Development and Characterization of Breast Cancer Reactive Monoclonal Antibodies Directed to the Core Protein of the Human Milk Mucin

Joy Burchell, Sandra Gendler, Joyce Taylor-Papadimitriou, Anne Girling, Angela Lewis, Rosemary Millis, and Derek Lamport

ABSTRACT

A mucin molecule, which has a molecular weight of greater than 400,000 and which carries tumor associated epitopes recognized by monoclonal antibodies HMFG-1 and HMFG-2, has been purified from human skimmed milk by affinity chromatography followed by passage through a size exclusion column. While treatment of the mucin with hydrogen fluoride for 1 h at 4°C removed the peripheral oligosaccharides, treatment with HF for 3 h at room temperature removed all of its lectin binding ability and revealed a dominant polypeptide of about 68,000. This appears to be the size of the mucin core protein. Monoclonal antibodies have been developed that react with the stripped and partially stripped molecule but not with the intact mucin. From the initial screening on histological sections one of these antibodies, SM-3, reacts with 91% of breast carcinomas but shows little or no reactivity on benign mammary tumors, normal resting, pregnant, or lactating breast. It appears that this monoclonal antibody is reacting with an epitope that is usually masked by oligosaccharide moieties in normal cells but which is exposed, perhaps due to aberrant glycosylation, in malignant cells.

INTRODUCTION

Many of the monoclonal antibodies raised against carcinoma cells or human milk fat globule membranes have been shown to react with epitopes present on large molecular weight glycoproteins (1–7). It has recently been shown that many of these antibodies react with the same group of molecules (8), which appear to be mucins. One such component, which contains about 50% carbohydrate oxygen linked to serine and threonine and has a molecular weight of greater than 400,000, is found in large amounts in the human milk fat globule membrane and is secreted into milk (9, 10). These mucin glycoproteins are also secreted by a number of other normal epithelial cells (11, 12).

The monoclonal antibody HMFG-1 (13) (original nomenclature 1.10.F3) is highly reactive with the milk mucin, and evidence suggests that the epitope recognized by this antibody is abundant on the fully processed mucin, characteristic of normal differentiation (14, 15). In tumors, the molecular weight of the molecules carrying the antigenic determinants differs among individual tumors and in the case of the components recognized by the HMFG-2 antibody (13) (original nomenclature, 3.14.A3) can range from 80,000–400,000 (1). Although it appears that the differences observed in the mobility of the high molecular weight bands are due to genetic polymorphism (12), this probably does not explain variations in the size of the lower bands observed. It has been proposed that these may be the result of aberrant processing occurring in the tumor cell possibly within the glycosylation pathways (1).

For the majority of the monoclonal antibodies reacting with this group of molecules, the exact nature of the antigenic epitopes remains undefined, although circumstantial evidence has suggested that carbohydrate may at least be partly involved in many of the epitopes. Moreover, from data so far available it is unknown whether the mucin found in the normal differentiated cells and that observed in the tumors contain the same core protein or merely carry common carbohydrate determinants.

The purpose of the present study was to develop monoclonal antibodies to the milk mucin core protein with a view to using the antibodies as tools to study processing of the mucins and to isolate the gene coding for the core protein from an expression library. The question as to whether the mucins produced by many epithelia and by cancers of epithelial origin are coded for by a single gene or by a family of genes could then be addressed. In addition, if aberrant glycosylation of the normal mucin is occurring in the tumor cells, areas of the core may become exposed during transformation, thus unmasking epitopes that may be recognized by antibodies to the core protein.

To this end the mucin was purified from skimmed milk using an HMFG-1 affinity column and stripped of its carbohydrate by treatment with hydrogen fluoride. This preparation was then used as the immunogen for the generation of monoclonal antibodies to the mucin core protein.

MATERIALS AND METHODS

Purification of the Milk Mucin. The milk mucin was purified from human skimmed milk by passage through an HMFG-1 affinity column followed by size exclusion chromatography. The HMFG-1 monoclonal antibody was purified from tissue culture supernatant using a Protein A column (1). The purified antibody was coupled to cyanogen bromide activated Sepharose (Pharmacia) as described in the manufacturer's instructions. Human skimmed milk was passed in batches of 100 ml through the antibody column followed by extensive washing with phosphate-buffered saline (153 mM NaCl-3 mM KCl-10 mM Na2HP04-2 mM KHP04, pH 7.4). Bound antigen was eluted from the column using 0.1 M glycine, pH 2.5, and the fractions registering absorbance at 280 nm were pooled, dialyzed against 0.25 M acetic acid, and lyophilized. Batches of about 20 mg were dissolved in 0.25 M acetic acid and passed through a G75 Sephadex column (1 x 100 cm) which had been previously equilibrated with acetic acid. The column was washed with 0.25 M acetic acid and 1-ml fractions were collected. The peak fractions which were eluted in the void volume were pooled, lyophilized, and the dry powder stored at 4°C. Amino acid analysis was performed using a Beckman 6300 amino acid analyzer.

Deglycosylation of the Milk Mucin. To remove the oxygen-linked carbohydrate from the milk mucin the molecule was treated with anhydrous hydrogen fluoride as described by Mort and Lamport (16), for either 1 h at 4°C which produced a partially stripped preparation or 3 h at room temperature which produced the extensively stripped mucin.

Iodination of the Milk Mucin. Iodinations using 2.5 μg of protein, of the purified mucin, and the partially or extensively stripped mucin were carried out using the Bolton and Hunter method (17). Free Bolton and Hunter reagent was removed from the reaction by passage through a G25 Sephadex column (PD10 columns; Pharmacia) previously equilibrated in phosphate-buffered saline.
Iodination of Lectins. WGA, PNA (Vector Labs), and HPA (Boehringer) were iodinated as described by Karlsson et al. (18) using the chloramine-T method.

Polyacrylamide Gels and Western Blots. Polyacrylamide gel electrophoresis and immunoblotting was performed as described (19). For lectin binding studies the Western blots were reacted with the iodinated lectins as described by Swallow et al. (12).

Production of Monoclonal Antibodies. A female BALB/c mouse was immunized with 5 μg of the partially stripped milk mucin in Freund’s complete adjuvant and 3 months later boosted with a further 5 μg of the same preparation in Freund’s incomplete adjuvant. After a further 20 days, 5 μg of the mucin extensively stripped of its carbohydrate were given i.v. in saline solution. The spleen was removed 4 days later and fused with the NS2 mouse myeloma cell line (20).

Screening of Hybridoma Supernatants and Immunoprecipitations. Screening of hybridoma supernatants and immunoprecipitations was performed according to the assay described by Melero and Gonzalez-Rodriguez (21) and modified by Shearer et al. (22).

Staining of Tissue Sections. Tissues from primary mammary carcinomas, benign breast biopsies, pregnant and lactating breast, normal breast (see Table 3), and a variety of other normal tissues were fixed in methacarn (methanol:chloroform:acetic acid, 60:30:10) and embedded into paraffin wax. Sections were stained with the antibodies using the indirect immunoperoxidase method as previously described (23).

RESULTS

Purification of the Milk Mucin. The milk mucin was purified from human skimmed milk on a HMFG-1 antibody affinity column. Iodination of the eluted material revealed the presence of a large molecular weight component and a Mr 68,000 band (Fig. 1, track 5). Precipitation of the affinity purified material with antibodies HMFG-1 and HMFG-2 (tracks 1 and 2) followed by gel electrophoresis showed that both the high molecular weight components and the Mr 68,000 component were precipitated by both antibodies (less effectively by HMFG-2). Although the Mr 68,000 component always copurified with the milk mucin it’s relationship to the high molecular weight component was unclear. It was not evident on immunoblots of the purified material probed with HMFG-1 (Fig. 2A) and could be precipitated by a control antibody to α-interferon and by the medium control (Fig. 1, tracks 3 and 4). Thus the Mr 68,000 component was removed by molecular sieve chromatography on a G75 column. The final purified product showed a major high molecular weight band, with only a trace of the Mr 68,000 component and some small molecular weight material running with the dye front (Fig. 2B).

The amino acid composition of the purified HMFG-1 reactive component was determined and compared to the amino acid composition of PAS-O (periodic acid-Schiff-O), a mucin isolated from human milk fat globule membranes and which contains 50% carbohydrate in oxygen linkage (9). Table 1 shows that there is good correspondence between the two sets of data, indicating that the core proteins of PAS-O and the mucin purified here are the same.

Isolation of the Core Protein of the Milk Mucin. To remove the oxygen-linked oligosaccharides the milk mucin was treated with anhydrous hydrogen fluoride which has been shown by...
Mort and Lamport (16) to be effective in removing sugars from pig submaxillary mucin without damaging the protein core. Amino acid analysis of the material produced after HF treatment of the milk mucin suggested that the protein core was also in this case undamaged, since the composition was very similar to that seen in the intact mucin (Table 1).

The milk mucin was exposed to hydrogen fluoride for 1 h at 4°C or for 3 h at room temperature. Fig. 3 shows an autoradiograph of the iodinated products after treatment for 1 h at 4°C (track 2) or 3 h at room temperature (track 1). It can be seen from Fig. 3 that the milder treatment results in a mixture of products made up of high molecular weight material and a number of smaller bands. After longer exposure to HF at room temperature, the high molecular weight bands disappeared resulting in polypeptide bands of about M, 68,000 and 72,000.

To test for the presence of sugars on the intact mucin and on the components produced after the two different HF treatments, each preparation was subjected to acrylamide gel electrophoresis, transferred to nitrocellulose paper, and reacted with ¹²⁵I-labeled lectins. The lectins used were PNA, which reacts with galactose linked to N-acetylgalactosamine, WGA, reactive with N-acetylgalactosamine, and HPA, which reacts with the linkage sugar N-acetylgalactosamine. Fig. 4 shows autoradiographs of the reacted blots, and it can be seen that while PNA binds strongly to the intact mucin (track 1) its binding to the mucin treated with HF for 1 h at 4°C is considerably reduced (track 2). This reduction in PNA binding is accompanied by the appearance of binding of the linkage specific lectin HPA. Thus it appears that treatment of the mucin for 1 h at 4°C partially strips the mucin of its carbohydrate, removing the peripheral oligosaccharides and so unmasking the linkage sugar. The smaller component seen in both the intact mucin (track 1) and in the partially stripped preparation (track 2) is a glycoprotein which reacts with WGA, although not with PNA. This may correspond to the component of similar molecular weight (around M, 68,000) seen after affinity chromatography of the mucin and may represent an intermediate precursor molecule.

Fig. 4 shows clearly that the M, 68,000 and 72,000 components produced after extensive treatment with HF (3 h at room temperature) show no reactivity with the lectins (track 3), including the N-acetylgalactosamine specific lectin HPA. This observation constitutes strong evidence that all the sugars have been removed from at least the majority of the molecules, and we will refer to this preparation as the extensively stripped mucin. It is of course formally possible that the molecule contains some nitrogen-linked sugars that would not be effected by treatment with HF; however, this seems unlikely because the extensively stripped material shows no reactivity with the wheat germ lectin.

Generation of Monoclonal Antibodies to the Milk Mucin Core Protein. A fusion was carried out using the spleen of a mouse that had been immunized with two injections of the partially stripped milk mucin followed by a boost with the extensively stripped mucin. The clones were screened against the extensively stripped mucin, the partially stripped mucin, and the intact molecule using the Protein A plate assay (see “Materials and Methods”). Three hybridomas that secreted antibodies showing no reactivity with the intact molecule but which bound strongly to the partially and extensively stripped mucin (Table 2) were selected and cloned. These appeared to be good candidates for monoclonal antibodies to the protein core and one, SM-3 (SM for stripped mucin) selected to be characterized further, was shown to be of the IgGl subclass. It can also be seen from Table 2 that the HMFG-1 and HMFG-2 antibodies reacted very strongly with the mucin...
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stripped of its carbohydrate. These two antibodies were, in fact, developed using the intact mucin (from the milk fat globule) as immunogen and, in the case of HMFG-2, growing mammary epithelial cells (13). Their reaction with the stripped mucin was unexpected, because circumstantial evidence had previously led to the belief that carbohydrate might form at least part of their antigenic epitopes.

Molecular Weight of Molecules Carrying Antigenic Determinants. Immunoprecipitation of the extensively stripped material with SM-3 showed a reaction with the lectin unreactive M, 68,000 component (Fig. 5A, track 3). The monoclonal antibody HMFG-2 can also be seen to immune precipitate the lectin unreactive M, 68,000 component (track 2). The antibodies were reactive with antigen on immunoblots, and Fig. 5B shows the reaction of antibody SM-3 with the dominant M, 68,000 band of the extensively stripped mucin (track 2).

We have previously shown that the molecular weight of the components in breast cancer cells carrying determinants found on the milk mucin is lower than 400,000 and can vary from one tumor to another (1). Reaction of antibody SM-3 with Western blots of gel separated extracts of breast tumor cells shows that this antibody reacts with components of similar molecular weight to those reactive with antibody HMFG-2 (data not shown). Because antibody SM-3 differs from antibodies HMFG-1 and 2 in that it does not react with the intact mucin processed by the lactating gland and yet reacts with molecules processed by breast cancer cells, it was appropriate to examine the reaction of SM-3 with a range of breast cancers.

Reactivity of SM-3 with Breast Tissues and Tumors. Antibody SM-3 reacted with paraffin embedded tissues provided that these were fixed in methacarn (not formalin). Using this method for preparation of tissue sections, the reaction of the antibody was compared to that of HMFG-2 on breast tissues and tumors with an indirect immunoperoxidase staining method. This analysis showed a dramatic difference in the staining pattern of SM-3 compared to that seen with HMFG-2. Thus, although a strong positive reaction was seen in 47 of 50 breast cancers stained with SM-3 (compared with 50 of 50 for HMFG-2), normal resting breast, pregnant, or lactating tissues, and most benign lesions were largely unstained with SM-3 but were stained with HMFG-2. Details of the staining are shown in Table 3, and some examples of staining patterns observed with breast tissues and tumors are illustrated in Fig. 6.

It should perhaps be noted that the intensity of staining with HMFG-2 seen with normal breast tissues and benign lesions fixed in methacarn was somewhat higher than that reported previously using formalin fixed material (11, 23).

In a preliminary study SM-3 stained 10 of 10 colon carcinomas, 2 of 3 ovarian carcinomas, and 1 of 3 lung carcinomas. However, SM-3 was shown to be negative on sections of normal liver, small intestine, lung, thymus, testes epididymis, prostate, bladder, thyroid, and skin (epidermis and sweat gland). Very weak positive staining was observed in normal pancreas, stomach, colon, ovary, and fallopian tube. Somewhat stronger staining was seen in salivary gland, sebaceous glands, and the distal tubules of the kidney.

Table 3 Reactivity of SM-3 and HMFG-2 with breast tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of specimens</th>
<th>No. positive</th>
<th>Intensity</th>
<th>Type of staining when positive</th>
<th>SM-3</th>
<th>HMFG-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast carcinomas</td>
<td>50</td>
<td>50</td>
<td>+</td>
<td>Strong cytoplasmic and membranous staining; heterogeneous</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>34</td>
<td>34</td>
<td>+</td>
<td>Strong cytoplasmic and membranous staining</td>
<td>34</td>
<td>Strong but heterogeneous</td>
</tr>
<tr>
<td>Infiltrating lobular carcinoma</td>
<td>13</td>
<td>13</td>
<td>++</td>
<td>Strong cytoplasmic and membranous staining</td>
<td>13</td>
<td>Strong but heterogeneous</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>3</td>
<td>3</td>
<td>++</td>
<td>Strong cytoplasmic and membranous staining</td>
<td>3</td>
<td>Strong but heterogeneous</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>18</td>
<td>18</td>
<td>+</td>
<td>Focal staining of only one or two glandular elements</td>
<td>18</td>
<td>Strong but heterogeneous</td>
</tr>
<tr>
<td>Papilloma</td>
<td>6</td>
<td>6</td>
<td>++</td>
<td>Weak, membranous</td>
<td>6</td>
<td>Strong, cytoplasmic and membranous staining</td>
</tr>
<tr>
<td>Cystic change</td>
<td>8</td>
<td>8</td>
<td>++</td>
<td>Membranous staining; weak and heterogeneous</td>
<td>8</td>
<td>Strong, cytoplasmic and membranous staining</td>
</tr>
<tr>
<td>Normal breast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>20</td>
<td>20</td>
<td>±</td>
<td>Extremely weak and usually confined to one or two acini/section</td>
<td>20</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>Pregnant and lactating</td>
<td>7</td>
<td>7</td>
<td>±</td>
<td>Very weak, staining confined to an occasional cell</td>
<td>7</td>
<td>Strong homogeneous</td>
</tr>
</tbody>
</table>

Fig. 5. Immunoprecipitation and immunoblots of the partially and extensively stripped mucin. In A, the 125I extensively stripped mucin was immunoprecipitated with SM-3 (track 3). HMFG-2 (track 2), or NS2 medium as a control (track 1) by the Protein A plate method (see "Materials and Methods"). In B, the partially (track 1) or extensively (track 2) stripped mucin was run on sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose paper. The blot was then reacted with SM-3 and the binding detected using an enzyme-linked immunosorbent assay method. KD, kilodaltons, expressed as molecular weight throughout the paper.
Fig. 6. Reactivity of monoclonal antibodies SM-3 and HMFG-2 with methacarn fixed breast tissue and tumor sections using an indirect immunoperoxidase staining method. Illustrated are infiltrating ductal carcinoma showing strong reactivity with both SM-3 (a) and HMFG-2 (b); fibroadenoma showing no reactivity with SM-3 (c) and strong heterogeneous staining of the epithelium with HMFG-2 (d); papilloma showing very weak reactivity with SM-3 (e) and strong positivity with HMFG-2 (f). Lactating breast (g) was negative when stained with SM-3, but stained positively with HMFG-2 (h). Bars, 70 μM.
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DISCUSSION

Large molecular weight mucin molecules are expressed by many carcinomas and carry many of the tumor associated antigenic determinants recognized by monoclonal antibodies. These epitopes may also be expressed by some normal epithelium, and some monoclonal antibodies like HMFG-1 react particularly well with a mucin found in normal human milk (1, 24). As long as the study of the mucins is restricted to their detection with antibodies reactive with undefined epitopes, the knowledge of their structure, expression, and processing will also be restricted. We have begun to investigate the structure and expression of the mammary mucin by isolating the core protein and developing antibodies which have allowed us to select partial complementary DNA clones for the gene coding for the core protein (25). This paper describes the production and characterization of these antibodies.

Treatment of the HMFG-1 affinity purified milk mucin with hydrogen fluoride resulted in the appearance of a dominant band of about Mr 68,000 and a minor species of about Mr 72,000 on sodium dodecyl sulfate acrylamide gels. These bands showed no reactivity with lectins, including H. pomatia agglutinin which is specific for N-acetylgalactosamine, the first sugar in oxygen-linked glycosylation (26). It therefore seems probable that this Mr 68,000 polypeptide represents the core protein of the mucin. Supportive evidence for this comes from the observation that the antibodies described here, which are reactive with the stripped Mr 68,000 component, can precipitate a molecule of this size from the in vitro translation products of mRNA isolated from breast cancer cells expressing the mucin (25).

As the milk mucin contains at least 50% carbohydrate (9), a protein core of only Mr 68,000 appears too small if the intact molecule has an observed molecular weight greater than 400,000. However, mucins can be composed of small subunits which aggregate and are held together by some form of noncovalent interactions, as yet not understood. For example, although the molecular weight of the ovine submaxillary mucin has been reported to be greater than 1 x 10^6 (27), it has a protein core of only 650 amino acids with a molecular weight of 58,300 (28).

An unexpected finding was that the antibodies HMFG-1 and HMFG-2 which react with the milk mucin also show a positive reaction with the extensively stripped material which showed no lectin binding capability. Previous indirect evidence had led to the belief that carbohydrate present on the milk mucin was involved in these epitopes. While it is possible that some nitrogen-linked sugars, which are resistant to HF and are undetected by the lectins, remain on the extensively stripped mucin described here, this cannot explain the reactivity of the antibodies HMFG-1 and 2. This can be said since both antibodies have recently been shown to react positively with β-galactosidase fusion proteins expressed by phage carrying DNA coding for the core protein of the mammary mucin. Furthermore, HMFG-1 and HMFG-2 can immune precipitate proteins translated in vitro from mRNA purified from a breast cancer cell line (25). It appears therefore that at least part of each of the epitopes recognized by HMFG-1 and HMFG-2 contain amino acids, but it must be assumed that these epitopes on the core protein are exposed, i.e., not masked by carbohydrate in the fully glycosylated molecule.

In this paper we have reported the development of new antibodies which are reactive with the protein core of the mucin and with the partially deglycosylated molecule but which are unreactive with the fully processed mucin produced by the lactating mammary gland. Furthermore, like HMFG-1 and 2 these antibodies have been shown to react with β-galactosidase fusion proteins from complementary DNA clones coding for the milk mucin and can immunoprecipitate proteins translated in vitro from MCF-7 cells (25). This provides conclusive evidence that the epitopes recognized by these antibodies are formed of amino acid sequences within the core protein of the milk mucin.

One of these antibodies, SM-3, has been studied in more detail. It has been shown to react with the mucin molecules which are produced by breast cancer cells and are recognized by many antibodies developed against the intact milk mucin. It should be emphasized however that the epitope recognized by SM-3 which is on the core protein and is exposed in the mucin as processed by tumor cells is not exposed on the normally processed milk mucin. This feature offers the possibility of enhanced tumor specificity, and a pilot immunohistochemical study of breast tumors and tissues has shown that indeed the SM-3 antibody reacts strongly with the majority of primary breast cancers (91%) but shows little or no reaction with benign breast tumors, resting or lactating breast, and most normal tissues.

There are several implications of the work described here which may be important for both basic and clinical studies in breast cancer. The observation that parts of the core protein (detectable by antibodies) are exposed on the mucins as processed by breast cancer but masked on the mucin as processed by cells in normal breast and benign tumors implies that there is an alteration in the processing of the mucin in malignancy. A more detailed study of the processing of the mucin in normal and malignant cells may then give basic information for defining the malignant cell. Moreover, since the specificity of the reaction of the antibody SM-3 for tumors is better than that of antibodies developed against the intact mucin, this antibody may prove to be a more effective diagnostic tool for the detection of breast cancer cells in tissue sections, tissue fluids, and cells. The reactive components are membrane associated as well as intracellular (data not shown), and in vivo localization of tumors may also be possible.

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