Gastric cancer associated structure in mucus glycoproteins shown as a clinically useful marker

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Abstract

A new monoclonal antibody has been developed which is capable of detecting structures in gastric mucus glycoproteins expressed in the fetus and in adult gastric mucosa in conditions such as gastric carcinoma. Cancer associated monoclonal antibodies were selected by testing them against various mucous glycoprotein samples from the alimentary tract, including salivary glycoproteins from both secretory and non-secretory subjects, and cancerous and normal gastric juice glycoproteins. They were tested against 1000 samples of gastric juice from an unselected population. Immunochemical characterisation suggested that the glycoproteins picked up by P₄ and i₁₁ include one of the compounds reacting with rabbit anti-fetal sulphoglycoprotein antigen serum. On the basis of a clinical trial and immunohistological evaluation further evidence was obtained of P₄ as the most promising antibody for further experimentation. A total of 302 gastric juice specimens from patients with various gastric symptoms were analysed using the enzyme linked immunosorbent assay technique and P₄ antibody. Of 10 gastric cancers, nine had P₄ in the gastric juice. A positive correlation was found between gastric ulcer and the appearance of P₄. Duodenal ulcers were not correlated to P₄. Atrophic gastritis and P₄ coincided less frequently. Raised P₄ values were found in between 3% and 9% of subjects, depending on the population. Cancer cases showed high P₄ values, which allows adjustment of the lower limit of a positive result to high level whereby a considerable number of non-cancerous P₄ positives are omitted.

Gastric cancer cells and their precursors, being of epithelial origin, have characteristics of epithelial mucous cells such as the capacity to secrete mucus glycoproteins. Consequently, we find these secretory products in the gastric juice. Earlier studies on fetal sulphoglycoprotein antigen (FSA), where a rabbit antiserum was used to detect qualitatively these gastric cancer associated blood group type glycoproteins, were based on this idea.^{1,2} It was stated that the antibodies detected structures other than known blood group determinants in these blood group type glycoproteins.³ Immunohistological data suggested that FSA structures were expressed in normal ontogenic fetal development of the gut.⁴

Detection of the FSA for the diagnosis of gastric cancer among rural populations with a high incidence of gastric cancer was promising in the early 1970s but became less important later, with a decline in incidence of this geographically

associated type of cancer. More precisely, although the method was satisfactory in showing early cases of cancer, the FSA test was plagued by a high rate of non-cancerous FSA positives, about 8% in an unselected population.⁵

In experimental studies gastric carcinomas can be produced by administration of a carcinogen.⁶ When the dose of the carcinogen was reduced a stage was found where the fetal glycoprotein markers were expressed but a morphological cancer did not develop.⁷ A relatively short exposure to carcinogens, such as chemicals in our surroundings, may cause an analogous situation, and thus explain the recently observed tendency in our population towards an increase in non-cancerous FSA positives.

The FSA glycoprotein, characterised by polyclonal rabbit antiserum, necessarily contains a multitude of determinants which in individual samples can be present in incomplete combinations. The possibility exists that some of the FSA determinants are more strongly cancer associated – that is, expressions triggered later in carcinogenesis. To investigate this, monoclonal antibodies were produced, then characterised and selected for use in a clinical trial. The present study gives immunochemical and histoimmunological data on the most promising of these monoclonal antibodies, P₄. A clinical study using P₄ was included to support our concept of its association with cancer.

Methods

PREPARATION OF GLYCOPROTEIN ANTIGENS FOR IMMUNISATION

The cancerous gastric juice sample was taken from a patient with gastric cancer (age 67 years, blood group O+, histological type: diffuse carcinoma). The specimen was taken by oral tube after an overnight fast, the patient having received 100 ml of phosphate buffered saline pH 6.5. The antigen was prepared following the procedure described earlier.⁸ Polyanions from the gastric juice were separated using cetyl pyridinium chloride (CPC) (Merck, Germany) and ethanol precipitations. The supernatant of the CPC precipitation containing the neutral glycoproteins was further treated with four volumes of ethanol, and the precipitate was dissolved in saline, to be used in immunisation as fraction 1. CPC precipitated polyanions were divided into two fractions by a further CPC-salt precipitation procedure, and they were used in immunisations as fractions 2 and 3.

PRODUCTION OF MONOCLONAL ANTIBODIES

Preparation of monoclonal cell fusions and the

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cloning procedure was as described elsewhere.⁹ To measure the specific reactivity of the mouse antibodies to the antigen, the enzyme linked immunoassay technique was used, with anti-mouse immunoglobulin conjugated with alkaline phosphatase. The antigenic preparation used in immunisations was coated in microplate wells by treatment with 0.2% glutaraldehyde in 0.1 mol/l phosphate buffer pH 5, at +4°C overnight.¹⁰

The quantitative assay for FSA using the monoclonal antibodies produced was developed by detecting the antigen on the solid phase with the monoclonal antibody. Attempts to coat native gastric juice samples met with difficulties. This was probably caused by competition for the fixing sites by too many kinds of unrelated proteins. Half an hour's incubation at 65°C in 0.1% papain-cysteine solution buffered in 0.2 mol/l acetate to pH 5.6 split most of the proteins and solubilised the FSA antigen molecules without destroying the antigenic properties. Four volumes of ethanol added subsequently precipitated the glycoproteins, which in aqueous solution could then be coated onto the solid phase.

TESTS FOR SPECIFICITY OF THE CLONES

The specificity of clones was tested by measuring their reactivity with (i) non-secretory saliva, (ii) secretory saliva, (iii) 10 samples of gastric juice from normal control subjects, (iv) 10 samples of cancerous gastric juice, and (v) blood group ABH, Lewis^{ab} systems, performed as in routine blood group serology. In addition, selected antibodies were further used to test 1000 specimens of gastric juice from an unselected population aged 49–74 years which had been preserved frozen from an earlier study.

Selected specific monoclonal antibodies were immobilised using CNBr₂ activated Sepharose (Pharmacia-LKB Biotechnology, Uppsala, Sweden). A small antibody column was prepared for affinity purification of the glycoprotein antigen. Gas chromatography for sugar analysis of the affinity purified glycoprotein was performed at the Department of Medical Chemistry, University of Helsinki. A gel filtration profile of the glycoproteins in various preparations was obtained on a Sephacryl S-200 column, detected by the specific monoclonal antibody.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis¹¹ was performed using 6% or 8% vertical slab gels either under reducing or non-reducing conditions. For immunoblotting,¹² the polypeptides were transferred onto nitrocellulose sheets (Trans-Blot Transfer Medium, Bio-RAD, Richmond, CA). Immunoblotting was performed and detected by using peroxidase coupled antimouse immunoglobulins (Dakopatts, Glostrup, Denmark).

IMMUNOHISTOLOGICAL STUDY

The immunochemically selected monoclonal antibody P₄ (see Results) was tested. A standard immunoperoxidase procedure was used on formaldehyde fixed tissue sections. Parallel negative controls were included in all assays. The following organs and conditions were studied:

non-cancerous stomach, gastric carcinoma, pancreatic carcinoma, small intestine, normal colon, colonic carcinoma, gall bladder, and sub-mandibular and parotid glands.

CLINICAL STUDY

The clinical study included 302 patients referred for gastroscopy at the gastroenterology unit of the University Hospital, Medical Department, Helsinki. The cases were deliberately not selected in any respect, the aim being to get an overall picture of specimens from the different groups of patients. To standardise the gastric juice specimens each patient drank 100 ml of 0.1 mol/l phosphate buffered saline pH 6.5 immediately before the gastroscope was introduced. The stomach was then emptied by suction through the gastroscope. The samples were preserved in sealed tubes at –20°C.

P₄ analysis was performed using the enzyme linked immunosorbent assay (ELISA) technique, the papain treated samples being coated onto solid phase as described above. Since in the preliminary tests i₁₁ (see Results) gave negative results in some of the cancer cases, P₄ was selected as the more promising antibody, and patient analyses are reported for P₄ only. To control the daily variation in the ELISA results, a standard antigen prepared from a P₄ positive sample was included in every run. The results are given as absorbance figures. The lower limit of a positive result was set at 0.40 absorbance, nearly the cut off of the background value.

Results

All three antigen preparations caused an immune response in mice. Cloning of the fusions gave 20 active clones in fusion 1 (antigen preparation 1), 23 in fusion 2, and 18 in fusion 3. The tests performed are listed in Table I. Two individual clones, i₁₁ in fusion 1 and P₄ in fusion 3, were selected to be the most promising as regards their cancer association. Unlike i₁₁ and P₄, most of the other clones reacted to salivary and normal gastric glycoproteins. The two selected clones were further tested against 1000 specimens of gastric juice derived from an unselected rural population. P₄ antibody gave a positive reaction in 30 samples, whereas i₁₁ reacted in only 14 of the same 30 P₄-positive samples. All i₁₁ positive samples were also P₄ positive.

Affinity purified glycoprotein from a P₄ anti-

TABLE I Tests for characterisation of the most promising monoclonal antibodies, P₄ (from immunisation with acid cancerous gastric glycoproteins) and i₁₁ (from immunisation with neutral cancerous gastric glycoproteins)

Tests performed	P ₄	i ₁₁
Enzyme linked immunoassay technique:		
Reactivity with non-secretory saliva	—	—
Reactivity with secretory saliva	—	—
Reactivity with 10 cancerous gastric juice samples	10/10	10/10
Reactivity with 10 non-cancerous gastric juice samples	—	—
Reactivity with 1000 samples from unselected population (aged 49–74 years)	30/1000	14/1000
Reactivity with blood group antigens:		
ABH	—	—
Lewis ^{ab}	—	—

TABLE II Gas chromatography analysis of sugars of P₄ affinity-purified cancerous gastric glycoprotein

	Percentage
Mannose	18.7
Galactose	38.8
Fucose	11.7
Glucosamine	20.1
Sialic acid	3.7
Galactosamine	14.9

body column indicated that besides the P₄ epitope, the i₁₁ epitope could also be situated in the same macromolecule, but the picture varied from one sample to another so that macromolecules with P₄ epitopes but not i₁₁ appeared. On the other hand, i₁₁ alone without the P₄ epitope was not found. In the clinical study, i₁₁ was lacking in some of the P₄ positive cancer cases. On these grounds, P₄ was considered to associate with a maximum number of the cancer-associated glycoproteins.

Table II gives the results of sugar analyses of the P₄ antibody affinity purified glycoprotein. A similar preparation from a subject with blood group A gave a positive reaction with monoclonal anti-A, indicating that P₄ positive macromolecules contain a blood group determinant. This is consistent with earlier studies on FSA.³ Rabbit anti-FSA also reacted with P₄ immunopurified FSA in immunodiffusion. P₄ reacted with samples of meconium.

Figure 1 shows the gel filtration profile of a P₄ positive sample of gastric juice glycoprotein, both untreated and after papain treatment. No appreciable alteration in the molecular size of the P₄ molecules can be observed. The profile indicates some polydispersity of the P₄ active macromolecules, the molecular size ranging from 40 000 to 150 000. It further suggests that the papain treatment solubilises some other glycoproteins.

The immunoplotting pattern of a P₄-i₁₁ positive sample after sodium dodecylsulphate-polyacrylamide gel electrophoresis is given in Figure 2. Compared with the profile from the Sephacryl S-200 run (Fig 1), no discrepancy exists since 6% electrophoresis gel apparently obstructs the free movement of the largest molecules. Figure 3 shows the standard dilution curve of the P₄ antibody enzyme linked immunoassay, the papain treated antigen on the solid phase.

Immunohistologically, P₄ was localised in gastric cancer cells in the cytoplasm and in the secretion (Fig 4A). In none of the sections did all cancer cells show the P₄ structure. The staining picture varied also in its intensity. As a rule the

Figure 2: Immunoblotting pattern of a P₄-i₁₁ positive sample after sodium dodecylsulphate-polyacrylamide gel electrophoresis. Lines A and B: molecular size standards; line C: protein staining; line D: P₄ antibody; line E: i₁₁ antibody; line F: an unrelated monoclonal antibody.

normal gastric mucosa of the same section – that is, adjacent to a tumour – was negative as was the mucosa from non-cancerous stomachs. Immunohistologically, metaplasia and atrophic gastritis were always negative, both in the gastric cancer cases and in non-cancerous cases. Exceptionally, some single glands stained positively (Fig 4B), less frequently in non-cancerous stomachs than in cancer cases. Some dysplastic glands adjacent to a cancer were positively stained in about two thirds of the cases (Fig 4C). Table III summarises the immunohistological results for various gastrointestinal materials.

Table IV gives the results of the P₄ analysis of

Figure 1: Gel filtration on Sephacryl S-200 of a pooled sample of gastric juice mucus glycoproteins. (A) After treatment with papain; (B) no enzymatic treatment. Detection at 210 nm (●); detection at 405 nm with P₄ monoclonal antibody using enzyme immunoassay (○).

Figure 3: Dilution curve of papain treated glycoprotein antigen of gastric juice. P₄ antibody enzyme linked immunoassay. Antigen on the solid phase.

clinical material, according to their absorbance figures; 83.1% of the samples were P₄ negative (0.00–0.39 absorbance). Slight elevation (0.40–0.79 absorbance) was found in 7.9%. The rate of high positives (absorbance 0.80 or over) was 8.9%. Out of this group, a subgroup of high values over 1.0 absorbance is shown separately.

Table V correlates the P₄ results to clinical gastric diagnoses. Nine of the 10 gastric cancer cases showed P₄ in the gastric juice. Only six of the 30 cases of atrophic gastritis were P₄ positive. Among benign gastric disorders, peptic ulcer was definitely associated with P₄. On the other

hand, P₄ and duodenal ulcer showed no positive correlation.

Discussion

All active clones reacted with the glycoprotein structures. Immunisation with a restricted selection of glycoproteins possibly leaves more reactive capacity in mouse in the immune system to identify different components compared with immunisation where whole cancer cells are used.¹³

Monoclonal antibodies P₄ and i₁₁ were selected using reactions to glycoproteins of the upper alimentary canal. A positive reaction to all 10 samples of cancerous gastric juice and a negative reaction to controls (Table I) was a promising result. Among 1000 gastric juice specimens collected earlier, 30 were positive to P₄ and 14 to i₁₁. These incidence rates only illustrate the rate of marker-positive samples in an unselected population of high cancer risk age groups. In the absence of clinical data no correlation to cancerous or precancerous states can be made from them.

P₄ and i₁₁ reacted with meconium, which indicates that the antigen structures belong to normal fetal development, unlike the mutation based aberrations such as blood group A-like structures.^{14,15} Blood group specificity was absent, which points to the assumption that other antigen structures are involved in P₄–i₁₁ expressions in the blood group-like gastric glycoproteins of FSA. The P₄ immunopurified glycoprotein reacted in immunodiffusion with the polyclonal anti-FSA, thus connecting the P₄ glycoprotein to the FSA.⁸ However, it is not yet clear whether true P₄ specificity is also present in rabbit anti-FSA.

Immunohistologically P₄–i₁₁ antibodies detected only structures of gastric origin among all alimentary organs and diseases studied. Our clinical data indicate that non-cancerous P₄–i₁₁ production also exists, although it is not easy to show this immunohistologically.

P₄ analysis from gastric juice seems to detect 80% to 90% of gastric carcinomas. In this study the cancer cases were not selected but were from a group of gastroenterology patients and in various clinical states. Whereas the rate of P₄ positivity in the unselected population was about 3%, both low and high positives included, the rate in selected cases from a gastroenterology unit would be expected to be higher. It actually varied between 9% and 16%, depending on how high the lower limit of a positive test was set. Gastric carcinomas gave high values of P₄, which allows a rise of the lower limit of positivity, and consequently the cutting off of an appreciable number of non-malignant P₄ positives. A larger group of cancer patients is, however, needed to fix this limit definitely. For instance, raising the limit from 0.40 to 0.80 in our series cuts off one cancer case but at the same time 23 non-cancerous P₄ positive cases.

In our cases gastric ulcer showed the highest rate of non-cancerous P₄ positivity. On the other hand, atrophic gastritis, which is known to be associated with gastric cancer, showed less frequent correlation to P₄ positivity. In addition, we

Figure 4: (A) Gastric cancer cells stained positively using the P₄ monoclonal antibody and the peroxidase technique (original magnification $\times 550$). (B) Morphologically normal gastric mucosa adjacent to a gastric carcinoma. Some glands stained weakly positively. P₄ antibody peroxidase technique (original magnification $\times 400$). (C) Positively stained dysplastic gland in the mucosa adjacent to a gastric carcinoma. P₄ antibody peroxidase technique (original magnification $\times 400$).

TABLE III Material studied immunohistologically using the P₄ antibody

Specimen	No of specimens	Immunohistological findings	P ₄
Non-cancerous stomach	5	1 of 5 cases: single positive glands	+
		4 or 5 cases: totally negative	—
Gastric carcinoma*	22	14 of 22 cases: part of cancer cells positive	+
		16 of 22 cases: a few positive dysplastic glands	+
		3 of 22 cases: totally negative	—
Pancreatic carcinoma	2		—
Small intestine	2		—
Normal colon	10		—
Colonic carcinoma	10		—
Gall bladder	4		—
Submandibular gland	2		—
Parotid gland	2		—

*12 were of intestinal and 10 of diffuse type.

TABLE IV Results of P₄ analyses grouped by absorbance figures

Absorbance	P ₄
0.00–0.39	251 (83.1%)
0.40–0.79	24 (7.9%)
0.80+	27 (8.9%)
1.00+	18 (6.0%)*
Total	302

*Included in the 0.80– column.

found no immunohistological verification of P₄ being localised in atrophic gastritis. In fact there was a P₄ positive rate of about 10% in acute gastritis (Table V). It thus seems plausible that the origin of biochemical alteration is to be found outside a morphological atrophic gastritis. Some benign conditions, gastric ulcer, and to a less extent gastritis apparently can include parallel factors, being linked to carcinogenesis at the biochemical level. The positive results associated with benign gastric ulcer and gastritis could limit its use as a marker for gastric cancer.

Gastric emptying is a routine part of gastroscopy, and sampling gastric juice only requires a minute to remove the yield to a preserving container. However, to guarantee a proper specimen for quantitative P₄ analysis, it is essential that the buffer is swallowed. By natural contraction movements it will properly mix.

Patients' attitudes to gastric examination depend greatly on motivation. The inconvenience caused by gastroscopy is widely accepted, thanks to the availability of information on its superior accuracy. In the 1970s, acceptance of gastric intubation in our population study of 40 000 patients was 75%.⁵ We have to bear in mind that at present there is no serum marker which is capable of detecting gastric malignancy reasonably early. A gastric juice marker has a far better potential for this. In mass screening or clinics, P₄ could be considered as a signal of the existence of a dysplastic condition or a malignancy.

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TABLE V P₄ measurements from gastric juice using the ELISA technique and correlated to clinical diagnoses

Diagnosis	Absorbance			
	0.0–0.39	0.40–0.79	0.8+	1.0+*
Acute gastritis	75	5	3	3
Atrophic gastritis	30	2	4	1
Erosion	6	4	0	0
Gastric ulcer	15	5	6	4
Duodenal ulcer	17	1	0	0
Hyperplastic polyp	5	3	1	1
Gastric carcinoma	1	1	8	7
Oesophageal carcinoma	0	0	1	1
Gastric lymphoma	0	0	1	1
Diagnosis total	149	21	24	18
No disorder verified	102	3	3	0
Total	251	24	27	18

*Included in the 0.80+ column.

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