Genetically Determined Polymorphism of the Circulating Human Breast Cancer-Associated DF3 Antigen

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The murine monoclonal antibody (MAb) DF3 was prepared against a human breast carcinoma. Previous studies have demonstrated that DF3 antigen levels are elevated in plasma of patients with breast cancer. Furthermore, MAb DF3 reacts with circulating glycoproteins of different molecular weights ranging from approximately 300 to 450 kd. The present study demonstrates that plasma DF3 antigen is comprised of at least four moieties with slow (S), intermediate (I), rapid (R) and very rapid (VR) electrophoretic mobilities. The electrophoretic mobility patterns for circulating DF3 antigen differ among individuals. Moreover, breast tumors and human milk. These findings suggested that the size difference for DF3 antigen could be due to a genetically determined polymorphism.

Previous studies have demonstrated a genetically determined polymorphism for a series of mucin-type high molecular weight glycoproteins detectable in human urine. This genetic polymorphism has also been detected in urine using MAb DF3 light chain.

In the present study we demonstrate that a similar genetic polymorphism accounts for the different molecular weight species of DF3 antigen detectable in plasma. DF3 antigen is detectable in urine, and the electrophoretic mobility of the urinary moieties is similar, but not identical, to that in the plasma. Studies in family members suggest that the electrophoretic heterogeneity of plasma DF3 antigen is determined by codominant expression of multiple alleles at a single locus. This locus may code for the core protein of DF3 antigen. These findings thus identify a genetically determined polymorphism of a circulating tumor-associated glycoprotein.

MATERIALS AND METHODS

Clinical materials. Plasma was separated from peripheral blood samples collected in EDTA-treated tubes. A malignant pleural effusion was collected from a patient with metastatic breast cancer. The effusion was centrifuged at 4,000 g for 20 minutes to remove cells and debris. Urine samples were centrifuged at 3,000 g for ten minutes and concentrated by ultrafiltration through an Amicon Diaflow membrane (cut-off:10,000 daltons). Human skim milk was separated from cream by centrifugation at 3,000 g for 30 minutes at 4°C. All samples were frozen at -70°C until analysis.

Immunoblotting. Plasma, urine, and/or milk from selected subjects was analyzed on 3% to 10% polyacrylamide gels in the presence of 4% dodecyl sulfate (SDS) and 3-mercaptoethanol. Transfer of the polyacrylamide gel to nitrocellulose paper was performed using a Bio-Rad Transblot apparatus with 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol buffer (pH 8.3). The nitrocellulose paper was incubated with MAb DF3 IgG and then with 125I-labeled sheep IgG antimouse immunoglobulin (Amersham, Arlington Heights, IL). Electrophoretic mobilities of the MAb DF3 reactive antigens were monitored by autoradiography. Myosin (200 kd), beta-galactosidase (116 kd), and phosphorylase (92.5 kd; Molecular Weight Standards-High, Bio-Rad Laboratories, Richmond, CA) were used as molecular weight markers.

Cell culture and extract preparation. The human breast carcinoma cell line ZR-75-1 was grown as a monolayer in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% HEPES. The cells were maintained in logarithmic phase, harvested, suspended in phosphate-buffered saline (PBS, pH 7.1), and disrupted by sonication. The resulting homogenate was used for analysis. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories).
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RESULTS

We previously demonstrated the heterogeneous electrophoretic mobility of MAb DF3 reactive antigens in plasmas from breast cancer patients and normal subjects. Although MAb DF3 reactivity was found predominantly with one or more antigens of molecular weights ranging from 300 to 450 kD, the results of that study pertained to the higher levels of DF3 antigen in patients with metastatic breast cancer. In the present study we have examined the heterogeneity of DF3 antigen expression to determine whether certain patterns are detectable in different individuals.

Plasma samples from breast cancer patients were monitored by transblot analysis for variations in electrophoretic mobility of the DF3 antigens. The results demonstrate several DF3 antigen phenotypes (Fig 1A). Certain individuals with a single MAb DF3 reactive species had either a relatively rapid (R, lanes 1, 3, 6, 14) or slow (S, lanes 4, 7, 12, 13) electrophoretic mobility. Other subjects had circulating DF3 antigens with both the slow (S) and rapid (R) mobility patterns (lanes 2, 5, 8, 10). Furthermore, one individual expressed another MAb DF3 reactive antigen with an electrophoretic mobility intermediate (I) between that of the S and R moieties (Lane 9). In this patient the I moiety was detected in combination with the R mobility DF3 antigen. Another individual expressed the S moiety and a fourth MAb DF3 reactive antigen that migrated very rapidly (VR, lane 11). Furthermore, certain DF3 antigens with S, R, or VR electrophoretic mobilities were comprised of two distinct components. Taken together the results suggest that DF3 antigen consists of moieties with at least four distinct electrophoretic mobilities and that further heterogeneity may exist within these species. Finally, the S-R electrophoretic mobility pattern was detected in the ZR-75-1 breast cancer cell line (Fig 1B).

The electrophoretic pattern of plasma DF3 antigen was compared to that detected in a malignant pleural effusion from a patient with metastatic breast cancer (Fig 2A). These
patterns were comparable and consisted of DF3 antigens with S and R mobilities. Although a similar pattern was observed in the urine of this patient, the urinary R band did not migrate as rapidly as either the plasma or pleural effusion R band (Fig 2A). We also compared the pattern of circulating DF3 antigen with that in milk and urine from a lactating woman. In this subject the electrophoretic mobilities of DF3 antigens in plasma and urine were similar (phenotype S-R, Fig 2B). However, as in the patient with breast cancer, the R band in the urine migrated slightly slower than that in plasma. Moreover, the DF3 antigen in milk from this subject consisted of bands with slow (S) and intermediate (I) mobility (Fig 2B). These findings would suggest that the DF3 antigen polymorphism detected in plasma, urine, and milk may be similar to the polymorphism previously described for high molecular weight mucins in urine.9,10

The electrophoretic heterogeneity of circulating DF3 antigen could be genetically determined. Consequently we have studied patterns of DF3 antigen expression in a patient with metastatic breast cancer and ten of her family members. Members of this family expressed DF3 antigens with S, I, and R electrophoretic mobilities (Fig 3). The DF3 antigen phenotype for the patient with breast cancer (subject 6) and her husband (subject 7) was S-R. One daughter (subject 11) had the same S-R mobility pattern as her parents, while the other daughter (subject 10) had a DF3 antigen with only the R mobility pattern. Thus subject 10 was homozygous for the R mobility DF3 antigen. Furthermore, the DF3 antigen phenotype for subject 8 was I-R, while that for her father (subject 2) was S-I. This would suggest that subject 8 inherited DF3 antigen with the I, but not the R, electrophoretic mobility from her father (Fig 3). Thus the data illustrated in Fig 3 suggest that DF3 antigen polymorphisms are genetically determined and expressed in an autosomal, codominant fashion.

The findings in this single family led us to further examine the possibility of a genetic basis for the DF3 polymorphisms. The electrophoretic mobility patterns of DF3 antigen were determined by Western blot analysis of serum from members of 17 additional Caucasian families (Table 1). The findings confirm that the DF3 antigens are inherited as codominant autosomal alleles. In this regard the observed DF3 antigen phenotypes agreed with those expected from the Hardy-Weinberg equilibrium with random allele segregation. In one family both parents were homozygous for the S mobility DF3 antigen. The only offspring also displayed this phenotype. DF3 antigens with S and R mobilities were expressed most frequently in 56 unrelated individuals. The relative frequencies of the four alleles were: S, 45%; I, 13%; R, 38%; and VR, 4%. Therefore the S-R mobility pattern was the commonest phenotype. This phenotype was expressed in 4 of 14 (29%) unrelated patients and in 47 of 122 (38%) individuals from 21 of 23 (91%) families.

**DISCUSSION**

We have previously demonstrated that DF3 antigen in human breast tumors and milk is comprised of high molecular weight mucin-like glycoproteins with sizes ranging from approximately 300 to 450 kDa.4 The DF3 antigen is susceptible to neuraminidase and protein digestion.7 These results have suggested that sialyl polysaccharides present on a peptide backbone are required for maintaining DF3 antigenicity. The heterogeneity of DF3 antigen in terms of electrophoretic mobility has been detected in human breast tumors, human breast cancer cell lines, and human milk.1,2,4 A similar heterogeneity was previously found in plasma of breast cancer patients and normal subjects.7 Patients with breast cancer and certain other carcinomas had detectably higher levels of DF3 antigen, although the electrophoretic mobilities were similar to those in normal subjects.6,7,11 These findings suggested that the pattern of DF3 antigen expression may be genetically determined.

DF3 antigen has at least four different electrophoretic mobilities, which we have designated slow (S), intermediate (I), rapid (R), and very rapid (VR). Further variations may exist within each of these categories in that certain subjects had two detectable components for each of the antigens. The patterns of DF3 antigen expression among family members suggest that the variation in electrophoretic mobility is related to a genetically determined polymorphism. Furthermore, these data suggest that DF3 antigen production is controlled by multiple alleles at a single locus expressed in an autosomal codominant fashion.

The heterogenous electrophoretic mobility of DF3 antigen could be related to differences in core proteins, glycosylation, or both. The separate alleles might code for core proteins with different sizes. In contrast, core proteins of similar size but with certain amino acid substitutions might direct alternative glycosylation patterns and thereby result in varied electrophoretic mobilities. The heterogeneity within alleles coding for the S, I, R, or VR moieties could be related to

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**Fig 3. Relationship of DF3 electrophoretic mobilities among family members.** (A) Family pedigree and schematic representation of DF3 antigen mobilities for each member. (B) Actual immunoblot results obtained for plasma samples (6 μL) from the members. Electrophoretic mobilities are categorized as slow (S), intermediate (I), and rapid (R). Lane numbers correspond to subject designation in family pedigree. Subject 6 is a patient with metastatic breast cancer.
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Table 1. DF3 Antigen Types in Families

<table>
<thead>
<tr>
<th>Parental Types</th>
<th>No. of Families</th>
<th>Offspring Types</th>
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<td>S-S x S-S</td>
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<td>S-S I-I R-R VR-VR</td>
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<td></td>
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<tr>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>S-I x R-VR</td>
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<td>(0) (0) (0) (0) (0) (1) (1) (1) (1) (1) (0)</td>
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<tr>
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<tr>
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<td>S-R x S-R</td>
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</tr>
</tbody>
</table>

Table 1.


References

References

- tissue specific glycosylation patterns. For example, the electrophoretic mobility of DF3 antigen in milk appears to differ from that in plasma from the same individual (Fig 2B). Moreover, the migration of the urinary R band was slightly slower than that in plasma (Figs 2A and B). This heterogeneity within alleles has not been detected in human breast cancer cell lines (Fig 1 and unpublished data). Taken together these findings would suggest that separate alleles code for the core proteins of different sizes and that heterogeneity within the S, I, R, or VR moieties is related to variations in glycosylation patterns. In this regard data with a cDNA probe for the core protein gene of the DF3 antigen suggest that the major differences in electrophoretic mobility (V, I, R, VR) are the result of variations in transcript size.13

Previous studies have demonstrated that mucin-type glycoproteins are detectable in human urine by lectin staining and exhibit a genetically determined polymorphism.9 Several MAbs that recognize high molecular weight mucin-type glycoproteins on malignant cells have been shown to react with the same urinary molecules identified by lectin binding.10 One of these MAbs, designated Cal, reacts with DF3 antigen but at an epitope distinct from the MAb DF3 and with human malignant versus benign breast tumors.9

Although several other plasma proteins exhibit electrophoretic polymorphisms,11 DF3 antigen appears distinct on the basis of (1) electrophoretic migration; (2) presence in human milk; (3) expression on the surface of epithelial cells; and (4) absence on erythrocytes and granulocytes (unpublished data). Thus DF3 antigen appears to represent a previously undescribed circulating polymorphism.
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