Coexpression of Cytokeratins Characteristic for Myoepithelial and Luminal Cell Lineages in Rat 13762NF Mammary Adenocarcinoma Tumors and Their Spontaneous Metastases


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ABSTRACT

We used immunohistochemical procedures to study the cellular expression and distribution of cytokeratins (CKs) in rat 13762NF mammary adenocarcinoma cells growing at mammary fat pad sites and at spontaneous lymph node and lung sites. In order to establish CK distribution in normal rat mammary epithelia, immature, resting, and lactating rat mammary glands were probed with a panel of monospecific antibodies that recognize individual CKs. Basal/myoepithelial cells were distinguished by expression of CKs 5 and 14 and coexpression of vimentin from luminal cells, which expressed CKs 8, 18, and 19. Antibody to CK 7 recognized luminal epithelium of immature and resting, but not lactating, mammary glands. Myoepithelial cells of lactating mammary gland were weakly recognized by antibodies to CKs 7 and 19. Tumors formed by cell lines and clones derived from parental 13762NF tumor (MTPa, MTC, MTA, and MTF7) were not recognized by any of the anti-CK antibodies. Only vimentin was expressed in these tumors and their metastases. In tumors and metastases generated from cell lines and clones derived from lymph node (MTLY) and lung metastases (M1112i) of the 13762NF tumor we observed heterogeneous CK phenotypes. Expression of CKs 5 and 18 was greatly reduced or lacking, while CK 14 was coexpressed with CKs 7, 8, and 19 with or without vimentin. Tumors from the highly metastatic clone MTLn3 had a dominant cellular phenotype, expressing CKs 7, 8, 14, and 19 and vimentin, a pattern that did not match normal mammary epithelia, whether luminal, basal/myoepithelial, or the dual-phenotype stem cell, in which CKs 5, 8, and 14 were coexpressed. MTLn3 lymph node and lung metastases expressed the same cellular phenotype as the s.c. growing MTLn3 tumor. The results appear to contradict the belief that malignant mammary tumors may be distinguished from benign tumors or hyperplastic growths by the lack of basal/myoepithelial markers.

INTRODUCTION

Analysis of CK expression is frequently used in tumor diagnosis, particularly of epithelial tumors (1–3). The definition of a tumor as a carcinoma is often based on the presence of CKs, and detection of specific CKs has allowed the subclassification of certain tumors, such as those arising in such complex epithelial tissues as the mammary gland (4–7). It is assumed that, in general, the profile of CK expression in the tumor cells qualitatively reflects the phenotype of the cell from which the tumor originated, but substantiation of this assumption necessitates precise definition of the normal and malignant phenotypes.

As defined by morphological, biochemical, and functional criteria, the mammary gland contains at least four types of epithelial cells: luminal ductal, basal ductal, luminal alveolar (secretory), and myoepithelial cells. The predominant phenotypes of these cell populations are distinct in the mature mammary gland. Luminal ductal and luminal alveolar epithelia are recognized by antibodies specific for CKs found in simple epithila, whereas basal ductal and myoepithelial cells are recognized by antibodies specific for CKs of stratified squamous epithila (8, 9) and coexpress vimentin (10, 11). There also exists a limited population of cells in the basal epithelia that coexpresses simple and stratified CKs (5, 10, 12). It has been suggested that these constitute a population of pluripotent stem cells from which the four distinct epithelia are derived (12) and generate genetically unstable cells during the carcinogenic process (13). In humans the majority of carcinomas lack cells expressing the basal/myoepithelial CK phenotype (5, 7, 14–18), and this also appears to characterize the acquisition of metastatic potential in rat mammary tumor cells (13, 19).

We recently reported that cell extracts enriched for intermediate filaments of double cloned or uncloned cell lines isolated from the rat 13762NF mammary adenocarcinoma, and its spontaneous lymph node and lung metastases varied phenotypically in the expression of CKs (20). These rat tumor cells failed to express CKs or expressed CKs characteristic for both simple and stratified epithila. This was an unexpected finding. To obtain positional information about the cells expressing CKs we used monospecific antibodies on frozen sections of tumors. We have demonstrated that CK-positive rat mammary tumor cells exhibit pronounced phenotypic heterogeneity of CK expression at the cellular level.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The tumor cells used in this study were derived from the 13762NF mammary adenocarcinoma syngeneic to F344 rats (21, 22). The 13762NF cell lines and clones vary reproducibly in their metastatic properties during in vitro growth (23) and display heterogeneity in a number of other properties (24, 25). Tumor cells were used at passages T14–16 (MTLn3), T11–13, (MTLY), T42–44 (MTLn2), and T8–12 (MTPa, MTC, MTF7). Tumor cells were grown as described previously (21, 25) in α-modified minimum essential medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT) without antibiotics. At 60–80% cell confluency, tumor cells were harvested by using 0.125% trypsin–2 mM EDTA in phosphate-buffered saline, pH 7.4 (containing, in g/liter: NaCl, 8; KCl, 0.2; Na2HPO4, 1.14; KH2PO4, 0.2), and diluted in fresh medium without fetal bovine serum. Cells (1 × 106) were injected into the mammary fat pads of F344 rats. Within 2–3 weeks tumors developed, reaching a size of 1 to 2 cm diameter after 4 weeks.

Histochemical Procedures and Immunofluorescence. Tumors were excised, snap frozen in liquid nitrogen, and stored at −20°C. Normal...
rat mammary tissue was obtained from 21-day-old female rats or mature lactating female rats. Tissue sections of 4-μm thickness were cut on a cryostat (International Equipment Co., Needham, MA) and processed as described previously (26).

Antibody Source and Description. Anti-CK 5, anti-CK 6, and anti-CK 14 are monospecific polyclonal antibodies generated in rabbits against peptide sequences located in the carboxyl termini that are unique to CKs 5, 6, and 14, respectively (20, 27–29). They were the gift of Dr. Robert G. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA). X-260, a polyclonal rabbit antibody recognizing CK 18 and CK 19, was the gift of Dr. Warren N. Schmidt (Vanderbilt University School of Medicine, Nashville, TN) and was characterized by us. In probes of human or rat tissues it recognized simple epithelia; in rat mammary gland this antibody recognized luminal epithelia. Although X-260 has been shown biochemically to recognize CKs 18 and 19, in extracts of tumor cells in the present study it detected only CK 19 (20). To demonstrate coexpression of CKs we performed double labeling by pairing a monospecific mouse MAb (antibody to CK 7, 8, 19, or Vim) with a monospecific rabbit polyclonal antiserum (to CK 5, 6, 14, or 18). Fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG and Texas red-conjugated sheep anti-mouse IgG were species specific antibodies purchased from Amersham Corp. All designations of CKs in this study correspond to the numbering system used for human CK studies (1) to facilitate comparison between human and rat. Microscopic observations were performed by using an Orthoplan Leitz photomicroscope equipped for transmitted and incident light fluorescence.

RESULTS

Normal Rat Mammary Glands. Comprehensive biochemical or immunohistochemical studies on CK expression and cellular distribution are not available for the rat mammary gland. To evaluate CK expression by rat mammary tumor cells, it was necessary first to establish the pattern of CK expression and distribution in normal rat mammary gland by using the same panel of antibodies subsequently applied to the tumors.

Frozen sections of mature and immature rat mammary glands were probed with combinations of the monospecific mouse MAb and monospecific rabbit polyclonal antibodies. Antibodies to CK 5, 6, 14, or 18 were paired with antibodies to CK 7, 8, 19, or Vim, respectively; Table 1 summarizes our findings. Coexpression of CKs defining simple epithelial (CKs 8 plus 18) or basal epithelial (CKs 5 plus 14 and Vim) cells was restricted to luminal or myoepithelial cells, respectively, in immature rat mammary glands (Fig. 1). However, tissue distribution patterns of the other CKs investigated were less clear cut or in some cases more variable when the two tissues were compared. For example, CK 19 in luminal epithelia of the immature and resting adult mammary glands was uniformly distributed (Fig. 1F), whereas in lactating mammary glands expression of CK 19 varied from cell to cell (data not shown). In both tissues basal and myoepithelial cells were weakly stained by antibody to CK 19. Similarly, luminal cells of both ducts and terminal end buds of immature mammary glands were positive for CK 7 expression (Fig. 1H), whereas in adult lactating mammary glands antibody to CK 7 reacted weakly with basal and myoepithelial cells and was unreactive with luminal cells (Fig. 1J). Occasional cells in the terminal end buds of immature glands and in ducts of resting adult mammary glands coexpressed CKs 5 and 8 normally restricted to the basal/myoepithelial or luminal cell types, respectively (data not shown). These cells may represent a subpopulation of dual phenotype stem cells like those described by Allen et al. (31). All epithelia of both both mature and immature mammary glands were negative for CK 6 expression (data not shown); however, low levels of CK 6 were detected in hair follicles of adjacent skin, indicating that the antibody could bind to CK 6 in the same tissue section.

Table 1 Expression of CKs and Vim in the mature lactating and immature resting rat mammary gland

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CK type (No.) or Vim</th>
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<tbody>
<tr>
<td>Lactating gland</td>
<td></td>
</tr>
<tr>
<td>Basal and myoepithelial cells</td>
<td>+* + + - (+) (+) +</td>
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<tr>
<td>Luminal cells</td>
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<tr>
<td>Immature gland</td>
<td></td>
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<tr>
<td>Basal and myoepithelial cells</td>
<td>+ + - - - (+) +</td>
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<td>Luminal cells</td>
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*+, positive for antibody staining; -, negative for antibody staining; (+), weak staining for antibody or expression varying from cell to cell.

CK Expression and Distribution in Locally Growing Mammary Tumors. Four cell lines and clones (MTPa, MTF7, MTC, and MTA) isolated from the 13762NF parental tumor were previously shown to be CK negative in studies using an antibody that recognized a single CK (26) or in biochemical analyses (20). Tumors generated by injection of these cell lines and clones into syngeneic rats were probed with the entire panel of antibodies to detect any low-level CK expression that went undetected in the previous studies. Tumor cells were negative with all CK antibodies tested, and Vim was the only intermediate filament protein expressed in these tumors (data not shown). The small clusters of CK-positive cells previously found in the mesenchyme at the tumor margin (26) were identified in the present study as residual normal mammary gland (data not shown). The structures possessed luminal cells positive for CKs 7, 8, 18, and 19, and they were surrounded by basal/myoepithelial cells positive for CKs 5 and 14 and Vim. Spontaneous lymph node metastases arising occasionally in rats given injections of the MTC clonal line were also negative for CK expression, using the same panel of antibodies (data not shown).

CK Expression and Distribution in Locally Growing Mammary Tumors Generated from Spontaneous Metastases. Three cell lines and clones, isolated from a spontaneous lymph node metastasis (cell line MTLY) and lung metastases (clones MTLn2 and MTLn3) in the same animal as the parental tumor, were found to be CK positive (26), expressing CKs tentatively identified as CKs 7, 8, 14, 15 or 17, and 19 (20). When probed with an antibody recognizing CK 8, the three lines varied in the proportion of CK-positive cells in the total cell population in vitro (26). In the present investigation, serial sections of tumors generated by injection of MTLY, MTLn2, or MTLn3 cells were probed with paired antibodies to specific CKs, which allowed us to locate the CK-positive cells and estimate their fraction of the cell population. In general, tumors exhibited a variety of CK expression phenotypes, while the CK-negative cells in these tumors expressed Vim. In CK-positive tumor cells, the combination of individual CKs varied considerably, and detailed staining patterns for individual tumor cells are presented only for MTLn3 tumors (Fig. 2) (summarized in Table 2). Based on the estimated frequency of cells that coexpressed individual CKs, a dominant CK-positive phenotype could be established in MTLn3 tumors but not in tumors formed from MTLn2 or the uncloned line MTLY. This domi-

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Fig. 1. Expression and distribution of CKs and Vim in immature (A-H) and lactating (I, J) rat mammary glands demonstrated by fluorescence microscopy. Sections of tissues were simultaneously probed with pairs of antibodies and detected by either fluorescein isothiocyanate-donkey anti-rabbit IgG or Texas red-sheep anti-mouse IgG in order to demonstrate exclusive or coexpression of CKs in single cells. The antibodies used are: anti-CK 14 (A, E); anti-Vim (B); Endo B, recognizing CK 18 (C); anti-CK 8 (D); anti-CK 19 (F); anti-CK 5 (G, I), and anti-CK 7 (H, J). x 400.
Fig. 2. Expression and distribution of CKs and Vim in locally grown (mammary fat pad) rat MTLn3 mammary adenocarcinoma tumors demonstrated by fluorescence microscopy. Sections of MTLn3 tumors were simultaneously probed with paired antibodies as in Fig. 1 in order to demonstrate exclusive or coexpression of CKs in the same cell. The antibodies used were: anti-CK 14 (A) and anti-Vim (B); anti-CK 14 (C) and anti-CK 7 (D); anti-CK 14 (E) and anti-CK 8 (F); anti-CK 14 (G) and anti-CK 19 (H). × 400.
A dominant CK-positive MTLn3 phenotype contained CKs 7, 8, 14, and 19, and these cells also expressed Vim. Heterogeneity in the cellular amounts of individual CKs was evident (Fig. 2). Although CKs 7, 8, and 19 were detected in the absence of CK 14 in <10% of CK-positive cells, CK 14 was always found coexpressed with CKs 7, 8, or 19, and usually with Vim (data not shown). The lack of X-260* or Endo B*, Vim* cells implied that cells positive for CKs 7, 8, and 19 and negative for CK 14 were similar to that seen with the fat pad growing tumors, namely coexpression of CKs 7, 8, 14, and 19 with Vim (Table 2).

A dominant CK phenotype could not be determined for tumors generated from the MTLn2 clone. Again, CK 14 was always found coexpressed with CK 7, 8, and 19. However, in contrast to MTLn3 tumors, coexpression of CK 14 with Vim was decreased. Briefly, six cellular phenotypes could be distinguished on the basis of CK coexpression: CK 14+, 19+, Vim+ (~20% of cells); CK 14+, 19+, Vim− (~4% of cells); CK 14+, 19+, Vim− (~30% of cells); CK 14+, 19+, Vim− (~6% of cells); CK 14+, 19+, Vim− (~24% of cells); and CK 14+, 19+, Vim− (~16% of cells).

Tumors arising from the uncloned lymph node metastasis-derived cell line MTLY also exhibited a variety of CK expression phenotypes (Table 2). Two cellular phenotypes were the most frequently seen: CK 7+, 8+, 14+, 19− (~60% of cells) and CK 7+, 8+, 14+, 19− (~40% of cells). In contrast to MTLn3 and MTLn2 tumors, only ~10% of the cells that expressed the CK 14+ phenotype were Vim+ in MTLn3 tumors. The CK 14+, Vim+ cells appeared fibroblastic and most likely were mesenchymal cells. These cells surrounded foci of tumor cells exhibiting a mixture of the two dominant cellular phenotypes. CK 14 was not present in the absence of CK 7, but CK 14 was occasionally found (~2% of cells) in the absence of expression of CKs 8 and 19. Thus, a small cell population exhibiting the phenotype CK 7+, 14+ (Vim+ or Vim−) was also present.

A Small Subpopulation of CK 5+ and 18+ Cells in Mammary Tumors and Their Metastases. In normal cells of the basal/myoepithelial lineage, CK 14 is coexpressed with CK 5. Although the only type II CKs detected in extracts of tumor cells were CK 7 and CK 8 (20), additional type II CKs could have been expressed by a limited population of cells in biochemically undetectable quantities. When tumor sections were probed with antibody to CK 5, a limited population of cells were positive. For example, in fat pad MTLn3 tumors less than 2% of CK-positive cells were positive for CK 5, whereas CK 5* cells did not exceed 20 cells per section of MTLn3 inguinal lymph node metastases (Fig. 3A). However, cells from axillary lymph nodes or lung metastases were negative for CK 5. Less than 5% of CK-positive cells were positive for CK 5 in fat pad MTLn2 tumors, while positive cells could not be identified in local MTLy tumors. When CK 5 was detected, it was coexpressed with CKs 7, 8, and 19, which appeared to be expressed at reduced but detectable levels. All of these tumors were negative for CK 6.

Various tumors were also probed with Endo B to test for the presence of CK 18, a type 1 CK normally coexpressed with CK 8 in the luminal cells of rat mammary glands. A low level of diffuse, positive staining for CK 18 was present in 10% of CK∗ cells in local MTLn3 tumors, but the staining pattern of CK 18 appeared filament-like in only a few cells (Fig. 3C). All CK 18* cells were Vim*, indicating that they were probably a subpopulation of the dominant CK* phenotype. Lymph node metastases of MTLn3 cells exhibited the same pattern of CK 18 expression (data not shown). MTLy tumors (fat pad) exhibited a low level of diffuse CK 18* staining in ~20% of tumor cells and anti-CK that had a filament-like distribution in less than 2% of CK 18* cells. MTLn2 tumors did not contain cells positive for CK 18 (data not shown).

Our findings are summarized in Table 2. It is obvious that most cells in MTLn3 local tumors and metastases express a mixed CK phenotype (Table 2, Section A). However, with the combination of antibodies used in this study, information as to coexpression of Vim with simple or basal CKs could not be obtained. Thus, the phenotypes described in Table 2 (Sections B and C) can only be classified as basal/myoepithelial- or luminal-like. Whereas in MTLn3 local tumors and their metastases a mixed phenotype dominates (Table 2, Section A), a significant proportion of basal/myoepithelial-like cells could only be demonstrated in local MTLn2 tumors (Table 2, Section B). Furthermore, luminal-like cells (Table 2, Section C) and an undefined phenotype (Table 2, Section D) were frequently seen in MTLy and MTLn2 local tumors. However, due to the lower metastatic potential of MTLy and MTLn2 tumors we did not have access to their spontaneous metastases to test if the pattern of CK expression was similar to the respective fat pad tumors or similar to the metastases from MTLn3 fat pad tumors.

### DISCUSSION

The utility of any animal tumor model in understanding the corresponding human malignant disease process necessarily depends upon the degree of similarity in the tumorigenic and metastatic process that occurs in each species. Fundamental to an evaluation of cell types involved in these processes is a definition of the normal cell types. The classification of cell types in complex tissues, such as mammary gland, has been facilitated by the availability of monospecific antibodies to individual CKs. In situ analysis of human mammary gland revealed that CKs 8 and 18 are restricted to luminal cells (6–8, 9, 32, 33), whereas CKs 5 and 14 are exclusively expressed in basal/myoepithelial cells (5, 9, 34). CKs 7 and 19 are primarily expressed in luminal cells (4, 8, 35), although antibodies monospecific for either CK also react weakly with basal/myoepithelial cells (8, 9). CK 17 and Vim are detected exclusively in
basal/myoepithelial cells (7, 11). The intensity of expression varies, with ductal basal cells that strongly express CK 17 and occasionally express Vim, whereas myoepithelial cells of alveoli exhibit the inverse relationship between CK 17 and vimentin expression. Although CK 15 has been biochemically detected in human mammary gland extracts (1), determination of its cellular distribution awaits development of a monospecific anti-CK 15 antibody. As established by the present study, CK expression in normal rat mammary gland, with the exception of CKs 15 and 17, which were not included because of the unavailability of monospecific antibodies, appears to be similar to that of normal human mammary gland. Indeed, the only notable difference in the normal mammary glands of the two species is the location of putative stem cells, that is, cells coexpressing CKs characteristic of the luminal or basal/myoepithelial lineages. In human mammary glands, these cells were found in the luminal cell population (discussed in Ref. 8), whereas they were located in the basal cell population of the rat mammary gland (12).

The mere presence of an individual determinant does not define a particular cellular phenotype, because each determinant may be shared by several cell types. Rather, it is the coordinated expression of several determinants combined with particular morphological or ultrastructural characteristics that define a distinct phenotype. The basal/myoepithelial cellular phenotype, in addition to expressing CKs 5, 14, 17, and possibly 15 (plus low levels of CKs 7 and 19), coexpresses Vim (7, 10, 13), smooth muscle α-actin (36), smooth muscle myosin, laminin, and collagen IV (10, 12, 37). Stem cells coexpress CKs 5 and 14, characteristic of the basal/myoepithelial lineage, with CKs 8 and 18, characteristic for luminal cells, but do not express most of the other basal/myoepithelial markers (12, 13). Since the dominant cellular phenotype in the metastatic cell line, MTLn3, expressed a mixed CK profile intermediate between myoepithelial and stem cell phenotype, no strict definition of the cell of origin of MTLn3 tumor cells can be made on the basis of CK expression. It might be argued that derivation of MTLn3 tumor cells from a lung metastasis and the subsequent cloning resulted in selection of a unique cellular phenotype not generally present in the primary tumor or normal cells (38). The dominant MTLn3 cellular phenotype (expressing CKs 7, 8, 14, and 19 but lacking CKs 5 and 18 and coexpressing Vim) could represent a myoepithelial or basal cell that has partially dedifferentiated, or alternatively, a stem cell which has progressed to an intermediate stage. MTLn2 and MTLY cells showed even greater phenotypic heterogeneity than MTLn3 cells; therefore, the cell of origin of these two cell lines also cannot be assessed. Examination of these tumors for other markers of the basal/myoepithelial cell lineage may resolve this issue. A preliminary survey has detected significant amounts of laminin around cells coexpressing Vim in MTLn3 and MTLn2 tumors, particularly in regions containing CK+ cells. In MTLY tumors laminin surrounded tumor cell foci.7

Several investigators have suggested that characteristics of the basal/myoepithelial lineage might serve as the criterion for a differential diagnosis of human mammary tumors when histological analyses are not sufficient (39–41). This suggestion was supported by the observation that dysplastic proliferations and benign tumors of the mammary gland contained a variety of normal and intermediate CK expression phenotypes, whereas

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7 Unpublished observations.
the majority of carcinomas lack cells expressing the basal/myoepithelial CK phenotype (5, 7, 14–18). The loss of cells expressing the basal/myoepithelial CK phenotype also appeared to characterize the acquisition of metastatic potential in rat mammary tumor systems (13, 19, 39). However, in a mouse mammary tumor model using an antibody that recognized CK 14 the basal/myoepithelial phenotype was detected in the fat pad tumors and cells in the hematogenously disseminated metastases (42). Furthermore, 10% of invasive human mammary ductal carcinomas were positive for CK 14 (43). Thus, this correlation remains a controversial issue, particularly in studies on human tumors. A new designation of “adenomyoepithelioma” has recently been suggested for human mammary tumors containing primarily spindle cells that exhibit a subset of CK markers characteristic for the basal/myoepithelial lineage (44), some of which exhibit metastatic potential (45–48).

The results of the present study indicate that the acquisition of metastatic potential does not necessarily correlate with the disappearance of CK markers for the basal/myoepithelial lineage in the rat mammary tumor system. The dominant CK phenotype present in the most metastatic of the three CK-positive cell lines, MTLn3, was CK 14* and Vim*, both of which distinguish basal/myoepithelial from luminal cells in normal mammary gland. This CK phenotype proved to be mixed, however, matching neither the normal luminal nor basal/myoepithelial CK phenotypes, since CK 14 and Vim were coexpressed with CKs 7, 8, and 19. The dominant MTLn3 tumor phenotype was distinguished by the negligible levels of expression of CKs 5 and 18, which have been detected in normal cells coexpressing determinants of both cell lineages (putative stem cells). Thus, the MTLn3 cellular phenotype matched neither the stem cell, luminal lineage, nor the basal/myoepithelial lineage. Retention of a subset of determinants characteristic of an intermediate developmental stage for mammary epithelia may ultimately define the malignant phenotype of the 13762NF tumors.

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