Correlation of Surface Fucoprotein Patterns with Tumorigenicity of Mouse Mammary Epithelial Cells

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ABSTRACT

The plasma membrane fucoproteins of tumorigenic and non-tumorigenic mouse mammary epithelial cells were studied. The types of cells analyzed included (a) cell lines derived from mouse mammary carcinomas of varying etiologies (viral, hormonal, chemical carcinogen), (b) a series of clonal cells lines which were nontumorigenic at lower passage levels and tumorigenic at higher passage levels, (c) normal primary cells derived from the mammary glands of pregnant or lactating animals, and (d) primary cells from tumors produced by s.c. injection of cultured mammary tumor cells into syngeneic animals. A distinctive difference was observed in the size distribution of the trypsin-sensitive surface fucoproteins from tumorigenic and nontumorigenic mouse mammary cells; the tumorigenic cells were relatively enriched in the larger fucoproteins. The size distribution of the trypsin-sensitive surface fucoproteins was not markedly influenced by the physiological state of the cells or by cell population density. It appears that the trypsin-sensitive surface fucoprotein size pattern may be a distinguishing characteristic between tumorigenic and nontumorigenic mouse mammary epithelial cells.

INTRODUCTION

Since the majority of human cancers are epithelial in origin, the characteristics which distinguish tumorigenic and nontumorigenic epithelial cells are of use in predicting the tumorigenicity of epithelial cells and in pinpointing those cellular properties which may be of functional significance in the development of carcinomas. Studies of normal and transformed fibroblasts have defined a family of altered properties typically associated with transformation. These include growth properties (e.g., Refs. 17 and 32), level of fibronectin (for reviews, see Refs. 20, 34, and 44) and size distribution of trypsin-associated fibronectin (30, 45). Whenever growth properties of normal and neoplastic epithelial cells from mouse mammary glands were compared, no consistent difference was observed (8, 11, 37). Similarly, cells derived from normal mammary glands and mammary carcinomas were not found to differ consistently with regard to fibronectin content (30, 45).

In contrast, a significant difference has been observed in the fucoprotein patterns of cells derived from a spontaneous mouse mammary tumor as compared to the fucoprotein patterns of cells derived from either normal mammary gland or a hormone-dependent tumor (29). The trypsin-sensitive surface fucoproteins from cells derived from the spontaneous tumor were enriched in the larger-molecular-weight components. In another epithelial cell system, rat hepatoma and rat liver cells, the same type of difference in size distribution of fucoproteins was observed (35). Furthermore, when the fucoprotein patterns of a rat liver epithelial cell line temperature sensitive for the transformed phenotype were analyzed, the fucoprotein size distribution was also found to be temperature sensitive (23). Prompted by these findings, this study was undertaken to determine the relationship between the fucoprotein size patterns and tumorigenicity of cultured mouse mammary cells, derived from normal mammary glands and carcinomas of varying etiologies.

MATERIALS AND METHODS

Cell Culture. The ESD/BALB and MTV-L/BALB cells and clonal derivatives originated from BALB/c mouse mammary tumors which have hormonal and viral etiologies, respectively (8). The DMBA/BALB and DMBA-2/BALB cell lines were established from chemical carcinogen-induced mammary tumors (8, 14). The ESD/BALB, MTV-L/BALB, DMBA/BALB, and DMBA-2/BALB cell lines were cloned between passages 30 and 40. The parental DMBA/BALB cell line was also cloned at passage 16 to produce the DMBA/BALB-16 clones. The ESD-M/BALB cell line is a clonal isolate from ESD/BALB Cl 3 cells, which had been exposed to the murine sarcoma-leukemia virus complex. The ESD/BALB CI 1 PA 1 cells were derived from a tumor produced by s.c. injection of ESD/BALB Cl 1 cells. The cell lines differ significantly with regard to expression of retrovirus information. For example, the DMBA-2/BALB cells are negative for both B- and C-type viruses, as judged by several criteria (13, 14), while the MTV-L/BALB cells appear to express the most viral information, i.e. detectable C-type reverse transcriptase activity (8) and intracellular murine mammary tumor virus RNA sequences (15). All cells were cultured in Dulbecco's modified medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (v/v; Grand Island Biological Co.), 100 units penicillin per ml, 100 μg streptomycin per ml, and 5 μg bovine insulin per ml (Sigma Chemical Co., St. Louis, Mo.). The culture medium for the DMBA/BALB and DMBA-2/BALB cells was also supplemented with 5 μg/ml hydrocortisone (Sigma Chemical Co.). Cells derived from normal mammary glands were grown in standard medium and medium supplemented with hydrocortisone. Subculturing of cells derived from the mammary glands of lactating or bearing BALB/c mice were sacrificed by cervical dislocation. The mammary glands or tumors were removed, washed in phosphate-buffered saline (0.15 M NaCl:0.005 M sodium phosphate, pH 7.4) and replating in fresh medium.

Preparation of Primary Cells Derived from Normal Mammary Glands and Tumors. Midpregnant, lactating, or tumor-bearing BALB/c mice were sacrificed by cervical dislocation. The mammary glands or tumors were removed, washed in phosphate-buffered saline (0.15 M NaCl:0.005 M sodium phosphate, pH 8.0), minced very finely, and centrifuged at 80 x g for 10 min. The resulting material derived from normal mammary glands was distributed into 2 fractions: tissue which floated (which, after further treatment, yielded predominantly...
epithelial cell cultures) and tissue which pelleted. The pellet fraction from the tumors and the floating fraction from normal glands were suspended in phosphate-buffered saline containing 0.2% trypsin (1: 250, w/v; Difco Laboratories, Detroit, Mich.) for 15 min at room temperature. After removal of the trypsinized cells, the remaining tissue was digested twice in Dulbecco's modified medium, containing 100 units penicillin per ml, 100 μg streptomycin per ml, and 2 mg collagenase per ml (type III; Worthington, Freehold, N. J.), for 1 hr each at room temperature. The cells, from the collagenase digestions, usually small aggregates, were washed and resuspended in complete culture medium.

Tumorigenicity. Tumorigenicity in vivo was assayed in syngeneic mice by transplantation s.c. of 1 or 2 × 10⁶ cells or by transplantation of 10⁵ cells into the cleared inguinal mammary fat pads (12). The cytchalasin B multinucleation assay is an in vitro assay which is positively correlated with tumorigenicity of mammary cells (21, 31). This assay was performed as described by Steiner et al. (31).

Preparation and Analysis of Trypsin-sensitive Cell Surface Fucopeptides. Cells were radiolabeled by culturing in medium supplemented with 5 μCi L-[6-¹⁴C]fucose per ml (25 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.) or 0.5 μCi L-[1-¹³C]fucose per ml (60 mCi/mmol; Amersham/Searle or 600 mCi/mmol; New England Nuclear, Boston Mass.) with [¹³C]fucose with a specific activity of 60 mCi/mmol was used in all the experiments depicted in the charts). Cells were harvested 48 to 72 hr after the addition of medium supplemented with radiolabeled fucose. Surface glycopeptides were isolated and analyzed by a modification of the method of Buck et al. (5). Briefly, cells on tissue culture dishes were subjected to trypsin treatment (1 mg 3x trypsin per ml; Worthington) for 15 min at 37°. After the addition of soybean trypsin inhibitor (Sigma), the trypsinate was centrifuged (1100 x g, 10 min; 75,000 x g, 45 min), and the resultant high-speed supernatant fraction was dialyzed against water. The sample was lyophilized, resuspended in 1 mg pronase per ml (predigested for 2 hr at 37°; Calbiochem, San Diego, Calif.) in 0.1 M Tris:1.5 mw CaCl₂, pH 7.6, and incubated for 96 hr at 37° with fresh pronase being added daily. Pronase-digested samples were lyophilized; resuspended in a minimal volume of 0.1 M Tris-acetate, pH 9.0:0.1% sodium dodecyl sulfate (w/v) and 0.1% β-mercaptoethanol (v/v); and analyzed by gel filtration on a Sephadex G-50 fine column equilibrated with the same buffer. The column fractions were analyzed by scintillation spectrosopy. Sialic acid groups were removed by incubation of the pronase-digested samples in 0.1 N H₂SO₄ at 80° for 1 hr. The samples were neutralized, lyophilized, and analyzed by gel filtration as above.

RESULTS

Surface Fucopeptide Size Distribution and Tumorigenicity. The size distributions of the trypsin-sensitive surface fucopeptides from primary cells derived from normal mammary tissue and from a mammary tumor induced by DMBA/BALB CI 1 cells were compared (Chart 1). The cells derived from the mammary glands of lactating animals (Chart 1A) had relatively more of the lower molecule weight species as compared to the cells derived from the tumor (Chart 1B). This pattern represented the same type of change in size distribution of surface fucopeptides observed in previous comparisons of normal and transformed fibroblasts (39).

In order to test for a possible positive correlation between an altered fucopeptide size distribution and tumorigenicity, closely related cells which differed significantly in their oncogenic potential were examined. Clonal isolates of the DMBA/BALB cells, at low passage levels after cloning, do not produce tumors upon s.c. injection, whereas these cells at higher passage levels do produce tumors (31). As shown in Chart 2B, the higher-passage-level cells of clonal isolate 16a (passage 19 after cloning) exhibited a gel filtration profile very similar to that of the tumor-derived cells shown in Chart 1B. In contrast, the lower-passage-level nontumorigenic cells (Chart 2A) were relatively enriched in the lower-molecular-weight species.

In view of the relative increase in the larger fucopeptides in the tumorigenic cells, the fucopeptides from a range of mammary gland-derived cells were examined. Cells derived from mouse mammary carcinomas of varying etiologies and at high and low passage levels were analyzed (Table 1). With a single exception, the cells which produced tumors had elution profiles of surface fucopeptides similar to those of the tumorigenic cells illustrated in Charts 1B and 2B. The exception was the ESD/BALB CI 1 cells which were tumorigenic when tested at passage level 61, while the fucopeptide pattern obtained from cells at passage level 70 was similar to that of the nontumorigenic cells. Since those cells could not be successfully recovered after being frozen, additional and simultaneous fucopeptide and tumorigenicity analyses could not be performed. However, cells which were derived from a tumor produced by s.c. injection of ESD/BALB CI 1 cells, the ESD/BALB CI 1 PA 1 cells,
were analyzed and were found to be both tumorigenic and enriched in the larger fucoproteins. It should be noted that the other cell lines were analyzed on multiple occasions with highly reproducible results. In addition, when the fucoproteins from normal cells, cultured in medium supplemented with [14C]fucose, were compared to the fucoproteins from MTV-L CI 2 cells cultured in medium supplemented with [3H]fucose, the elution profiles were the same as that seen with the reverse labeling procedure (data now shown).

**Surface Fucoproteins, Cell Density, and Cell Physiology.**

![Chart 2](chart2.png)

**Table 1**

<table>
<thead>
<tr>
<th>Fucoproteins</th>
<th>Passage level analyzed</th>
<th>Tumorigenicity</th>
<th>Passage level analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary cells from mammary carcinomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBA/BALB CI 16a</td>
<td>- 10</td>
<td>- 5, 6</td>
<td></td>
</tr>
<tr>
<td>DMBA/BALB CI 16b</td>
<td>+ 19, 20</td>
<td>+ 19</td>
<td></td>
</tr>
<tr>
<td>DMBA/BALB CI 1</td>
<td>- 12</td>
<td>- 13</td>
<td></td>
</tr>
<tr>
<td>DMBA-2/BALB CI 4</td>
<td>+ 7, 17, 24</td>
<td>+ 7, 11, 31</td>
<td></td>
</tr>
<tr>
<td>MTV-L/BALB CI 2</td>
<td>+ 27, 51, 74</td>
<td>+ 15, 86</td>
<td></td>
</tr>
<tr>
<td>ESD/BALB CI 3</td>
<td>+ 33, 49, 93</td>
<td>+ 12, 105</td>
<td></td>
</tr>
<tr>
<td>ESD/BALB CI 1</td>
<td>- 70</td>
<td>+ 61</td>
<td></td>
</tr>
<tr>
<td>ESD/BALB CI 1 PA 1</td>
<td>+ 22</td>
<td>+ 22</td>
<td></td>
</tr>
<tr>
<td>Primary cells derived from mammary glands and tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBA/BALB CI 1</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td>MTV-L/BALB CI 2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBA-2/BALB CI 4</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESD/BALB CI 3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammary glands, lactating animals</td>
<td>-</td>
<td>(3rd passage level)</td>
<td></td>
</tr>
</tbody>
</table>

Mammary glands, pregnant animals

- F, pattern similar to that of tumor-derived cells illustrated in Charts 1B and 2B; - , pattern similar to that of normal mammary gland-derived cells illustrated in Chart 1A; ±, pattern intermediate between those of Chart 1A and B. All methods are as indicated in "Materials and Methods."

- Tumors observed in all animals, after s.c. injection of cells, with latent period of 2 weeks; - , tumors not observed after s.c. injection of cells.

- Tumorigenicity assayed by implantation of cells in cleared mammary fat pads.

- Passage level 10 and 13 cells assayed by in vitro assay for tumorigenicity (cytochalasin B-induced multinucleation); cells exhibited a low percentage of cytochalasin B-induced multinucleation, i.e., the response of nontumorigenic cells.

- One of 5 animals tested developed a tumor after a latent period of 8 weeks.

- Tumors were produced by the s.c. injection of the indicated cells. At least 3 separate independent analyses of each of the following were performed except for the single analysis of passage level 3 cells from the mammary glands of lactating animals.
Since the physiological status of the mammary gland can vary, the possible effect that this might exert on the fucoseptide size distribution was examined. The trypsin-sensitive surface fucoseptides from cells derived from the mammary glands of pregnant and of lactating animals were analyzed (Table 1). The elution profiles of the fucoseptides from both groups of cells were quite similar and were distinctive from those of tumorigenic cells. In addition, the same type of normal fucoseptide pattern was observed for the fucoseptides from subcultured cells (passage 3) derived from the mammary glands of lactating animals (Table 1). When the cells derived from the normal mammary gland are seeded at high cell densities, they produce cultures which contain domes, i.e., raised areas believed to reflect transport functions of the cells (e.g. 24). Cell cultures which contained domes had fucoseptides which gave the same elution profile as that of fucoseptides from cells seeded at lower cell densities (data not shown).

Cell density has been reported to affect the size distribution of the trypsin-sensitive surface fucoseptides of both normal and transformed fibroblasts (6). Fibroblasts at lower cell densities are growing and not touching, while normal epithelial cells and some of the tumorigenic cells, e.g., DMBA-2/BALB, grow in "islands" or groups. Therefore, it was possible that cell density might specifically and significantly affect the fucoseptides of mammary cells which can grow without touching. The ESD/BALB Cl 3 and MTV-L/BALB Cl 2 are 2 examples of cells which exhibit this type of growth pattern. As shown in Chart 3, there was not a marked change in the size distribution of the trypsin-sensitive surface fucoseptides as a function of cell density with these 2 cell lines. The change that was observed reproducibly was an increase in the larger components at higher cell density [the reverse of what was observed in the fibroblast system (6)]; however, in the rat liver cell line which is temperature sensitive for transformation, there was also a slight increase in the faster eluting species in confluent cultures (23)]. Thus, it appears that the difference in the size distribution of the trypsin-sensitive surface fucoseptides from tumorigenic and nontumorigenic mammary epithelial cells is not markedly influenced by the physiological state of the cells.

In a number of cell systems, the trypsin-sensitive surface fucoseptides from the transformed cells were found to be more highly sialylated than those from nontransformed cells such that, after desialylation, the fucoseptides from both cell types were observed to be similar in size (35, 40). Prompted by these observations, the trypsin-sensitive surface fucoseptides from mammary gland-derived cells were analyzed after removal of
Typically, the tumorigenic cells are enriched in the larger gland-derived cells generally differ in a consistent manner.

Peptides from tumorigenic and nontumorigenic mammary DISCUSSION
desialylated surface fucopeptides from MTV-L/BALB CI 2 cells and from cells derived from the mammary glands of pregnant animals also exhibited size distributions similar to those illustrated in Chart 4. Thus, it appears that the trypsin-sensitive surface fucopeptides from tumorigenic cells are more highly sialylated as compared to the fucopeptides from normal cells.

**DISCUSSION**

The size distributions of the trypsin-sensitive surface fucopeptides from tumorigenic and nontumorigenic mammary gland-derived cells generally differ in a consistent manner. Typically, the tumorigenic cells are enriched in the larger fucopeptides. The possible exception noted was with the ESD/BALB CI 1 cells; however, since the tumorigenicity and fucopeptide analyses were performed at different passage levels, it may be that the cells were not tumorigenic at the passage level at which the fucopeptides were analyzed. Changes in the oncogenic potential of cultured mammary carcinoma cells have been observed. For example, the RAMA 25 cells, derived from a rat mammary adenocarcinoma, are tumorigenic and give rise to the RAMA 29 cells which have not been observed to be tumorigenic (3). There is also a marked difference in the oncogenic potential of the DMBA/BALB cells as a function of "in vitro" cell passaging (31). Although the basis for this change in transplantability is not known, the DMBA/BALB cells provide a useful control of subcultured nontumorigenic mammary cells and closely related tumorigenic cells.

Other epithelial cell systems which have been studied, namely, cells derived from a hormone-independent mammary tumor and hepatomas as compared to control cells (23, 29, 35) have been found to exhibit the same type of difference in fucopeptide size distribution. The size distributions of the trypsin-sensitive surface fucopeptides have also been reported to differ in transformed and normal fibroblasts (for review, see Ref. 39) as well as in neuroblastoma (19), melanoma (41), and leukemic cells (36) as compared to appropriate control cells. Thus, an altered size distribution of the trypsin-sensitive surface fucopeptides appears to be a general feature of tumorigenic cells.

The biochemical basis for the difference(s) in the fucopeptides is not known in detail, although in many cases, including the tumorigenic mammary cells examined, the fucopeptides from the transformed cells are more highly sialylated (e.g., Refs. 35 and 40); furthermore, these fucopeptides appear to be more highly branched (22, 27). In a study of trypsin-sensitive surface fucopeptides from Rous sarcoma virus-transformed and control hamster cells, the differences between the fucopeptides of the 2 cell types were found to be largely quantitative (4, 18). The functional significance of this alteration has not been elucidated; however, in those same virus-transformed and control hamster cells, it was determined that the size difference in pronase-generated glycopeptides was exhibited by multiple-membrane proteins (33). A significant functional role for specific oligosaccharide chains on a glycoprotein is suggested by the observed association of alterations in the oligosaccharide chains of a major glycoprotein and xenotransplantability of a rat mammary adenocarcinoma (7).

The relationship of the difference in fucopeptide size distribution observed in this study to other differences observed between cells derived from normal mammary glands and mammary carcinomas remains to be determined. However, it is notable that the alterations which have been observed in mammary carcinoma cells may all be related to the surface membrane. These alterations include an increase in Na+ permeability in the tumor cells (28), deviations in morphology or disposition of the basal lamina between mammary tumors and surrounding nonepithelial tissue (26), loss of selectivity in junctional intercellular communication in tumor cells (16), and changes in the glycosaminoglycan composition (10) and water nuclear magnetic relaxation times (2). In addition, the differential responses of the tumorigenic and nontumorigenic cells to cytochalasin B (31) and to the multiplication-stimulating activity of insulin (38) may be mediated via the cell surface, e.g., cell surface receptors. Some specificity to these surface membrane alterations is indicated by the lack of consistent difference in other surface membrane characteristics including those which differ in normal and transformed fibroblasts. These surface membrane characteristics include K+ permeability (28), fibronectin content (30, 45), and morphology of cell-cell junctions (25). Furthermore, there is a substantive list of other cellular characteristics which do not differ in a consistent manner between tumorigenic and normal mammary cells (9). These include growth rate and saturation density (8, 11, 37) level of plasminogen activator (45) and distribution and organization of microtubules and microfilaments (1).

All the studies summarized above point to the surface membrane as an important site in the transformation of epithelial cells. The findings that the transforming gene product of the avian and the murine sarcoma viruses appear to be localized on the inner surface of the plasma membrane (42, 43) suggest a model in which a primary alteration in the surface membrane may lead to the pleiotropic changes found in the transformed cells.
REFERENCES


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