Effects of Differentiating Agents on Cell Surface Expression of the Breast Carcinoma-associated DF3-P Epitope

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ABSTRACT

The DF3 antigen is a member of a family of high-molecular-weight glycoproteins aberrantly expressed in human breast carcinomas. Recent work has described the generation of a monoclonal antibody (MAb), designated DF3-P, that reacts with immature, underglycosylated precursors of DF3 antigen. Immunoperoxidase staining studies have demonstrated that MAb DF3-P exhibits selective reactivity with malignant mammary epithelium. Using flow cytometry and live cell radioimmunoassays, the present studies demonstrate that the epitope recognized by MAb DF3-P is expressed on the surface of MCF-7 and other human breast carcinoma cell lines. We also demonstrate that treatment of MCF-7 cells with 12-O-tetradecanoylphorbol-13-acetate, an agent known to induce a more differentiated mammary cell phenotype, is associated with increased expression of the DF3-P epitope. Similar findings were obtained with sodium butyrate. The results indicate that these agents increase both cell surface DF3-P antigen density and the percentage of DF3-P-positive cells. Immunofluorescence studies performed on chamber slides further demonstrate that MAb DF3-P-reactive cells are detectable in small clones or clusters. Similar studies with 12-O-tetradecanoylphorbol-13-acetate- and butyrate-treated cells demonstrate increases in the size and number of these clusters. Taken together, these results indicate that the DF3-P epitope is expressed on the surface of human breast carcinoma cell lines and that the heterogeneity of this expression is related to the presence of differentiating signals.

INTRODUCTION

Previous studies have demonstrated that DF3 antigen and related members of this family of high-molecular-weight glycoproteins are aberrantly expressed in human breast carcinomas (1). DF3 antigen is detectable on the apical borders of secretory mammary epithelial cells and at high levels in the cytoplasm of malignant breast cells (2). This apical and cytoplasmic staining pattern has also been described for MAb3 prepared against human milk fat globule membrane and breast cancer cell lines (3-6). The mature glycoproteins are generated through the synthesis of several lower-molecular-weight precursors. Pulse chase labeling experiments have demonstrated the translation of core proteins ranging in size from M; 160,000 to M; 220,000 (7, 8). Modification at the posttranslational level involves proteolytic cleavage of the peptide core and addition of O-linked glycans (7, 8). Other findings have also suggested the presence of N-linked glycans (7, 8). The demonstration that the major carbohydrate component of the DF3 glycoprotein in BT-20 breast cancer cells is the Thomsen-Friedenreich antigen (Galβ1, 3GalNac) and that this structure is undetectable in the related milk glycoprotein has supported differential glycosylation patterns in transformed mammary cells (9).

Recent work has shown that DF3 antigen expression in Stage II breast carcinomas is predictive of both disease-free and overall survival (10). These findings have suggested that expression of the DF3 epitope may be associated with certain biological characteristics of human breast tumors. Indeed, analysis of primary breast tumors has demonstrated that DF3 antigen expression is related to degree of differentiation and estrogen receptor status (11). Moreover, certain maturational agents, such as TPA and sodium butyrate, have been shown to both induce a more differentiated phenotype in breast cancer cell lines and increase DF3 antigen expression (12, 13). These findings and the demonstration that DF3 antigen is present in human milk (14) have suggested that the DF3 glycoprotein is a differentiation antigen aberrantly produced by malignant mammary epithelium. However, the basis for aberrant expression of this antigen in breast carcinomas remains unclear.

Sequence analysis of complementary DNA clones coding for the core protein of this antigen family has revealed the presence of highly conserved (G + C)-rich 60-base pair tandem repeats (15, 16). These repeats code for epitopes identified by MAb DF3 and other MAbs prepared against the intact glycoproteins or the deglycosylated protein core (15-18). The availability of complementary DNA clones coding for the repeats has also provided an opportunity to prepare antibodies against recombinant nonglycosylated peptide. In that regard, recent studies have demonstrated the generation of one such MAb, designated DF3-P, which binds to an epitope on the protein core (19). In contrast to MAb DF3, MAb DF3-P exhibits little if any reactivity with the mature glycoprotein. The present studies demonstrate that the DF3-P epitope is detectable on the surface of breast cancer cells and that DF3-P antigen expression is regulated by differentiating signals.

MATERIALS AND METHODS

Cell Culture. The human MCF-7, BT-20, ZR-75-1, T47-D, and MDA-MB-231 breast carcinoma cell lines were obtained from the American Type Culture Collection (Bethesda, MD). MCF-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO), while the four other cell lines were grown in RPMI 1640 (Sigma Chemical Co.). The media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 0.25 IU insulin/ml, and 2 mm l-glutamine. ZR-75-1 cells were also grown in the presence of 0.252 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (TPA (10 nm; Sigma Chemical Co.) and sodium butyrate (2 mm; Sigma Chemical Co.) were added 1 day after the cells were plated. The medium was changed every other day.

Live Cell Radioimmunoassay. MCF-7 cells were harvested with a rubber policeman and resuspended in cold PBS. A single cell suspension was obtained by passing the cells through a 21-gauge needle. Dead cells were then separated by centrifugation in a Ficoll gradient (Ficoll-Paque; Pharmacia) Cell viability was monitored by trypan blue exclusion. Viable cells after Ficoll gradient separation accounted for 85-95%
MODULATION OF DF3-P EPITOPE EXPRESSION

of the MCF-7 population. Cells were pelleted and resuspended in 3% BSA in PBS for 1 h at 4°C. Cells (5 × 10⁴) were incubated for 1 h at 4°C with MAb DF3, MAb DF3-P, or isotype-identical control antibodies (IgG1, MOPC 21; IgG2a, UPC 10; Sigma Chemical Co.) diluted in 3% BSA in PBS. After washing with 1% BSA in PBS, cells were then exposed to ¹²⁵I-conjugated rabbit anti-mouse antibody (1 × 10⁶ cpm/5 × 10⁴ cells) for 1 h at 4°C (12).

Cell Surface Binding Assay Using Flow Cytometry. Cells were harvested and reacted with MAb DF3, MAb DF3-P, or isotype control antibodies at 4°C for 1 h. After exposure to FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co.) at 4°C for 1 h, cells were washed and then resuspended in 1% BSA and 5 μg/ml propidium iodide. Fluorescence was determined for 10,000 stained viable cells capable of excluding propidium iodide using an EPICS 750 series flow cytometer (Coulter Electronics, Hialeah, FL).

Cell Surface Binding Assay Using Cells Grown on Chamber Slides. Cells (1–2 × 10⁵) were plated on plastic chamber slides (Lab-Tek; Nunc, Inc., Naperville, IL). Staining with MAbs DF3, MAb DF3-P, or isotype-identical control antibodies was performed at 4°C. After exposure to FITC-conjugated goat anti-mouse antibody, cells were washed with cold PBS and then fixed in formalin for 10 min. Slides were kept in the dark at 4°C until analysis by fluorescent microscopy.

RESULTS

Five human breast cancer cell lines were studied for expression of the DF3-P epitope by immunoblot analysis. MAb DF3-P reacted with extracts of each line, although the intensity of the signal was considerably less for MDA-MB-231 cells (Fig. 1A). The pattern of reactivity also varied considerably among the lines. For example, MAb DF3-P reactivity was predominant against M, ~160,000–250,000 proteins (Fig. 1A). These findings were consistent with binding to core proteins or partially glycosylated species (7, 8, 19). In contrast, binding of MAb DF3 was detectable against the higher-molecular-weight or more mature glycoproteins (Fig. 1B). The MDA-MB-231 cell line was an exception, however, with little if any MAb DF3 reactivity (Fig. 1B). Taken together with the previous demonstration that MAb DF3-P reacts with the DF3 core peptide, while MAb DF3 reacts principally with the glycosylated protein (19), the present results provide support for accumulation of underglycosylated DF3 antigen in breast cancer cell lines.

Previous studies have demonstrated that the DF3 epitope is detectable on the surface of human breast cancer cell lines (12, 20). While these findings are consistent with the properties of a shed antigen, less is known about cell membrane expression of immature precursors of glycoproteins. In order to address this issue, we compared flow cytometry patterns using MAbs DF3 and DF3-P. As previously shown, MAb DF3 reacted strongly with intact ZR-75-1 cells (Fig. 2A). Cell surface reactivity was also detectable when using MAb DF3-P, although the fluorescence intensity was less than that obtained with MAb DF3 (Fig. 2B). Similar studies were performed with the other cell lines. Expression of the DF3 epitope was also detectable on MCF-7, T-47D, and BT-20 cells, while there was little if any MAb DF3 reactivity with the MDA-MB-231 line (Table 1). MAb DF3-P also reacted with MCF-7, T-47D, and BT-20 but not with MDA-MB-231 cells (Table 1). However, in each of the

![Fig. 1. Reactivity of MAbs DF3-P and DF-3 with breast cancer cell lines by immunoblot analysis.](image)

![Fig. 2. Cell surface reactivity of MAbs DF3 and DF3-P.](image)

![Fig. 3. Reactivity of MAbs DF3 and DF3-P by live-cell radioimmunoassay.](image)
Table 1 Reactivity of MAbs DF3 and DF3-P with human breast cancer cell lines by flow cytometric analysis

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MAb DF3</th>
<th>MAb DF3-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>T-47D</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>BT-20</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

* Reactivity: +++, strong; ++, intermediate; +, moderate; -, no reactivity.

Fig. 4. TPA and sodium butyrate increase cell surface expression of the DF3-P epitope. MCF-7 cells (A) were treated with TPA for 3 days (B) or sodium butyrate (butyric acid) for 5 days (C). Reactivity of MAb DF3-P (■) and an IgG2a isotype identical control antibody (○) was determined by flow cytometry.

Four DF3-positive cell lines, the reactivity of MAb DF3-P was less than that observed with MAB DF3 (Table 1). These findings indicated that the DF3-P epitope is expressed on the surface of some but not all breast cancer cell lines.

We and others have previously demonstrated that exposure of MCF-7 cells to TPA or sodium butyrate is associated with the appearance of a more differentiated phenotype and increased DF3 antigen expression (12, 13, 20). Similar live cell radioimmunoassay studies were performed to determine the effects of TPA on cell surface levels of the DF3-P epitope. As demonstrated previously for sodium butyrate (12), TPA treatment was associated with the appearance of an epithelial configuration and projection of cytoplasmic processes (data not shown). TPA also increased expression of both the DF3 (2.3-fold) and DF3-P (3.5-fold) epitopes (Fig. 3A). Similar findings were obtained with sodium butyrate (Fig. 3B). Compared to TPA, sodium butyrate induced similar increases in both DF3 (2.4-fold) and DF3-P (3.5-fold) expression. Furthermore, while the level of MAB DF3 reactivity was higher than that with MAB DF3-P in both control and treated cells, the magnitude of the increases for both DF3 and DF3-P expression was comparable after drug treatment (Fig. 3).

Flow cytometry was performed to further determine the basis for TPA- and butyrate-induced increases in DF3-P expression. Compared to control cells (Fig. 4A), TPA treatment was associated with an increase in cell membrane MAB DF3-P reactivity (Fig. 4B). Similar increases in surface fluorescence were obtained with butyrate-treated cells (Fig. 4C). The percentage of MAB DF3-P-reactive cells in two separate experiments was 14 ± 6.5% (mean ± SD) after TPA and 22 ± 9.5% after butyrate exposure, compared to 3.3 ± 0.6% for the untreated cells.

Fig. 5. Immunofluorescence staining patterns with MAbs DF3-P and DF3. MCF-7 cells were grown on chamber slides to ~90% confluence (A and C), reacted with MAb DF3-P (B) or MAb DF3 (D) and then a FITC-conjugated second antibody. The cells were fixed in formalin and visualized for fluorescence staining.
Moreover, treatment with both agents was associated with an increase in the heterogeneity of antigen expression (Fig. 4). These results and the patterns as determined by flow cytometry indicate that the enhancement in DF3-P expression observed with live cell radioimmunoassay is due to both an increase in MAb DF3-P-reactive cells and an increase in antigen density/cell. Similar results were obtained for the DF3 antigen (data not shown).

In order to identify patterns of DF3-P and DF3 expression, immunofluorescence was performed on MCF-7 cells growing as monolayers on chamber slides. In these studies, cells at ~90% confluence (Fig. 5, A and C) were reacted with MAbs DF3-P, MAb DF3, or isotype-identical control antibodies and then exposed to a FITC-conjugated second antibody. These cells were then fixed in formalin and visualized for fluorescence staining. MAb DF3-P-reactive cells were detectable in small clusters of cells (Fig. 5B). A similar pattern was observed with MAb DF3, although the intensity of this reactivity and the number of positive cells were greater (Fig. 5D). In contrast, there was no detectable reactivity with the isotype-identical control antibodies (data not shown). Treatment with TPA was associated with an increase in both the size and number of MAb DF3-P-reactive clusters per field (Fig. 6A). However, certain cells remained DF3-P antigen-negative (Fig. 6A). This effect was even more apparent when staining with MAb DF3 (Fig. 6B). In contrast, treatment with sodium butyrate resulted in a more diffuse increase in DF3-P and DF3 expression throughout the population without the formation of MAb-reactive cell clusters (Fig. 6, C and D).

**DISCUSSION**

We have recently shown that the DF3-P epitope is similar to that defined by MAb DF3 (19). Both epitopes reside within positions 7 to 13 (TRPAPGS) of the 20-amino acid tandem repeat of the peptide core. The DF3 epitope includes a threonine at position 7 and a serine at position 13. In contrast, the DF3-P epitope has been mapped to RPAPG (19). Thus, glycosylation of the threonine and/or serine sites could contribute to the affinity of MAb DF3 binding. Moreover, glycosylation of these sites could inhibit the reactivity of MAb DF3-P by steric hindrance or a conformational change in the peptide. These findings have suggested that differential glycosylation of the protein core accounts for the selective reactivity of MAb DF3-P with human breast carcinomas. Underglycosylation or qualitative changes in carbohydrate composition during synthesis of the DF3 glycoprotein in malignant mammary epithelium could increase the accessibility of the DF3-P epitope. The observation

![Fig. 6. Effects of TPA and sodium butyrate on immunofluorescence staining with MAbs DF3-P and DF3. MCF-7 cells were treated with TPA for 3 days (A and B) or sodium butyrate (butyric acid) for 5 days (C and D). Reactivity of MAb DF3-P (A and C) or MAb DF3 (B and D) was determined as described in Fig. 5. Cells were ~90% confluent at analysis.](image-url)
that DF3-P expression is selective for primary breast cancers would support this hypothesis (19). The present results with different breast cancer cell lines also demonstrate that MAb DF3-P reacts with the underglycosylated species, while MAb DF3 detects the more mature glycosylated antigen.

Expression of the DF3 gene is regulated at the transcriptional level, and there appears to be little if any control of DF3 mRNA levels by posttranscriptional mechanisms (21). Moreover, levels of DF3 transcripts correspond with the amount of DF3 protein (21). These findings have indicated that overexpression of DF3 antigen in breast tumors is related to aberrant control of DF3 gene transcription in malignant cells. While the DF3 promoter has been cloned and sequenced (22), little is known about the cis elements that are functional in regulating transcription of this gene. Nonetheless, differentiating agents, such as TPA, have been shown to increase the rate of DF3 transcription and thereby antigen levels (21). Moreover, the finding that treatment with TPA or butyrate increases cell surface expression of DF3-P is in concert with increased synthesis of the peptide and saturation of glycosylation during transport of the DF3 peptide from the endoplasmic reticulum to the Golgi and cell membrane (23). While the induction of a more differentiated mammary cell phenotype would predict increased production of the mature DF3 glycoprotein, the results suggest that defective posttranslational processing of the DF3 peptide in breast cancer cells persists despite treatment with differentiating agents.

Detection of the DF3-P epitope on the surface of breast cancer cells suggests that the DF3 glycoprotein reaches the cell membrane in an incompletely glycosylated state. Similar findings have been described for the sialomucin ASGP-1 synthesized by ascites 13762 rat mammary carcinoma cells (24). Incompletely glycosylated ASGP-1 molecules at the cell surface undergo a recycling process with entry into a second O-glycosylation pathway and the addition of new oligosaccharides (23, 24). This model predicts that secreted carcinoma glycoproteins will contain less carbohydrate than their cell surface counterparts. Of interest is the finding that while DF3 antigen is secreted in substantial amounts into the culture supernatants of the breast cancer lines used in the present studies, only low to undetectable levels of the DF3-P epitope were detectable in these preparations (data not shown). These findings would suggest that the incompletely glycosylated DF3 peptides at the cell surface are probably first subject to recycling and then more extensive glycosylation before release.

Another aspect of the present studies is the finding that DF3-P and DF3 expression is restricted to certain populations of breast cancer cells. Heterogeneity of both DF3-P and DF3 staining has been recognized in immunoperoxidase studies of primary breast carcinomas (10, 19). Moreover, while flow cytometry analysis of breast cancer cell lines reflected both DF3-positive and negative cells, the present results using chamber slides indicate that clusters of MCF-7 cells are responsible for MAb DF3 reactivity. Similar patterns of reactivity were obtained with MAb DF3-P. These findings suggested that surface expression of the DF3-P epitope and glycosylation to the mature glycoprotein is restricted to specific cell clones. However, we have isolated single MCF-7 cell clones that are initially DF3-negative or positive and after expansion found patterns of antigen expression similar to those found in the parent line. Flow cytometry for DNA content has demonstrated that DF3 expression is not restricted to specific phases of the cell cycle (data not shown). Thus, other factors must contribute to the growth of antigen-positive clusters. The demonstration that TPA and sodium butyrate increase the expression of both the DF3-P and DF3 epitopes suggests that antigen-positive clusters may reflect the presence of differentiated clones. Of interest is the finding that both recombinant human α-interferon and γ-interferon increase DF3 antigen expression on the surface of MCF-7 cells (25). However, further studies are needed to determine whether these agents also increase DF3-P expression and whether these effects are associated with a more mature phenotype.

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REFERENCES

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