Tropomyosins of Human Mammary Epithelial Cells: Consistent Defects of Expression in Mammary Carcinoma Cell Lines

Basudev Bhattacharya, Gaddamanugu L. Prasad, Eva M. Valverius, David S. Salomon, and Herbert L. Cooper

Cell and Molecular Physiology Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland 20892 [B. B., G. L. P., D. S. S., H. L. C.] and Vincent Lombardi Cancer Research Center, Georgetown University Hospital, Washington, DC 20007 [E. M. V.]

ABSTRACT

Suppression of synthesis of specific tropomyosin (TM) isoforms occurs commonly in human, murine, and avian fibroblasts transformed by retroviral oncogenes or other modalities. The resulting deficiency or altered distribution of TMs may predispose the cells to microfilament instability and contribute to expression of the transformed phenotype. In this study we have asked whether defects in TM expression had relevance to human neoplasia, which arises most often in cells of the epithelial lineage rather than in fibroblasts and often is unrelated to demonstrable expression of oncogenes. TMs were characterized in normal primary human mammary epithelial cells (HMEC) and in an immortalized nontumorigenic cell line derived from them. Seven TM isoforms were identified in primary HMEC, two of which may be unique to epithelial cells. Immortalized nontumorigenic HMEC expressed the same array of isoforms. Of six established human breast carcinoma cell lines studied, all failed to express the M, 39,000 TM isoform and five of six also lacked expression of either the M, 38,000 or 35,000 isoform. Northern blot analysis with probes specific for the 1.1-kilobase mRNA of fibroblast TM1 detected a mRNA of this size in normal HMEC. This mRNA, which probably encodes the M, 39,000 TM missing from all the carcinoma lines, was absent from five of the six breast cancer cell lines. These results indicate that abnormalities in TM expression in neoplastic cells are not limited to fibroblasts. The high frequency and consistent nature of such abnormalities among cell lines derived from human breast cancer raises the possibility that such abnormalities in expression of a major cytoskeletal protein may play a role in human neoplasia.

INTRODUCTION

A number of studies have shown that synthesis of certain members of the TM family of microfilament-associated cytoskeletal proteins is suppressed when cultured human, murine, and avian fibroblasts undergo neoplastic transformation as a result of expression of various transforming retroviral oncogenes (1–5), due to transformation by carcinogens (6), or following treatment with growth factors that can induce the transformed phenotype (7). The resulting deficiency of TM in the formation of actin microfilaments has been proposed as a basis (1, 2, 5, 8) for the prominent disorganization seen in these cytoskeletal elements in cells transformed by retroviral oncogenes (reviewed in Ref. 9). It has been suggested that such cytoskeletal derangement, ubiquitous in neoplastic cells, may play a causal role in oncogenesis (5, 9, 10, 11). Transforming growth factor α, which is elaborated by cells expressing various retroviral oncogenes and which can induce a transformed phenotype, causes suppression of synthesis of the same TM isoforms affected in retrovirally transformed cells and also inhibits incorporation of newly synthesized TM into cytoskeletal structures (7), thus suggesting one possible pathway through which oncogene expression might act.

These studies with fibroblast cell cultures raise the question of whether such observations may be relevant to human malignancies, the great majority of which arise not on cells of the fibroblast lineage but in those of epithelial origin. Previous workers, using immunohistochemical techniques, have reported disorganization of actin microfilaments in association with increased metastatic potential in cell lines from melanoma (12, 13) and from prostatic carcinoma (14). It is germane, therefore, to ask whether derangements in TM expression, which may provide a biochemical basis for microfilament disorganization in transformed fibroblasts, may also occur in neoplastic cells of epithelial derivation, with similar implications. As an initial step in answering this question, we have characterized the TMs expressed by normal human mammary epithelial cells in culture and compared them with those expressed in a panel of six established cell lines derived from human mammary carcinomas. We found consistent abnormalities of TM expression in all of the mammary carcinoma lines studied, indicating that derangement of TM expression in transformed cells is not limited to fibroblasts and may play a role in human neoplasia.

MATERIALS AND METHODS

Cell Cultures. Human diploid mammary epithelial cell strain 184, generously provided by Dr. M. Stampfer (University of California, San Francisco), was maintained at ≤1% CO2 in serum-free MCDB 170 medium (University of California, San Francisco, Tissue Culture Unit) supplemented with EGF, bovine pituitary extract, and insulin, as described (15, 16). Line 184A1N4 is a subclone of the line 184A1, which is a derivative of the 184 strain that was immortalized by treatment with benz(a)pyrene (17, 18) and was obtained from the same source. It was maintained at 5% CO2 in improved modifed Eagle's medium (GIBCO) supplemented with 0.5% fetal bovine serum, insulin (10 μg/ml), hydrocortisone (0.1 μg/ml), and EGF (5 ng/ml; Collaborative Research).

Cell lines derived from human breast carcinomas (MCF-7, BT-20, MDA-MB-231, BT-474, T-47D, and ZR-75.1) and diploid human skin fibroblasts (WS1) were obtained from the American Type Culture Collection (Rockville, MD) and were maintained at 5% CO2 in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum.

Radiolabeling and Preparation of Proteins for Analysis. To estimate relative synthesis rates of various proteins, cells were incubated for 4 h in methionine-free Dulbecco's modified Eagle's medium supplemented with 50 μCi/ml [35S]methionine (1100 Ci/mmol; Amersham) and 10% dialyzed fetal bovine serum.

For preparation of cytoplasmic proteins, cells were chilled on ice, scraped from culture vessels, and washed by centrifugation in phosphate-buffered isotonic saline. After resuspension in 0.5–1.0 ml lysis buffer [0.01 M NaCl/0.01 M Tris-HCl, pH 7.4/1 mM EDTA/1% (w/v) Nonidet-P-40/1.2 mM PMSF], the cell suspension was vortexed for 30 s and then placed in an ultrasonic bath (Branson) for 30 s. Insoluble material was removed by centrifugation for 10 min at 10,000 × g. Acid-insoluble radioactivity in the supernatant was assayed and the supernatant was then stored at −80°C. For electrophoretic analysis of total cytoplasmic proteins, 4 × 106 cpm of labeled protein were precipitated from an appropriate volume of the supernatant by addition of 5 volumes of ice-cold 10 parts acetone/0.57 parts NH4OH. After standing on ice for 5–10 min, precipitated proteins were recovered by centrifugation at

Received 4/11/89; revised 11/16/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed.

2 The abbreviations used are: TM, tropomyosin; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; cDNA, complementary DNA; EGF, epidermal growth factor.

3 H. Cooper, unpublished data.
DISORDERED TROPOMYOSIN EXPRESSION IN BREAST CANCER CELL LINES

10,000 x g for 20 min. Supernatant acetone was aspirated completely and the precipitate was dried exhaustively under vacuum to remove residual acetone and volatile salts.

For preparation of heat-stable proteins, cytoplasmic extract was adjusted to 0.1 M NaCl and then placed in a boiling water bath for 5 min. After chilling, insoluble material was removed by centrifugation at 10,000 x g for 20 min and supernatant proteins were recovered by acetone precipitation as above.

For preparation of cytoskeletal proteins, washed cell pellets, prepared as above, were gently resuspended in 8 ml of SOL buffer [100 mM NaCl/300 mM sucrose/10 mM piperezine-N,N,N'-bis(2-ethanesulfonic acid), pH 6.8/3 mM MgCl₂/0.5% (v/v) Triton X-100/1.2 mM PMSF] on ice for 5 min. After 5 min the suspension was centrifuged at 10,000 x g for 10 min and the supernatant was removed completely and discarded. The pellet was resuspended by vortexing in 200-400 µl cytoskeletal buffer [600 mM ammonium acetate/10 mM piperezine-N,N,N'-bis(2-ethanesulfonic acid), pH 6.8/3 mM MgCl₂/1.0% (v/v) Triton X-100/1.2 mM PMSF] and kept on ice for 5 min. Insoluble material was removed by centrifugation at 10,000 x g for 10 min and supernatant proteins were precipitated with acetone as above.

Anti-TM Antiserum. TM was purified from fresh chicken gizzard as described (19, 20). Purity of the product was estimated by one- and two-dimensional electrophoresis. Polyclonal rabbit antiserum was raised to the purified protein by standard immunological methods. The rabbit serum was used directly and was not further purified.

Immunoprecipitation. TMs were immunoprecipitated from cytoplasmic preparations using formalin-fixed heat-killed staphylococci (Calbiochem-Behring), as described (1).

Two-Dimensional Polyacrylamide Gel Electrophoresis. Samples were prepared and analyzed by two-dimensional polyacrylamide gel electrophoresis as described (1). First dimension amphyoles (LKB) were 1%, pH 3.5-10, and 1%, pH 4-6. First dimension gels were run for 12,000 V·h. Second dimension gels were 12% polyacrylamide.

Quantitation of Protein Labeling in Two-Dimensional Gels. After radioautography, radioactivity from [35S]methionine in dried gels was scanned directly with an AMBIS two-dimensional beta-scanning system. Quantitation of radioactivity from [35S]methionine in dried gels was determined, with suitable background subtraction.

RESULTS

Characteristics of Cell Lines Studied. The cell lines studied, and some of their relevant characteristics, are summarized in Table 1. 184 cells are primary diploid human mammary epithelial cells derived from a reduction mammoplasty (15, 16) and retain biochemical characteristics of mammary epithelial cells, including expression of epithelial-specific cytokeratin (15). They exhibit limited life-span in culture (about 20 passages) and have stringent medium requirements. These cells were used for our identification of TMs expressed by normal human mammary epithelium. Line 184A1N4 is a near-triploid subclone of line 184A1, which is an immortalized line derived from 184 cells by treatment with benz[a]pyrene (17, 18). These cells served as a control for the effects of immortalization of cell lines. Neither 184 cells nor 184A1N4 cells are tumorigenic in nude mice and they do not exhibit anchorage-independent growth in culture (17, 18); therefore, they are considered not to have undergone malignant transformation. All of the breast cancer-derived cell lines originated from ductal or adenocarcinoma cells either in pleural effusions or from solid tumor and they have been characterized as epithelial cells (22, 23).

Identification of Human Mammary Epithelial TM. TMs of normal human epithelial cells have not been previously characterized. We therefore performed two-dimensional polyacrylamide gel electrophoretic analysis of TM immunoprecipitated from radiolabeled extracts of both 184 cell and 184A1N4 cell cytoplasm by a polyclonal (rabbit) antiserum raised against chicken gizzard TM (Fig. 1). The antiserum, although raised against smooth muscle TM from another species, cross-reacts with TMs from human fibroblasts (Fig. 2). Its affinity is greatest, however, for the higher M, muscle-type TMs than for the lower M, non-muscle-type.

When applied to lysates of 184 mammary epithelial cells, the antiserum specifically precipitated a set of proteins in the M, range of the known TMs (M, 32,000-39,000) and of characteristic pi (approximately 4.75) (Fig. 1A). None of these proteins appeared in control immunoprecipitations with preimmune rabbit serum (not shown). The putative TMs have been tentatively numbered according to their apparent M, in gels, although final M, assignments must await cloning and sequencing studies. The 33a and 33b forms were difficult to detect by immunoprecipitation in 184 cell material (Fig. 1A), but they were prominent among the heat-resistant (Fig. 1C) and cytoskeletal (Fig. 1D) proteins, as were the M, 35,000, 38,000, and 39,000 forms, indicating that the M, 33,000 species were relatively poorly recognized by the antiserum. This, together with their lower M, is consistent with their being of the non-muscle-type. The M, 36,000 and 32,000 forms were recognized with difficulty by antisemur and also were poorly represented among the heat-resistant and cytoskeletal proteins, indicating that they are minor forms. However, the fact that they were detected at all in immunoprecipitates indicates that the antisemur has better affinity for them than for the non-muscle forms, suggesting that they share epitopes with the muscle-type TMs. TM-36 was often difficult to resolve from the more heavily labeled TM-35.

On the basis of their M, and pi, their characteristic heat resistance, their presence among the cytoskeletal proteins, and their specific reactivity with anti-TM antisemur, we identify the set of seven proteins with apparent M, from 32,000 to 39,000 as TMs of human mammary epithelium. Comparison of 184 and 184A1N4 cells (Fig. 1, A versus B) indicates that immortalization of cell lines, perhaps related to passage history variation in the relative level of expression of the various isoforms, and has stringent medium requirements. Although quantitative variation may exist. TM-36, although poorly resolved in the illustrated preparation of 184A1N4, was evident in other preparations. We also observed quantitative variation in the relative level of expression of the various isoforms, particularly of TM-35, in different preparations of both 184 and 184A1N4 cells, perhaps related to passage history or to cell density and growth conditions. At this time we cannot eliminate the possibility that other isoforms are expressed in human breast cancer cell lines.
**Table 1** Characteristics of cell lines studied

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Cytokeratin</th>
<th>Activated oncogenes</th>
<th>Receptors</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td>Primary mammary epithelium</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>184A1N4</td>
<td>Immortalized derivative of</td>
<td>+</td>
<td>+</td>
<td>b</td>
<td>17, 37</td>
</tr>
<tr>
<td>184</td>
<td>184 (benzo[a]pyrene), non-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumorigenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>Ductal carcinoma</td>
<td>+</td>
<td>Amplified N-ras</td>
<td>+</td>
<td>23, 37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>variable</td>
<td></td>
<td>37, 38</td>
</tr>
<tr>
<td>BT-20</td>
<td>Ductal carcinoma</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Adenocarcinoma</td>
<td>+</td>
<td>Point-mutated c-Ki-ras</td>
<td>-</td>
<td>23, 37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37, 39</td>
</tr>
<tr>
<td>BT-474</td>
<td>Ductal carcinoma</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D</td>
<td>Ductal carcinoma</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZR-75.1</td>
<td>Ductal carcinoma</td>
<td>+</td>
<td>Amplified N-ras</td>
<td>+</td>
<td>23, 37</td>
</tr>
</tbody>
</table>

* Unless otherwise noted, all lines (strains) are reported to lack amplification of c-Ha-ras, erbB2, c-myc, and N-ras.

**Fig. 1.** Identification of TMs of normal human mammary epithelial cells. Nearly confluent monolayers were labeled for 4 h with [35S]methionine, and TMs prepared in various ways were resolved by two-dimensional polyacrylamide gel electrophoresis. A, 184 cells, immunoprecipitated TMs; B, 184A1N4 cells, immunoprecipitated TMs; C, 184 cells, heat-stable proteins; D, 184 cells, cytoskeletal (detergent-resistant) proteins. Sample preparations were as in "Materials and Methods." Only regions of gels containing TMs are shown (M, 31,000-43,000, pI ~4.5). Acidic proteins are toward the right. TMs are labeled according to their apparent molecular weight \( \times 10^{-2} \) as estimated from gels.

**Fig. 2.** Comparison of two-dimensional electrophoretic mobilities of TMs from 184 human mammary epithelial cells and WS1 diploid skin fibroblasts. TMs were immunoprecipitated from [35S]methionine-labeled 184 and WS1 cell lysates. Preliminary two-dimensional electrophoretic analyses were performed to determine the amounts of each immunoprecipitate needed to give equivalent film densities for the two samples. These amounts were then analyzed singly and as a mixture. A, 184 cell TMs. B, Mixture of 184 and WS1 cell TMs. C, WS1 cell TMs. Only short film exposures were used to optimize resolution. Under these conditions, non-muscle-type TMs could not be visualized. 184 cell TMs are labeled according to their apparent molecular weight \( \times 10^{-2} \) as estimated from gels. WS1 cell TMs are labeled according to the scheme in general use for fibroblast TMs (16).

TM isoforms may occur in human mammary epithelial cells which are sufficiently divergent from chicken smooth muscle TM as to be unrecognized by the antiserum.

Comparison of Two-Dimensional Electrophoretic Mobilities of Epithelial and Fibroblast TMs. From data reported by various investigators (24–27), it has become apparent that, in skeletal muscle, smooth muscle, and fibroblasts, tissue-specific TM isoforms are expressed. These can be grouped into homologues that are derived from the same gene but are encoded by different mRNAs because of tissue-specific alternative exon-splicing pathways. To ascertain whether any of the epithelial cell TMs might be tissue specific, we determined the correspondence in two-dimensional electrophoretic mobility between the epithelial cell TMs and previously reported human fibroblast TMs. Immunoprecipitated TMs from radiolabeled 184 cells and from WS1 diploid human skin fibroblasts were analyzed singly and in mixture by two-dimensional electrophoresis (Fig. 2). The mixture (Fig. 2B) was a summation of the individually analyzed samples (Fig. 2, A and C). It was evident that fibroblast TM1 and TM3 comigrated with epithelial cell TM-39 and -35, respectively, since there was no evidence of doubling of any of those spots, and the relevant spots showed heightened intensity in the mixture due to overlap. (The numbering of the fibroblast TMs is that currently adopted by numerous workers, e.g., Ref. 6.) 6) Epithelial TM-36 was barely visible in this film exposure (Fig. 2A), but its mobility appears to be identical to that of fibroblast TM2. These epithelial and fibroblast TMs, therefore, may be homologous, but further study is required to determine the relationships among their mRNAs. Epithelial cell TM-38 did not comigrate with any of the fibroblast TMs and may, therefore, be a tissue-specific isoform.

As noted above, the antiserum recognizes non-muscle TMs poorly. Because of this, the fibroblast non-muscle TMs (TM4 and TM5) were not immunoprecipitated in amounts sufficient to determine their electrophoretic mobilities relative to the epithelial cell non-muscle-type TMs 33a and 33b, to which they are most likely to be homologous. TM-32 does not correspond to any detectable or reported fibroblast TM, suggesting that it, too, is a tissue-specific isoform.

Relative Levels of TM Synthesis in Epithelial Cells and in Fibroblasts. TMs are prominent components among the newly synthesized proteins of fibroblasts (1, 7). To determine whether mammary epithelial cells expressed TMs at similar levels, radiolabeled total cytoplasmic protein samples from 184 and WS1 cells were analyzed by two-dimensional electrophoresis (Fig. 3). In this preparation of 184 cell proteins, TM-38 migrated in close proximity to a slightly more basic, heavily labeled protein (Fig. 3B). In the photographic reproduction shown here, this juxtaposition makes identification of TM-38 difficult. However, on careful examination of the original autoradiogram the
Table 2 Synthesis of TMs and actin in 184 mammary epithelial cells and in WS1 skin fibroblasts

Radioactivity in TMs and actin in gels shown in Fig. 3 was quantitated and expressed as counts/10^6 counts of total protein resolved on the gel. Figures in parentheses indicate the presumptive homologies between epithelial cell and fibroblast TMs, for purposes of comparison. Levels of labeling for TM-36 and TM-32 were too low for quantitation in this experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>184 cells</th>
<th>WS1 cells</th>
<th>184/WS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM1 (39)</td>
<td>1,180</td>
<td>15,153</td>
<td>0.078</td>
</tr>
<tr>
<td>TM-38</td>
<td>1,740</td>
<td>8,773</td>
<td>0.20</td>
</tr>
<tr>
<td>TM2</td>
<td>1,625</td>
<td>10,442</td>
<td>0.155</td>
</tr>
<tr>
<td>TM3 (35)</td>
<td>562</td>
<td>1,076</td>
<td>0.522</td>
</tr>
<tr>
<td>TM4 (33b)</td>
<td>1,602</td>
<td>3,163</td>
<td>0.506</td>
</tr>
<tr>
<td>TM5 (33a)</td>
<td>6,709</td>
<td>38,607</td>
<td>0.174</td>
</tr>
<tr>
<td>Total TM</td>
<td>33,262</td>
<td>149,343</td>
<td>0.223</td>
</tr>
<tr>
<td>Actin</td>
<td>33,262</td>
<td>149,343</td>
<td>0.223</td>
</tr>
</tbody>
</table>

The 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

**TM-38 spot were evident.** Fig. 3 indicates that, under conditions of similar sample loading and film exposure, synthesis of the muscle-type TMs relative to total proteins was very prominent in WS1 fibroblasts (Fig. 3A) but appeared less so in the 184 mammary epithelial cells (Fig. 3B).

This observation was confirmed by quantitation of the labeling of the major TMs and actin relative to total proteins entering the gel in WS1 and 184 cell samples (Table 2). Labeling of TM-39 (184 cells) was less than 10% of the value for TM1 (WS1 cells), to which it may be homologous based on electrophoretic mobility. The comparison between TM-35 and TM3 similarly showed a diminution in the 184 cells to about 15% of the fibroblast labeling rate. However, the values for the nonmuscle TMs in epithelial cells (TM-33a and -33b) were not as markedly reduced, amounting to approximately half those for the corresponding TMs (TM4 and TM5) in fibroblasts. Combined labeling of all TMs relative to total proteins in the gel in the 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

**TM-38 spot were evident.** Fig. 3 indicates that, under conditions of similar sample loading and film exposure, synthesis of the muscle-type TMs relative to total proteins was very prominent in WS1 fibroblasts (Fig. 3A) but appeared less so in the 184 mammary epithelial cells (Fig. 3B).

This observation was confirmed by quantitation of the labeling of the major TMs and actin relative to total proteins entering the gel in WS1 and 184 cell samples (Table 2). Labeling of TM-39 (184 cells) was less than 10% of the value for TM1 (WS1 cells), to which it may be homologous based on electrophoretic mobility. The comparison between TM-35 and TM3 similarly showed a diminution in the 184 cells to about 15% of the fibroblast labeling rate. However, the values for the nonmuscle TMs in epithelial cells (TM-33a and -33b) were not as markedly reduced, amounting to approximately half those for the corresponding TMs (TM4 and TM5) in fibroblasts. Combined labeling of all TMs relative to total proteins in the gel in the 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

**TM-38 spot were evident.** Fig. 3 indicates that, under conditions of similar sample loading and film exposure, synthesis of the muscle-type TMs relative to total proteins was very prominent in WS1 fibroblasts (Fig. 3A) but appeared less so in the 184 mammary epithelial cells (Fig. 3B).

This observation was confirmed by quantitation of the labeling of the major TMs and actin relative to total proteins entering the gel in WS1 and 184 cell samples (Table 2). Labeling of TM-39 (184 cells) was less than 10% of the value for TM1 (WS1 cells), to which it may be homologous based on electrophoretic mobility. The comparison between TM-35 and TM3 similarly showed a diminution in the 184 cells to about 15% of the fibroblast labeling rate. However, the values for the nonmuscle TMs in epithelial cells (TM-33a and -33b) were not as markedly reduced, amounting to approximately half those for the corresponding TMs (TM4 and TM5) in fibroblasts. Combined labeling of all TMs relative to total proteins in the gel in the 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

**TM-38 spot were evident.** Fig. 3 indicates that, under conditions of similar sample loading and film exposure, synthesis of the muscle-type TMs relative to total proteins was very prominent in WS1 fibroblasts (Fig. 3A) but appeared less so in the 184 mammary epithelial cells (Fig. 3B).

This observation was confirmed by quantitation of the labeling of the major TMs and actin relative to total proteins entering the gel in WS1 and 184 cell samples (Table 2). Labeling of TM-39 (184 cells) was less than 10% of the value for TM1 (WS1 cells), to which it may be homologous based on electrophoretic mobility. The comparison between TM-35 and TM3 similarly showed a diminution in the 184 cells to about 15% of the fibroblast labeling rate. However, the values for the nonmuscle TMs in epithelial cells (TM-33a and -33b) were not as markedly reduced, amounting to approximately half those for the corresponding TMs (TM4 and TM5) in fibroblasts. Combined labeling of all TMs relative to total proteins in the gel in the 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

**TM-38 spot were evident.** Fig. 3 indicates that, under conditions of similar sample loading and film exposure, synthesis of the muscle-type TMs relative to total proteins was very prominent in WS1 fibroblasts (Fig. 3A) but appeared less so in the 184 mammary epithelial cells (Fig. 3B).

This observation was confirmed by quantitation of the labeling of the major TMs and actin relative to total proteins entering the gel in WS1 and 184 cell samples (Table 2). Labeling of TM-39 (184 cells) was less than 10% of the value for TM1 (WS1 cells), to which it may be homologous based on electrophoretic mobility. The comparison between TM-35 and TM3 similarly showed a diminution in the 184 cells to about 15% of the fibroblast labeling rate. However, the values for the nonmuscle TMs in epithelial cells (TM-33a and -33b) were not as markedly reduced, amounting to approximately half those for the corresponding TMs (TM4 and TM5) in fibroblasts. Combined labeling of all TMs relative to total proteins in the gel in the 184 cells was, nevertheless, less than 20% of the value for WS1 fibroblasts. Interestingly, the relative labeling of actin in 184 cells was also reduced to about 20% of the level in WS1 cells, suggesting coordinate regulation of production of these two major microfilament components. These findings suggest that microfilaments may play a more restricted role in epithelial cells than they do in fibroblasts.

All of the carcinoma lines lacked expression of TM-39. Three of them (MCF-7, BT-474, and ZR-75.1) also showed absence of TM-38 expression. These lines all expressed TM-35 prominently and TM-33a, -33b, and -32 in varying relative amounts. T-47D also expressed TM-35 strongly but, in addition, expressed TM-38 at a relatively reduced level, compared with TM-35. TM-33a and -32, but not -33b, could be detected in T-47D on prolonged film exposure. The remaining two carcinoma cell lines (BT-20 and MDA-MB-231) expressed TM-38, but
TM-35 expression was markedly reduced or absent. In addition, BT-20 failed to express TM-33b but prominently expressed TM-32. Because of its low level, the expression of the minor species TM-36 was difficult to assess but did not seem to be affected in the neoplastic lines except for a possible increase in T-47D.

As noted above, the antiserum used shows relatively poor affinity for the lower M, non-muscle TMs. To obtain more information about the latter forms and to corroborate the results obtained by immunoprecipitation, radiolabeled heat-stable cytoplasmic proteins derived from each of the breast carcinoma lines were analyzed by two-dimensional electrophoresis (Fig. 5). This experiment confirmed the results obtained by immunoprecipitation; all carcinoma cell lines failed to express M, 39,000 TM. MDA-MB-231 and BT-20 did not express TM-35, and expression of TM-38 could not be detected in the remaining lines. The presence of TM-38 expression in T-47D, which is indicated by immunoprecipitation (Fig. 4G), could not be confirmed among the heat-stable proteins because of streaky migration of heavily expressed TM-35, which obscured the relevant region. This is a problem we have frequently encountered specifically with this protein.

The relative expression of the various isoforms, particularly that of the non-muscle TMs, could be assessed in the heat-stable preparation without bias due to antiserum specificity. Thus the expression of TM-33a was relatively prominent in all cells while the expression of TM33b was highly variable, ranging from absent in BT-20 and T-47D to strongly expressed in MCF-7. Expression of TM-35 was relatively reduced in ZR-75.1, while expression of TM-32 was relatively increased in BT-20. The importance of such quantitative variations in relative expression of the different TM isoforms is uncertain and may be an example of 'isoform switching,' which has been described in transformed fibroblasts (6). In our view the consistent absence of expression of specific TM isoforms in the breast carcinoma-derived cells is a more significant and well-defined modification.

In summary, all of the carcinoma lines failed to express TM-39 and five of the six lines also failed to express either TM-38 or TM-35. Two lines also failed to express TM-33b. All expressed TM-32, one prominently. Thus, cell lines derived from human breast carcinomas exhibit consistent major deviations from normal mammary epithelial cells in their pattern of TM isoform expression. This is seen as virtually complete absence of certain isoforms, notably of the muscle type.

Expression of 1.1-Kilobase Tropomyosin mRNA. Epithelial cell TM-39 has two-dimensional electrophoretic mobility identical to that of fibroblast TM1. Human fibroblast TM1 is encoded by a 1.1-kilobase mRNA that is apparently an alternative splice product of the same gene that encodes skeletal muscle β-tropomyosin (24). If TM-39 and TM-1 are identical proteins encoded by the same mRNA, then normal human mammary epithelial cells would be expected to express 1.1-kilobase TM mRNA at a level consistent with their relatively low production of TM-39 (Table 2). Moreover, since all of the breast cancer cell lines lack expression of TM-39, those lines would be expected to lack expression of 1.1-kilobase mRNA.

Expression of 1.1-kilobase TM mRNA was studied by Northern blot analysis. The probe used was TMe1, which was cloned in our laboratory from a human colon carcinoma cell cDNA library. It is a full length cDNA including the 3′ untranslated region and a portion of the 5′ untranslated region. Except for a shorter 5′ untranslated segment, its nucleic acid sequence is identical, base for base, to that of TM1 of fibroblast origin previously reported by MacLeod et al. (24). Thus, although it was cloned from an epithelial cell library, TMe1 is a cDNA to a mRNA that encodes a major muscle-type TM of fibroblasts. Data obtained with TMe1 hybridization were checked with probe M1558, which is specific for fibroblast TM 1.1-kilobase mRNA and which was provided by Dr. A. R. MacLeod (Cambridge) (24). In every case, probe M1558 gave results identical to those obtained with TMe1.

The Northern blot analysis was performed using serial dilutions of applied 184 cell and WS1 RNA and the blot was probed sequentially with TMe1 and a β-actin probe (Fig. 6). At the lowest level of RNA loading, a 1.1-kilobase mRNA was just detectable in 184 cell material but was strongly expressed in WS1 RNA. Radioactivity hybridizing to the 1.1-kilobase and β-actin bands was quantitated and is displayed in Fig. 7, normalized to WS1 values. It is evident that 1.1-kilobase mRNA is expressed in 184 cells but at less than 10% of the level found in WS1 fibroblasts. Moreover, the relative expression of β-actin mRNA showed the same relationship, so that the ratio of 1.1-kilobase mRNA to β-actin mRNA expression remained con-
DISORDERED TROPOMYOSIN EXPRESSION IN BREAST CANCER CELL LINES

Fig. 6. Comparison of 1.1-kilobase and β-actin mRNA expression in 184 cell and WS1 total RNA preparations. Total RNA quantities ranging from 5 to 40 μg from each cell type were analyzed by agarose-formaldelyde gel electrophoresis on the same gel, blotted to a single nylon membrane, and hybridized successively with cDNA probe TMel and with β-actin cDNA. A and B, TMel probe. C and D, β-actin probe. A and C, 184 cell RNA. B and D WS1 cell RNA.

Fig. 7. Quantitative analysis of 1.1-kilobase and β-actin mRNA in total RNA from 184 cells and WS1 cells. TMel (1.1-kilobase mRNA) and β-actin cDNA (1.7-kilobase mRNA) hybridization signals in the Northern blot shown in Fig. 6 were quantitated and expressed as counts/μg total RNA for each lane. Values obtained from the different lanes for each cell type were averaged. Means and SEs were normalized to the values for WS1 and are shown as the fraction of WS1 values. For each group: □, 1.1-kilobase counts/μg total RNA; ●, β-actin counts/μg total RNA; * * * ratio of 1.1-kilobase:β-actin counts.

DISCUSSION

This study has demonstrated that epithelial cell lines derived from human mammary carcinomas commonly exhibit derangements in TM expression. Moreover, the nature of the derangement was consistent in the neoplastic cell lines studied; TM-39 expression was uniformly absent and was combined with the absence of either TM-38 or TM-35 in all but one case. With one exception, all of the breast carcinoma cell lines also failed to express a 1.1-kilobase mRNA which encodes TM-39. The affected TMs were all of higher Mr, and were well recognized by anti-TM antiserum raised against smooth muscle TM, indicating that they are muscle-type TMs. The two-dimensional electrophoretic mobilities of TM-39 and TM-35 corresponded to those of two of the muscle-type TMs of human fibroblasts (TM1 and TM3). In studies of fibroblasts in which the transformed phenotype was induced by retroviral oncogenes, carcinogens, and transforming growth factor α, suppression of TM synthesis uniformly has involved muscle-type TMs (1–7). Thus, the association between deranged expression of muscle-type TMs and neoplastic transformation continues to be a general one, now including cultured epithelial cells derived from spontaneously arising human breast carcinomas.

The relationship between abnormality of TM production and the expression of activated protooncogenes in the breast cancer cell lines is uncertain. Three of the cancer cell lines studied are reported to have amplification or point mutations affecting members of the ras oncogene family (Table 1). The remaining three lines, while showing no abnormalities among the oncogenes tested (Table 1), may be expressing as yet undetected or unknown activated protooncogenes. Known activated or amplified protooncogenes have been isolated from some human cancers and previously uncharacterized transforming DNA sequences have been recovered from others (28), including the tk oncogene, which includes a translocated portion of a TM-encoding gene (29). Nevertheless, only 15–20% of human carcinomas have yielded such elements (28). In a study of TM metabolism in a line of immortalized mouse mammary epithelial cells, we reported that expression of the activated c-Ha-ras oncogene did not demonstrably affect synthesis of TMs but enhanced actin synthesis. The ratio of TM relative to actin accumulation in the cytoskeleton was distorted, however, with a resulting relative deficiency in cytoskeletal TM (8). It is possible, therefore, that suppression of TM synthesis may not be a characteristic response to oncogene expression in epithelial cells as it is in fibroblasts. If, as seems likely, some of the breast...
cancer cell lines exhibit deranged TM production for a reason other than the expression of activated protooncogenes, then other mechanisms must be evaluated. Among possible alternative mechanisms, consideration must be given to genetic rearrangements which might delete or otherwise cause loss of expression of specific TM genes. Examination of this possibility is currently in progress.

The fact that all six breast carcinoma cell lines, selected at random from available cultures, showed major and consistent derangements in expression of one particular family of genes coding for a set of structural proteins conserved in all cells justifies serious consideration of the possibility that deranged TM expression may play a role in human mammary carcinogenesis. It is now essential to determine whether such alterations will be found in fresh tumor specimens that have not been adapted to long term cell culture. Such a study is presently in progress in this laboratory.

The way in which deranged TM expression might contribute to carcinogenesis is obscure, since the function of TM in non-muscle cells, other than as a structural component of microfilaments, is unknown. TMs may act to stabilize microfilaments against the action of depolymerizing factors (30–33). Formation of microfilaments deficient in muscle-type TMs (7, 8) or with atypical arrays of TM isoforms (6) may impede this protective action and lead to microfilament destabilization, which may alter cell morphology, motility, or other microfilament functions associated with normal cell growth control. Human mammary epithelial cells express relatively high levels of EGF receptors (34) and evidence of association of the EGF receptor with the cytoskeleton has been reported (35). Activation of the EGF receptor by transforming growth factor α results in rapid interference with the utilization of newly synthesized TM in the cytoskeleton (7), suggesting a functional as well as a structural connection between this growth-regulatory receptor and the TM-containing cytoskeletal elements. Interference with these connections through microfilament disorganization may provide a link between derangement of TM expression and disordered growth control.

Of the seven TMs detected in 184 epithelial cells, two (TM-38 and TM-32) have two-dimensional electrophoretic mobilities different from any of those of fibroblast TMs (6). These, therefore, may be differentiation-specific isoforms characteristic of human (mammary) epithelial cells. Future cloning and sequencing studies will be required before it can be determined whether these isoforms are alternative splice products of genes that encode differentiation-specific isoforms in other cell lineages.

Northern blot analysis suggests that TM-39, which was absent from all the breast cancer cell lines, is encoded by a 1.1-kilobase mRNA related to the 1.1-kilobase mRNA of fibroblast TM1. Probe TMe1, which is a full length TM1 cDNA, and probe M1558, which is specific for the 1.1-kilobase mRNA of fibroblast TM1, both recognize a 1.1-kilobase mRNA in 184 cells. The level of expression of the 1.1-kilobase mRNA in epithelial cells, per μg of total RNA, is around 10% of that of the fibroblast 1.1-kilobase mRNA. TM-39, which comigrates electrophoretically with TM1, is expressed at a similar low level when compared with fibroblast TM1 protein. With one exception the breast carcinoma cell lines, all of which failed to express TM-39, also failed to express 1.1-kilobase mRNA. It appears likely, therefore, that the 1.1-kilobase mRNA of mammary epithelial cells encodes TM-39. The exception, line MDA-MB-231, expressed a high level of a 1.1-kilobase mRNA despite its failure to express TM-39. The reason for this inconsistency is not known. It is possible that the 1.1-kilobase message expressed by MDA-MB-231 is defective and not translated or that it is modified so that its product migrates aberrantly.

From the above considerations, it appears possible that identical TM proteins, encoded by identical mRNAs, may be expressed in fibroblasts and in mammary epithelial cells. However, due to the high degree of amino acid conservation even among TMs derived from quite different mRNAs (36), identity of electrophoretic mobility is not proof of identity of encoding mRNA. Also, alternative exon splicing may produce mRNAs of identical size which differ in sequence, since alternative coding exons may be of identical size (27). Therefore, we cannot at present conclude that human mammary epithelial cells depart from the typical pattern for TM expression, which utilizes differentiation-specific alternative splice products of common
ACKNOWLEDGMENTS

The authors wish to thank Elwood McDuffie for excellent technical assistance.

REFERENCES


13. Stampfer, M. R., and Bartley, J. C. Induction of transformation and contin...
Tropomyosins of Human Mammary Epithelial Cells: Consistent Defects of Expression in Mammary Carcinoma Cell Lines

Basudev Bhattacharya, Gaddamanugu L. Prasad, Eva M. Valverius, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/7/2105

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.