Selection of Tumor-specific Epitopes on Target Antigens for Radioimmunotherapy

of Breast Cancer

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Abstract

Evidence is presented for two different breast epithelial antigens that some epitopes have greater tumor specificity and are more effective targets for radioimmunotherapy than others. The two antigens, which are major components of the human milk fat globule membrane, are breast mucin and a Mr 46,000 glycoprotein (BA46). Of five monoclonal antibodies (Mc5, Mc1, BrE-1, BrE-2, and BrE-3) against breast mucin, all recognize overlapping amino acid epitopes on the tandem repeat domain. However, each has unique and different tissue and tumor specificities and unique epitope structures on the fully glycosylated breast mucin. In preclinical studies, radioimmunoconjugates of all five monoclonal antibodies inhibit growth of transplantable breast tumors in immunodeficient mice. In human clinical trials, radiolabeled Mc5 was very poor in localizing breast tumor metastases. On the other hand, 111In-labeled BrE-3 imaged almost 90% of breast tumors and showed promise in radioimmunotherapy when labeled with 125I. The failure of Mc5 in clinical trials may be partly attributed to the high levels of its epitope on circulating mucin compared to the epitope of BrE-3. The Mc5 binding affinity increased significantly with glycosylation, while the BrE-3 epitope was masked by glycosylation. The BA46 glycoprotein is a breast tumor-associated membrane antigen containing an NH2-terminal, epidermal growth factor-like domain into which a cell adhesion sequence (RGD) is inserted and a COOH-terminal domain with homology to the phospholipid binding C1/C2 domain of coagulation factor V and VIII. It promotes cell attachment in an RGD-dependent manner. Monoclonal antibody Mc8, which binds to the C2-like domain, is only moderately effective in experimental radioimmunotherapy, while Mc3, which binds an epitope in the EGF-like RGD domain, was highly effective in destroying breast tumors in nude mice. With 125I-labeled Mc3, 6 of 7 mice are cured of the tumors. These results indicate that by selecting appropriate monoclonal antibodies, a normal antigen can be used as a target for radioimmunotherapy.

Introduction

The ideal MAb1 for use as a targeting agent for therapy of breast cancer would be one that targeted a tumor-specific antigen. However, the identification of such truly breast tumor-specific antigens has been elusive. Our approach has been to develop MAbs against normal breast epithelial antigens and select epitopes that are preferentially expressed in breast carcinomas. The two antigens described here are both major components of the normal HMFG membrane, which is derived from the apical surface of the breast epithelial cell during lactation.

The first antigen is a highly glycosylated, large molecular weight glycoprotein referred to as breast epithelial mucin. The breast mucin is highly immunogenic (1) in that the majority of MAbs that have been prepared against breast carcinoma cells, their membranes, and HMFG membranes have been found to identify epitopes on this molecule (1, 2). Nearly all MAbs that bind to the core peptide (encoded by the MUC-1 gene; Ref. 3) have epitopes on an internal tandem repeat domain of the breast mucin (4). In spite of this, however, they differ considerably in their biodistribution in normal and tumor tissues (1).

The second antigen is a Mr 46,000 glycoprotein (BA46) that, through cDNA sequencing and cell binding assays, appears to be a cell membrane-associated adhesion molecule. We have developed numerous MAbs against these two HMFG antigens and present evidence here that for both antigens, different epitopes can differ in their effectiveness as targets for radioimmunotherapy.

Materials and Methods

Cloning of the BA46 cDNA. The partial cDNA sequence published previously for the Mr 46,000 HMFG antigen (BA46; Ref. 5) was obtained from a normal lactating human breast Agtl cDNA library (Clonetech, Palo Alto, CA). The complete sequence was obtained by screening a ZR75 Agtl breast carcinoma cell cDNA library (Clonetech, Palo Alto, CA) and by PCR amplification from the normal lactating human Agtl library.

Epitope Mapping. Overlapping peptide hexamers, spanning residues 330–382 of the BA46 sequence and the 20-amino acid tandem repeat of the breast mucin sequence, were synthesized onto the ends of polystyrene pins using the Epitope Scanning Kit (Cambridge Research Biochemicals, Cambridge, United Kingdom; Ref. 6). Binding of the MAbs to the hexamer peptides was tested using the ELISA method with horseradish peroxidase-conjugated goat antimouse IgG (Promega, Madison, WI) and color development with 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, MO). Also, a cDNA construct containing the EGF-like RGD domain was prepared and expressed in Escherichia coli as inclusion bodies (7). The recombinant protein was bound to microtiter plates and tested for binding with MAbs against BA46 using a secondary radioiodinated goat anti-mouse IgG.

Preparation of HMFGs and Immunofluorescence Staining. For preparation of HMFGs, PBS was layered on top of human milk and centrifuged at 4°C at 10,000 × g. The cream fraction that floated up through the PBS was then collected and resuspended in PBS at its original concentration in milk. Immunofluorescent staining of the human milk fat globules and transfected cell lines was done using MAbs Mc3, Mc8, and Mc16, followed by FITC-conjugated rabbit anti-mouse IgG, as described previously (8).

Purification of BA46 Adhesion Molecule. Fresh human milk was centrifuged at 2,000 × g for 5 min at 4°C, and the cream fraction was removed, resuspended in PBS, and centrifuged again. The washed cream was resuspended in 2% Triton X-114 in PBS and incubated at 37°C for 1 h with occasional vortexing. It was then centrifuged at 10,000 × g for 20 min at 4°C, the top fat layer was removed, and the supernant was saved, discarding the pellet. The protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Cell Adhesion Assays. Adhesion assays of 3T3-L1E mouse fibroblasts were performed basically as described by others (9). The HMFGs, suspended in PBS diluted 1:500 from its original concentration in milk and Triton X-114 (1 mg/ml) diluted 1:1000, were added to untreated polystyrene, 96-well microtiter plates with flat-bottomed wells (Nunc, Naperville, IL) in duplicate (60 µl/well) and were allowed to bind overnight at 4°C. Control solutions PBS and 0.9% BSA were tested in parallel. On the next day, the solutions were removed, and the wells were blocked with DME containing 1% BSA at 37°C for approximately 45 min. 3T3-L1E cells were cultured in DMEM (Sigma), 10% fetal bovine serum, and 1% penicillin/streptomycin solution. On the day before the assay, DME + 1% penicillin/streptomycin solution was added to the cells. The next day, 3T3-L1E cells were removed from the flasks with 0.02% EDTA and suspended in DME + 1% BSA; then 106 cells in the presence of different concentrations of GRDGS or GRQESP peptides were added to the wells and incubated at 37°C for 1 h. Unbound cells were removed by washing twice with PBS. Bound cells were then fixed with 1% acetic acid in methanol.

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3 The abbreviations used are: MAb, monoclonal antibody; HMFG, human milk fat globule; EGF, epidermal growth factor; MXDTPA, 1-p-iodoquiatoxybenzyl-3-methyl diethylene triminepentacetic acid; RGD, arginine-glycine-aspartic acid.
Determination of Antibody Affinity. The affinities of the MAbs for the native antigen and fusion proteins were determined by an antibody displacement method described by Sheldon et al. (12). The antigen was bound to the solid phase of microtiter wells, and a constant amount of $^{125}\text{I}$-labeled MAb was added with increasing amounts of unlabeled MAb. The molar concentration of unlabeled MAb at which 50% maximum binding of labeled MAb occurred was taken as the dissociation constant ($K_d$) of the MAb. The affinity constant was calculated as $1/K_d$.

Results and Discussion

Epitopes of the Tandem Repeat of Breast Mucin and Radioimmunotherapy. Five MAbs [Mc5, BrE-1, BrE-2, BrE-3, and Mc1 (also known as HMFG-2)] against breast mucin have all been shown to be effective in arresting growth of breast tumors transplanted in immunodeficient mice (11, 13, 14). All of these MAbs recognize overlapping linear amino acid sequences on an immunodominant region comprising an 8-amino acid sequence of the 20-amino acid tandem repeat of breast mucin (Fig. 1). In fact, virtually all MAbs that recognize the core protein (MUC1) of the mucin (3) bind to this most hydrophilic region of the tandem repeat (4). In spite of this, the different MAbs exhibit considerable differences in their binding in immunohistochemistry among both normal and tumor tissue (1), exhibit cell heterogeneity of expression among and within breast tumors (15), and differ in their binding to circulating mucin. This epitope heterogeneity is apparently due to alterations in glycosylation that occur in breast carcinomas and selective release of a more glycosylated mucin (see below).

To investigate the epitope heterogeneity of the tandem repeat domain and the role of glycosylation on epitopic expression, competition studies were performed with these five MAbs on the native fully glycosylated mucin. GVTSAAPDTTRPGSTAPPHGVTSA

**Fig. 1.** Tandem repeat amino acid sequence (delineated between vertical lines) showing the linear amino acid epitopes for MAbs Mc1, Mc5, BrE-1, BrE-2, and BrE-3 determined by epitope mapping (6). * sites of potential O-linked glycosylation.

**Fig. 2.** Competition between MAbs against overlapping epitopes on the mucin tandem repeat on the fully glycosylated breast mucin in the HMFG membrane (A and C) and to a synthetic tandem repeat peptide (APPAHGVTSAPDTTRPGST; 125 ng/well) as described previously (10). MAbs Mc5 or BrE-3 were iodinated using the chloramine-T method, as described previously (11). Approximately $2 \times 10^6$ cpm of $^{125}\text{I}$-labeled Mc5 or BrE-3 were mixed with different concentrations of the unlabeled MAbs in RIA buffer (PBS, 0.3% Triton X-100, and 10% calf serum), added to the coated wells, and incubated overnight at room temperature. The wells were then washed extensively with RIA buffer, and the radioactivity was determined in each well cut from the plate.

**Fig. 2.** Competition between MAbs against overlapping epitopes on the mucin tandem repeat on the fully glycosylated breast mucin in the HMFG membrane (A and C) and to a synthetic peptide of the 20-amino acid tandem repeat (APPAHGVTSAPDTTRPGST) (B and D). A and B. $^{125}\text{I}$-labeled MAb Mc5 with competing amounts of unlabeled MAbs. C and D. $^{125}\text{I}$-labeled MAb BrE-3 with competing amounts of unlabeled MAbs. * Mc5; $\triangle$, Mc5; $\bigtriangleup$, BrE-1; $\blacktriangle$, BrE-2; $\blacklozenge$, BrE-3; $\blacklozenge$, Mc1 (also known as HMFG-2). See "Materials and Methods" for experimental details.
glycosylated mucin from HMFG and a nonglycosylated synthetic tandem repeat peptide (Fig. 2). When MAb Mc5 was labeled with 125I, cold unlabeled Mc5 competed for binding to the native breast mucin, while none of the other MAbs competed (Fig. 2). In contrast, on a synthetic peptide of the tandem repeat, equivalent competition was seen for cold Mc5, Mc1, BrE-2, and BrE-3. BrE-1 also competed but to a lesser degree. When BrE-3 was labeled, cold Mc1, Mc5, and BrE-1 showed little competition for binding to HMFG. However, BrE-2 competed equally or better than cold BrE-3 itself. This latter competition result is consistent with the observation that both the latter MAbs recognize the amino acid sequence TRP on the tandem repeat (Fig. 1), although their tissue distributions are different (1). When the competition was done using the synthetic peptide, cold Mc5, Mc1, BrE-2, and BrE-3 all competed equally well with labeled BrE-3, while BrE-1 did not. The lack of competition with BrE-1, although the amino acid epitope overlaps with BrE-3, could be due to its lower affinity for the peptide. It is interesting to note that BrE-1 is the most breast tumor specific of the five MAbs in that it shows no binding at all to normal breast (1). These competition results indicate that there is heterogeneous glycosylation of the tandem repeats in the native breast mucin, resulting in an array of unique and different epitopes identified by these MAbs. The distribution and prevalence of these epitopes appear to be different in breast tumor cells due to alterations in glycosylation, as described below.

Since Mc5 was the most extensively studied preclinically and since it bound strongly to breast carcinomas, it was our first candidate for human clinical trials. One advantage of the tandem repeat domain as a target for MAb therapy is its polyepitopic nature, thus providing multiple targets on the surface of breast cancer cells. Also, because of the cell heterogeneity of expression of such epitopes on breast carcinoma cells, radioconjugated MAbs are preferable, since every cell need not bind the MAb for achieving cytotoxicity for the localized tumor (11). As a prerequisite for radioimmunotherapy, clinical imaging trials were initiated in which 12 breast cancer patients were injected with 131I-labeled Mc5 (16). Disappointingly, in only one patient was there any significant imaging of metastatic disease (16). In an effort to explain this lack of imaging, serum levels of the Mc5 epitope on circulating antigen were determined, and it was found that all patients had detectable levels in their serum. In contrast, in the same serum samples from these patients, the level of the BR-E-3 epitope was 5-10-fold lower (4).

Therefore, differences between the epitopes of Mc5 and BrE-3 were explored further. Epitope mapping of the polypeptide epitopes of Mc5 and BrE-3 on the tandem repeat region of the breast mucin revealed that they have overlapping linear amino acid sequences of DTPAP and TRP, respectively, in their epitopes (Fig. 1; Ref. 17). In spite of this, the binding affinity of Mc5 was significantly affected by the degree of glycosylation of the breast mucin in that the affinity on the fully glycosylated, mature breast mucin isolated from HMFG was significantly higher (disassociation constant, 3.63 x 10^-9) than on the nonglycosylated mucin (hydrogen fluoride method; 1.62 x 10^-8) or on the nonglycosylated core protein produced in bacteria as a fusion protein (1.0 x 10^-7) (4, 7). This indicated that oligosaccharide structures on the mature mucin are either part of the Mc5 epitope or glycosylation alters the polypeptide configuration to yield the highest affinity peptide epitope structure on the breast mucin. In contrast, the affinity constant for BrE-3 was not affected by the degree of glycosylation of 1.66 x 10^-8 for the mature mucin, 1.56 x 10^-8 for the deglycosylated mucin, and 1.15 x 10^-8 for the fusion protein. Mc5 bound strongly to both normal breast (apical staining only) and breast carcinomas (membrane and cytoplasmic staining), while BrE-3 bound strongly to breast carcinomas (membrane and cytoplasmic staining) but only weakly to normal breast epithelial cells (1).

Because BrE-3 stained the majority of breast carcinomas immunochemically and its epitope was expressed at relatively low levels on circulating mucin compared to the Mc5 epitope, clinical trials with BrE-3 were initiated. In this case, the imaging trials were conducted with 111In-labeled BrE-3 using an MXDTPA chelate (18). Very successful imaging was obtained, where 86% of all known lesions were localized (18). In radioimmunotherapy trials using 90Y-MXDTPA-labeled BrE-3, with doses below maximal tolerable doses, there was a partial response in two of six patients (19), while supralethal doses with bone marrow support showed a partial response in 50% of the patients (20). The most straightforward interpretation of these results is that the cell-associated mucin is less glycosylated than that which is released into the circulation. Thus, Br-E-3, which identifies a less glycosylated tumor-associated mucin, is better for therapy, while Mc5, which identifies the more glycosylated mucin that is released into the circulation, is better as a diagnostic reagent for construction of serum assays (21).

Epitopes on the Adhesion Molecule, BA46, and Radioimmunotherapy. A novel antigen that we have identified in the HMFG membrane and that is expressed in breast carcinoma cells is a Mr 46,000 glycoprotein (BA46), recognized by four MAbs: Mc3, Mc8, Mc15, and Mc16 (1). The BA46 cDNA (to be published elsewhere) has a single open reading frame that codes for a polypeptide consisting of an NH2-terminal signal peptide sequence, followed by an EGF-like domain into which a cell adhesion sequence (RGD) is inserted, and a COOH-terminal domain with homology to the phospholipid binding C1/C2 domain of coagulation factors V and VIII (5).

Although the derived amino acid sequence suggests a soluble glycoprotein, BA46 is a major component of the HMFG membrane (22), separates in the membrane fraction of breast carcinoma cells (23), and is expressed on the surface of mammalian cells transfected with the complete BA46 cDNA. Also, it separates in the detergent phase, using Triton X-114 fraction procedures on breast cell lines (5). If HMFG is fractionated with Triton X-114, coagulation factor V is sedimented, while integral membrane proteins with transmembrane domains (e.g., breast mucin and butyrophilin) are found in the pellet. The COOH-terminal C1/C2-like domain is possibly involved in its membrane association. Other proteins have also been found to contain Factor V/VIII C-type domains (5, 24–26), including glycoproteins of milk.

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4 Unpublished data.
fat globules of mice, guinea pigs, and cattle (27, 28), that also appear to lack any transmembrane domain. The mechanism of membrane association for BA46 may involve covalent linkage to phospholipids or fatty acids. The C1/C2 domain of coagulation factors V and VIII appears to be involved in phospholipid binding, a necessary interaction involved in coagulation (29).

The cell adhesion sequence RGD (30) appears to be functional in that BA46 can mediate cell attachment to a solid surface. Both washed milk fat globules from human milk and nonionic detergent (Triton X-114)-extracted HMFG membrane proteins can promote attachment of mouse 3T3 cells to treated plastic surfaces, and this attachment is inhibited specifically with an RGD peptide (Fig. 3). Since these preparations were a mixture of several proteins besides BA46, BA46 was further purified from delipidated human milk fat globule membranes using preparative SDS-PAGE. This isolate BA46, as well as a complete recombinant BA46 produced and purified from BA46 transfected E. coli, also promoted 3T3 cell attachment in an RGD-dependent manner. These results suggest that BA46 is an adhesion protein that could interact with specific receptors on cell-associated integrins (30).

Two MAbs (Mc3 and Mc8) against BA46 were compared for their effectiveness in experimental radioimmunotherapy of human breast tumors transplanted in immuno-deficient mice. Mc8 was only moderately effective compared with Mc3, which was surprisingly effective (4). In experimental biodistribution studies, as much as 72% of 111In-MX-DTPA-labeled Mc3 was found to localize to the transplanted tumors. In experimental radioimmunotherapy studies, both 131I- and 90Y-labeled Mc3 were found to inhibit growth of the transplanted tumors. Moreover, with the latter radioimmunoconjugate, 6 of 7 of the mice were cured of their tumors (4).

To investigate the molecular basis for the different radioimmunotherapy effectiveness of these two MAbs against the BA46 antigen, additional studies were undertaken on their epitopes. Epitope mapping (6), using consecutive overlapping hexamer peptides encompassing the 53 amino acids of the COOH-terminal end (all within the C2-like domain), identified the epitopes of Mc8 and Mc16 as adjacent sequences DPRTG and SKKIF, respectively (Fig. 4). MAb Mc3 did not bind to any of these peptides. However, Mc3 bound to recombinant polypeptides produced in transfected E. coli, encompassing the entire mature BA46 amino acid sequence, as well as only the EGF-like RGD domain. Thus, all three MAbs bound to the polypeptide moiety of this glycoprotein: Mc3 to the EGF-like/RGD domain and Mc8 and Mc16 to adjacent linear amino acid sequences in the C2 domain (Fig. 4). The epitope of Mc3 did not appear to be in the cell adhesion sequence (RGD), since it would not inhibit recombinant BA46-promoted cell attachment.

The orientation of the BA46 antigen in the membrane was studied using indirect immunofluorescence. The surface of the HMFG stained in a punctuated or granular fashion, with both Mc3 and Mc8 using a secondary fluorescein-labeled anti-mouse IgG. However, Mc16 did not stain HMFGs. Similarly, surface staining of BA46-transfected mouse myeloma (SP2/0) and Chinese hamster ovary (CHO) cells was obtained with both Mc3 and Mc8 but not with Mc16. Untransfected control cells did not stain with any of the MAbs. In addition, the BA46 antigen was detected readily in untreated culture medium of BA46-transfected SP2/0 cells with both Mc3 and Mc8, using radioimmuno-binding assays, but hardly at all with Mc16. However, if the HMFG, BA46-transfected cells or culture medium of the transfected cells were extracted with SDS and analyzed by Western blotting, all three MAbs detected the antigen. These results suggest that the Mc16 epitope is masked when BA46 is associated with the membrane of HMFG and the transfected cells, while Mc3 and Mc8 are exposed. Also, it appears that the membrane-association site is proximal to the Mc16 epitope toward the COOH-terminal. Furthermore, a portion of the BA46 antigen released by the transfected cells is particulate, possibly associated with membrane fragments or micelles, since at least 50% of the released BA46 can be pelleted at 10,000 × g. These results suggest that BA46 antigen is oriented on the cell surface with attachment at the C2 domain and the EGF-like RGD domain exposed on the surface. The greater radioimmunotherapy effectiveness of Mc3 may be related to the positioning of its epitope on the membrane and/or its binding to a potential function domain of the adhesion molecule. The Mc8 epitope may be less accessible due to its proximity to the membrane attachment site.

These results demonstrate that with two different breast epithelial antigens, breast mucin and BA46 antigen, different epitopes on the same molecule can have considerable differences in their effectiveness as targets for radioimmunotherapy. In the case of the breast mucin, these differences are probably due to the role of oligosaccharides in the epitopes resulting from alterations in glycosylation that occur in breast carcinoma cells. In the case of the BA46 glycoprotein, it seems to be due to binding of the MAbs to different functional domains and the orientation of the molecule on the cell surface. The two MAbs that showed the most favorable biodistributions and effectiveness in radioimmunotherapy, BrE-3 for breast mucin and Mc3 for BA46, have both been humanized (31–33), and these humanized forms show identical affinities and biodistributions in an experimental mouse model (34) compared to their mouse counterparts. These detailed epitope structural studies clearly show that it is possible to develop MAbs against normal breast differentiation antigens that have appropriate breast tumor specificity for radioimmunotherapy.

References
BREAST TUMOR EPITOPES FOR RADIOIMMUNOTHERAPY


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