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High Molecular Weight Epithelial Mucins as Markers in Breast Cancer*

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INTRODUCTION

HIGH MOLECULAR weight glycoproteins, often described as mucins or mucin-like components, are frequently found associated with human breast carcinomas. These components are highly immunogenic and they have been identified as the target antigens for many monoclonal antibodies raised against human milk fat globule membranes or breast carcinoma cells. Such mucin antigens are clearly the products of normal epithelia as well as their malignant counterparts. Even so, despite this lack of tumor specificity, there is an increasing appreciation that epithelial mucins may be usefully exploited in both basic and clinical studies in breast cancer.

MUCINS AS MOLECULES

Molecular weights of 250,000 or more are often quoted for members of the major family of mucins recognised by monoclonal antibodies in breast tumours and milk fat globule membranes [1–5]. Compositional analyses of the mucin termed PAS-O (periodic acid Schiff-O) from human milk has indicated that the molecule contains approx. 50% carbohydrate by weight (in O linkage) and that the protein core is rich in the amino acids serine, theonine, proline, glycine and alanine [6]. Comparable analyses were later reported for affinity purified-milk mucin bearing epitopes for the antibody HMFG-1 [7].

Since epithelial mucins are extensively glycosylated, variability in the estimated molecular weights of the products of different tumours, as assessed by polyacrylamide gel electrophoresis, was initially attributed to microheterogeneity in oligosaccharide side chains, to incomplete or aberrant glycosylation in malignant cells or to the action of tumour glycosidases. These may well be contributory factors, but major differences observed in the mobility of the epithelial mucins from milk or urine appear to be due to a polymorphism which has a genetic basis and is determined by codominant Mendelian inheritance [8–10]. Variations in the size of the protein core (due to differences in the number of repeats of short sequences of amino acids) are likely to account for the observed polymorphism [11, 12].

Siddiqui *et al.* [13] have isolated a 309-base-pair cDNA clone, termed pDF9.3, coding for the epitope of the breast carcinoma-associated mucin antigen defined by the antibody, DF3. As with the related mucins from milk or urine, the electrophoretic heterogeneity of the DF3 antigen was determined by codominant expression of multiple alleles at a single locus [14]. Nucleotide sequence analysis of pDF9.3 revealed a highly conserved (C + G)-rich 60-base-pair tandem repeat. Again it was suggested that variation in the size of alleles coding for the polymorphic DF3 glycoprotein may represent different numbers of repeats [13].

The biological significance of this polymorphism as well as its relevance to malignant transformation and tumour development, is presently unknown but since mucins are assumed to offer certain protection at the surface of epithelial cells, the functional implications of mucin polymorphism deserve further attention.

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MUCINS AS ANTIGENS

Many monoclonal antibodies reactive with breast carcinoma-associated mucins have been produced independently in different laboratories. There appear to be subtle, but distinct, differences in the profile of immunohistochemical staining reactions of these antibodies which suggests that most, if not all, identify separate and distinct determinants on mucins [15, 16]. This is often taken to illustrate the complexity of determinants in the oligosaccharide side chains of the mucin, the assumption being that the epitopes defined by monoclonal antibodies reside within carbohydrate structures and not on the polypeptide core. However, the anti-milk fat globule membrane antibodies HMFG-1 and HMFG-2, which were originally thought to define carbohydrate epitopes [1], were found to react with deglycosylated milk mucin [7]. Furthermore, both of these antibodies reacted positively with β-galactosidase fusion proteins expressed by phage carrying DNA coding for the protein core of the milk mucin. Thus, the protein core of mucins is by no means totally obscured by carbohydrate and is accessible to the binding of antibodies [12]. Even the anti-breast carcinoma monoclonal antibody, NCRC-11, which is of the IgM class, belongs to that group of antibodies capable of recognising determinants within the protein core—NCRC-11 was recently found to react with a 20 amino acid synthetic peptide expressing epitopes for the antibodies HMFG-1, HMFG-2 and SM-3 (SM-3 being a monoclonal antibody prepared against the deglycosylated mucin [7]) (J. Burchell, personal communication).

The NCRC-11 antibody has been used in affinity columns to purify the target high molecular weight mucin antigen from breast and ovarian carcinomas, normal body fluids including milk and urine, and from pooled sera from patients with advanced breast cancer [3, 17–20]. Each of these antigen preparations bound both the original NCRC-11 antibody as well as a variety of other antibodies raised against human milk mucin or tumours. The co-expression of diverse monoclonal antibody-defined epitopes on epithelial mucins has been the subject of investigation in a number of studies [21, 22]. Clearly, some of the epitopes are located within carbohydrate structures—for example, binding of the Ca1 antibody or the W1 antibody is abolished by the removal of sialic acid with neuraminidase [23, 24]. Other epitopes reside in more cryptic sites—desialylation of breast carcinoma-associated mucin is required to reveal the oligosaccharide sequence of the I(Ma) blood group antigen Galβ1→4G1cNAcβ1→6–, defined by the antibody, M18 [18, 25]. Similarly, the binding of a panel of anti-mucin antibodies has been increased by neuraminidase treatment suggesting that the epitopes for these antibodies were unmasked by removal of sialic acid [24]. Antibodies reactive with the protein core of epithelial mucins may offer a more restricted specificity than those defining epitopes in the oligosaccharide side chains which may be commonly expressed in glycolipids and glycoproteins. Both McIllhinney *et al.* [26] and Linsley *et al.* [24] have identified antibodies recognising epitopes carried on glycolipids and mucin glycoproteins although the structural features of the epitopes have yet to be reported.

In summary, the complexity of epithelial mucins is reflected in the number and diversity of distinct monoclonal antibodies which have been raised against them. These define epitopes residing both in the carbohydrate side chains and in the protein core, as well as in sites involving their linkage so that there would therefore appear to be no single immunodominant region within these molecules.

MUCINS IN IMMUNOCYTOCHEMISTRY

In diagnostic pathology, anti-mucin antibodies (both monoclonal and polyclonal) have found applications in the identification of malignant cells in serous effusions [27], the detection of bone marrow and lymph node metastases from breast carcinomas [28–30] and for the differential diagnosis of anaplastic tumours [31]. Such antibodies have been used in the analysis of the nature of breast nodules by fine needle cytology [32] and the formulation of 'cocktails' of antibodies is being considered as one option for improving discrimination, and elimination of false-negative results in diagnostic cytology [33].

It should be emphasized that antigen expression in epithelial tumours is characteristically heterogenous, which in no ways simplifies the interpretation of antibody staining reactions [34]. Zotter *et al.* [16] have completed a systematic analysis of the reactivities of 20 monoclonal antibodies on serial sections of more than 200 epithelial and non-epithelial tumours and this therefore provides a reference antibody reactivity profile for comparative testing.

In early studies with the anti-breast carcinoma antibody NCRC-11, a highly significant relationship was found between the intensity of antibody staining of tumour tissue sections and patient survival (with a minimum follow-up of 5 years or to death) [35]. The relationship of staining to survival was independent of other known prognostic factors, and the findings were confirmed in a more extensive retrospective study of the staining of tissue from 444 breast cancer patients [36]. The biological significance of these observations is, as yet, unknown. One explanation is that the expression of NCRC-11 epitopes in tumours reflects an aspect of normal differentiation, the

retention of which is indicative of a favourable prognosis. It is a reasonable proposal that some epitopes on molecules as complex as the epithelial mucins may be characteristic of the fully processed glycoprotein while others, modified or generated by aberrant glycosylation in the transformed cell, are more closely associated with malignancy. It may be anticipated that antibodies prepared against products of normal tissues (e.g. milk fat globule membranes) would define epitopes of the former category, whereas raising antibodies against tumours would reveal a more diverse array of specificities.

MUCINS AS CIRCULATING TUMOUR MARKERS

A feature of neoplasia is the loss of functional polarity of cells within the tumour. In normal mammary tissue, for example, mucins are frequently expressed upon the apical aspect of glandular epithelia whence they may be secreted, or exfoliated in membranes of milk fat globules (by a pinocytotic process) into the lumenal space. The loss of functional polarity in tumour cells, coupled with disruption of normal tissue architecture by a developing and invasive neoplasm, will tend to facilitate access of tumour-associated mucins into the circulation.

Several investigations have confirmed that monoclonal antibody-defined mucins are indeed to be found in the sera of breast cancer patients. Quantitative double determinant or sandwich immunoassays have usually been employed for this purpose. The procedure involves immobilizing one antibody (the so-called 'catcher' or 'capture' antibody) to a solid phase such as the surface of wells in microtitre plates. Serum, suitably diluted, is added and after antigen binding and plate washing, a second anti-mucin monoclonal antibody (radiolabelled or enzyme-linked—the 'tracer' antibody) is used to quantitate bound antigen. The 'catcher' and 'tracer' antibodies may be the same monoclonal antibody provided that the relevant epitope is a repeated structure within the mucin (as may be the case both for epitopes expressed within the oligosaccharide side chains and for those found within the protein core). Alternatively, various combinations of pairs of antibodies, recognizing different epitopes, have been evaluated.

In homologous immunoassays (where the 'catcher' and 'tracer' antibodies are the same), elevated levels of circulating mucins have been detected in breast cancer patients using the anti-milk fat globule membrane antibodies HMFG-1, HMFG-2 [37], 115D8 [38–40] and W1 [24], and also in tests using the anti-tumor antibodies NCRC-11 [19], DF3 [41] and 3E1.2 [42]. Heterologous immunoassays have been developed using various combinations of antibodies (for example, 115D8 + DF3 [43], BC4E 549 + BC4N 154 [44], M29 + M38 [24]) and even polyclonal and monoclonal antibodies have been brought together in the same assay [45]. With each of these immunoassays, serum from patients with metastatic breast cancer showed raised levels of circulating antigen as compared with groups of non-malignant controls. The sensitivity for detection of elevated antigen levels in these investigations has been reported to be from around 30% up to 90% of patients although direct comparisons are somewhat difficult since there is no commonly used criterion for establishing the upper limit of the range for normal controls. Indeed, the choice of individuals comprising the 'normal' control group varies from 'normal female medical students' and 'healthy volunteers' to 'patients with benign breast disease'. The smoking habits of serum donors are considered to have little or no influence on marker levels [39, 42].

Despite the difficulties in establishing values for the normal cut-off, it is a general finding that elevated levels of epithelial mucins in the circulation of breast cancer patients' sera are indicative of extensive malignant disease, and rising or falling levels may reflect progression or regression of tumours [39, 42, 45]. Furthermore, measurement of mucin glycoprotein is considered to be at least as good as, and usually superior to, CEA determination [43, 45]. Hence, International Cis (France) who have produced the first assay kit for circulating mucins using the antibodies 115D8 and DF3 (the so-called CA 15-3 assay) recommend that measurement of the two markers, mucins and CEA provides an excellent combination for monitoring breast cancer, both with respect to early detection of metastases or recurrences and an appreciation of therapy efficiency.

Experience with the anti-breast carcinoma antibody, NCRC-11, has illustrated the potential of this reagent for measuring mucin glycoproteins in serum immunoassays. In the preliminary investigations, over 40% (28/69) of samples from advanced breast cancer patients displayed elevated levels of NCRC-11 antigen compared with 0/60 controls [19]. Subsequent tests have shown sera from patients with benign breast disease are not raised with respect to mucin glycoprotein and that mean levels of NCRC-11 antigen increase with increasing extent and severity of disease. There was no correlation between the NCRC-11 antigen levels and those of CEA which would indicate that the two markers could be used in combination to increase the accuracy in defining clinical status. Finally, the NCRC-11 antigen has been purified from advanced breast cancer patients' sera and identified as characteristic high molecular weight mucin glycoprotein [20]. This affords the oppor-

tunity of preparing new antibodies which may be selected for their reactivity with epitopes preferentially associated with serum mucins and these antibodies should be of particular use for the further refinement of serum assays for breast cancer.

MUCINS AS TARGETS FOR TUMOUR IMAGING

The fact that tumour-associated mucin glycoproteins are to be found in the circulation of cancer patients might be expected to limit their use as targets for tumour localization using radiolabelled antibodies. However, this may not necessarily be the case and anti-mucin antibodies have been employed for radiodiagnostic imaging of tumours. For example, the anti-HMFG antibody, M8, labelled with ^{111}In localized to primary breast tumours as well as their skeletal metastases, although soft tissue metastases were not identified [46]. Since breast tumours and their local recurrences are close to the central blood pool external radiodiagostic imaging is not without its difficulties. It may be possible to improve the resolution of tumours here by using antibody fragments since these will be cleared more rapidly from the circulation.

Ovarian tumours often express the mucin antigens common to breast tumours so that many of the anti-mucin glycoprotein antibodies have application in ovarian cancer [18, 37, 45, 47]. Ovarian tumours have been successfully detected by external imaging techniques in patients administered with [^{123}I]HMFG-2 antibody [48] and this procedure was further used to assess the response to chemotherapy and to provide evidence of the recurrence of known ovarian cancer [49]. HMFG-2 antibody has been employed as a vehicle to deliver therapeutic doses of radiation to tumours by administration directly into the appropriate body compartment [50]. This approach was continued with some success for the treatment of pleural and pericardial effusions [51]. The potential efficacy of 'guided' radiotherapy with [^{131}I]HMFG-2 antibodies for treatment of recurrent ovarian ascites was stressed by Ward *et al.* [52] although the limitations of the approach for treatment of solid tumours were emphasized.

FINAL COMMENT

High molecular weight epithelial mucins are proving to be of biological significance and clinical value in breast cancer. While these complex molecules are not tumour specific, but rather are related to differentiation of the tissue of origin, it is still feasible that there are epitopes, most likely in the oligosaccharide side chains, which are preferentially associated with the malignant state. Kenemans *et al.* [53] have emphasized that definition and exploitation of such determinants using monoclonal antibodies may offer a means to obtain greater sensitivity and specificity in immunodiagnostic assays. Even so, with the limitations in the tests available at the present time, it is apparent that the epithelial mucins that have been the subject of this report, have much to commend themselves as markers of breast cancer.

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