gradually decreases.⁶ Through staining of parallel sections with AM-3 and alcian blue (not shown) we corroborated and extended this finding: the overall decrease in mucin synthesis in colorectal adenomas is accompanied by increased expression of the antigen detectable with AM-3.

A family of high molecular weight glycoproteins has recently been defined by poly- and monoclonal antibodies raised against antigenic preparations from human milk, breast cancer cells, or transformed cells originating from other tissues.^{25–26} The best characterised members of this family are the MAM-6 antigen, recognised by monoclonal antibody 115D8,²⁷ the DF3 antigen, recognised by monoclonal antibody DF3,²⁸ and the Ca antigen, recognised by monoclonal antibody Ca 1.²⁹ The Ca antigen was found exclusively on almost all cell types of carcinomatous tissue investigated, but not in normal tissue.³⁰

The antigens DF3 and MAM-6 were detected in human breast carcinomas and also in carcinomas of other organs, including the large intestine, as well as in a number of normal tissues. Of these antigens, MAM-6, a high molecular weight epithelial sialomucin, exhibits tissue distribution resembling, in part, that described here for AM-3. MAM-6 was detected in 95–100% of the carcinomas of the breast, ovary, and colorectum.²⁷ The antigen detected by AM-3 was also found in all colorectal carcinomas investigated, but a positive reaction with ovarian and breast carcinomas was less common (50% and 78%, respectively) and less intense. The extensive immunohistochemical study showed that MAM-6 is expressed only in highly dysplastic colorectal adenomas but not in adenomas of mild or moderate dysplasia, which were positive with AM-3 (fig 6).³¹ Furthermore, the MAM-6 antigen was detectable in normal stomach mucosa,³² but was absent from the liver,²⁷ while AM-3 yielded the opposite result. These differences in staining between AM-3 and 115D8 were corroborated through direct comparison of parallel tissue sections, stained with each antibody (not shown). The tissue distribution as well as the apparent relative mass of the antigen therefore indicate that AM-3 detects an antigen which may be related to the family of mucins expressed in human carcinomas but is distinctly different from the antigens associated with colon carcinomas described to date.

In summary, AM-3 detects an antigen whose amounts in histologically normal colonic tissue are not detectable by immunohistochemistry. In colorectal adenomas its expression increases in a manner parallel to the morphological

changes defined by the grade of dysplasia. Its expression seems to be an early event in some of the adenomas. The antibody AM-3 will allow the biological importance of expression of this antigen during colon carcinogenesis to be investigated.

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