Polyclonal Development of Mouse Mammary Preneoplastic Nodules

William C. Kisseberth and Eric P. Sandgren

1Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio, and 2Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin

ABSTRACT

Studies of cellular interactions are critical to the understanding of tumorigenesis. Although many studies have demonstrated a monoclonal composition of advanced neoplasms in humans and mice, the clonal composition of smaller, antecedent lesions has been studied less thoroughly. To examine the clonal development of breast cancer, we generated chimeric mammary glands using mouse mammary epithelium with an inherited predisposition for neoplasia. Analysis of whey acidic protein-transforming growth factor-α transgenic mouse mammary glands, chimeric for two different cell lineage markers, revealed that mammary ducts and alveoli are polyclonal, and putative early preneoplastic lesions, hyperplastic alveolar nodules (HANs), frequently are polyclonal. Furthermore, the chimeric patch patterns in individual HANs were similar to the patterns observed in pregnant chimeric mammary glands. Thus, polyclonality in HANs appears to reflect persistence of the polyclonal architecture of ducts and/or alveoli, suggesting that hyperplasia formation can be the result of non-cell autonomous local tissue microenvironmental influences on groups of cells, rather than clonal progression of a single initiated cell.

INTRODUCTION

The multistage model of carcinogenesis emphasizes somatic mutation as the neoplastic initiating event, which then is followed by accumulation of additional genetic and epigenetic changes in the initiated cell or its progeny. Collectively, these events lead to the emergence of a clonal dominant cell population. This model predicts that the earliest lesions in this process will be monoclonal. Until recently, most evidence has supported a monoclonal origin for solid tumors, including tumors of the colon, skin, and breast in humans (1–8) and in carcinogen-treated rodents (9–11). However, studies have demonstrated polyclonality in at least some early preneoplastic lesions and epithelial tumors (12–14).

Although most analyses of animal and human tumors support a monoclonal lesion composition, interpretation of these studies is complicated by two related problems:

(a) When addressing the question of tumor clonality, it is important to differentiate between clonal origin and clonal evolution. A clonal origin would follow from the emergence of a single cell with a growth advantage that clonally expands into a neoplasm. In contrast, clonal evolution implies a later event that is the result of selective growth of a subpopulation of tumor cells (15, 16). Monoclone at a late stage of tumor progression thus may not reflect the cellular composition of the originating neoplastic cell population. Similarly, polyclonality at this late stage is difficult to interpret. Mammary neoplasms tend to be cytogenetically complex, with individual cells often having different, seemingly unrelated karyotypes (17, 18). This diversity may reflect the underlying genetic instability within advanced neoplasms rather than the original cellular composition. For these reasons, it is important to analyze the smallest recognized lesions, which are more likely to represent the initially transformed cell population.

(b) Most methodologies used to investigate clonality, especially in human tumors, involve biochemical or molecular evaluation of homogenized tissue. These techniques include analysis of patterns of inactivation of polymorphic X chromosome-linked genes such as glucose-6-phosphate dehydrogenase (19), glycerophosphate kinase, the androgen receptor gene (HUMARA; Ref. 2), and hypoxanthine phosphoribosyltransferase (20); detection of somatic mutations (21, 22); studies of viral integration site by Southern blot analysis (23); and detection of microsatellite instability (24). Using these methods, the genotype of the entire tissue sample is identified without determining the genotype for individual cells. The sensitivity of these procedures can be relatively low. For example, for X-inactivation studies using homogenized tissue, a monoclonal cell population must comprise at least 20% of the sample population to be recognized reliably (25).

Conversely, a polyclonal composition may be inferred mistakenly if nonneoplastic tissue contamination (e.g., stroma) is too great.

Evaluation of the clonal origin of breast cancer has yielded conflicting answers. Human breast neoplasms and preneoplastic proliferative lesions occasionally show cytogenetic abnormalities that suggest the presence of multiple clones (26). Recently, Going et al. (14) used a combined approach evaluating X-inactivation and androgen receptor exon I CAG polymorphism to propose that one-third of human mammary carcinomas analyzed were polyclonal. Nevertheless, other studies indicate that some of the same lesions appeared to be clonal (7, 27–29). Experimentally, mouse mammary tumor virus-induced preneoplastic hyperplastic alveolar nodules (HANs) and tumors have been suggested to be monoclonal, based on restriction fragment length polymorphism analysis of proviral insertion sites (30). Also, in rats, methyltestosterone-induced mammary carcinomas possessed monoclonal mutations in the ras oncogene (11).

Whey acidic protein-transforming growth factor-α (WAP-TGFα) transgenic mouse overexpress mature, soluble TGFα in mammary epithelium (31). Female mice that overexpress TGFα in mammary epithelium develop mammary carcinomas, temporally preceded by formation of HANs in the mammary gland (32–34). HANs histologically resemble lactating epithelium in a nonlactating gland. HANs are serially transplantable (in some cases indefinitely), display increased neoplastic potential compared with normal mammary epithelium, and also can be induced by mouse mammary tumor virus, hormones, and chemical carcinogens in rodents (35, 36). For these reasons, HANs have been considered to represent a preneoplastic mammary epithelial cell population. Here we describe studies of transplantation of chimeric mammary glands in which each gland is composed of two uniquely marked epithelial cell populations that both carry the same TGFα gene. We have used this cell marking system to determine the clonal composition of normal epithelium and of TGFα-induced epithelial hyperplasias (HANs) in the mouse mammary gland.

MATERIALS AND METHODS

Mouse Strains. The 804-2 line of R26-hPAP transgenic mice, which expresses the marker gene human placental alkaline phosphatase (hPAP) in all tissues (37), and the K18TG2 line of transgenic mice, which expresses human cytokeratin 18 (hCK18) in simple epithelia, including mammary epithelia (38,
39), were generated and maintained on the FVB/N background. The 3573-2 line of WAP-TGFα transgenic mice, [TgN(WAPTgα)215Bri], was described previously (31). WAP-TGFα mice originally were generated in a C57BL/6 × SJL background, but for this study were used after >10 generations of backcrossing into the FVB/N strain. The hCK18 mice were provided by Robert Oshima (The Burnham Institute, La Jolla, CA). All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities and handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experimental procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

**Genotyping.** Transgenic mice were identified by PCR analysis using the following primer pairs: R26-hPAP, SP-vpoly-1f (5′-CTGATGAAATGGGAGCAGTGGTGGAATG-3′) and SP-vpoly-2r (5′-GCAGACACTCTATGCCTGTGCAGTGGTGGAATG-3′); hCK18, hCK18a-2f (5′-GCATGAGCAACCACGCCCAGC-3′) and hCK18-6r (5′-CTGCTGAAGGCTGGAGGGCCAAAC-3′); and WAP-TGFα, TGF-PC3f (5′-TGTCAGGCTCTGGAGAAACAGC-3′) and TGF-E4r (5′-CACAGCGGAACACCCACGTACC-3′). Twenty-five μl of reaction mixture containing genomic DNA from tail were subjected to the following conditions for R26-hPAP and WAP-TGFα genotyping: (a) 92°C for 110 s; (b) 35 cycles of: 45 s at 92°C, 60 s at 60°C, and 60 s at 72°C; and (c) 72°C for 5 min. For hCK18 transgene genotyping, genomic DNA was subjected to the following conditions: (a) 92°C for 120 s; (b) 34 cycles of: 60 s at 92°C, 90 s at 60°C, and 110 s at 72°C; and (c) 72°C for 7 min. R26-hPAP mice alternatively were genotyped by enzyme histochemistry performed on peripheral blood (37).

**Generation of Mammary Gland Chimeras.** Chimeric mammary glands were produced by transplantation of 5 × 10^6 collagenase/trypsin-isolated mammary epithelial cells from each of two genotypes into cleared mammary fat pads of 3-week-old syngeneic FVB/N nontransgenic female recipients (Refs. 40, 41; Fig. 1). Four different genotypes in two combinations were used: bitransgenic R26-hPAP/WAP-TGFα and bitransgenic hCK18/WAP-TGFα co-transplanted, or marker-only R26-hPAP and hCK18 cotransplanted. Recipient mammary fat pads were “cleared” of host epithelium by surgical excision of the mammary rudiment before it extended into the fat pad. Transplanted mammary epithelial cells divide and differentiate to produce a morphologically normal mammary gland. We used this procedure to create chimeric mammary glands in which each mammary epithelial cell contains: (a) the WAP-TGFα transgene and one of two marker transgenes (R26-hPAP/WAP-TGFα → hCK18/WAP-TGFα); or (b) one of two marker transgenes only. For subsequent analyses, chimeric or control mammary glands were collected from virgin, day 18 pregnant, or nonpregnant multiparous females. Mice were not mated until at least 8 weeks after epithelial cell transplantation.

**hCK18 Immunohistochemistry and hPAP Histochemistry.** Reconstituted mammary fat pads or tumors were fixed in 4% paraformaldehyde at 4°C for 4 h. Fixed tissues were dehydrated through graded alcohols to Hemo-De and embedded in paraffin. Antigen retrieval and immunohistochemistry were performed as described previously (37). Sections were dewaxed in Hemo-De, rehydrated, heated at 65°C in substrate buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 5 mM MgCl2] for 30 min to block endogenous alkaline phosphatase activity, and then incubated with fresh buffer containing 0.17 mg/ml of the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, St. Louis, MO). Sections were incubated 18–48 h at 37°C, rinsed, dehydrated through graded alcohols to Hemo-De, and mounted under glass coverslips. Immunohistochemistry for human cytokeratin 18 using mouse monoclonal antibody (clone DC10; BioGenex, San Ramon, CA) at a dilution of 1:10–1:20 was used in a standard peroxidase-based protocol. Briefly, 5-μm tissue sections were dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed by microwaving the slides in 6 M urea (pH 6.0) for 10 min at 50% power in a 1450-W microwave. Endogenous peroxidase activity was quenched by incubation of the slides in 0.3% hydrogen peroxide in methanol for 20 min on ice. Immunohistochemistry then was performed with the Supersensitive peroxidase system according to the manufacturer’s directions (BioGenex). Sections were developed in diaminobenzidine substrate (Sigma). Sections were counterstained with Mayer’s hematoxylin for 2 min. Sections that were double-labeled first were stained with hPAP histochemistry with 48-h substrate incubation, followed by hCK18 immunohistochemistry.

**Determining Number of Chimeric HANs in Reconstituted Mammary Glands.** The number and clonal composition of HANS was determined in a single random hCK18 immunostained and hematoxylin counterstained sagittal section from each reconstituted gland. Individual anatomical structures (mammary ducts, alveoli) in the entire stained tissue section were analyzed for the presence of all brown (hCK18+), all nonbrown (hCK18–), or mixed brown/nonbrown (hCK18+/hPAP–) cells. HANS are defined by the presence of two or more alveolar lumenal profiles that are ≥40μm in luminal profile. HANS profiles with single random profiles were excluded. HANS profiles with ≥40 μm in luminal profile were included in this study. HANS profiles with single random profiles were excluded. HANS profiles with ≥40 μm in luminal profile were included in this study.
RESULTS

hCK18 and R26-hPAP as Cell Lineage Markers in Mammary Gland. We used the R26-hPAP and hCK18 transgenic lines as cell lineage markers in chimeric mice. R26-hPAP transgenic mice express human placental alkaline phosphatase in all tissues and have been used to mark transplanted cells in previous studies (37, 42, 43). hCK18 transgenic mice express human cytokeratin 18 under the control of its own regulatory elements (38, 39). The monoclonal antihuman cytokeratin 18 antibody that we used detected strong immunoreactivity in alveolar mammary epithelia of hCK18 transgenic mice but did not recognize the mouse cytokeratin 18 homolog, endoB, in nontransgenic mice. Ductal mammary epithelial cells also stained strongly in hCK18 transgenic mice but did not stain or stained very weakly in nontransgenic mice (data not shown). To determine whether R26-hPAP and hCK18 are expressed in transplanted WAP-TGFα mammary epithelium, we crossed WAP-TGFα transgenic mice with R26-hPAP or hCK18 marker mice and transplanted isolated mammary epithelial cell lines into cleared mammary fat pads of nontransgenic recipient female mice, 3 weeks of age, as described in “Materials and Methods.” Glands from virgin, pregnant, or nonpregnant multiparous females were removed at selected times, fixed, sectioned, and stained to localize the hCK18 and/or hPAP markers. Reconstituted mammary glands from WAP-TGFα/H9251 chimeric marker control recipient mice showed strong and spatially consistent hCK18 immunostaining in all epithelial cells and HANs (Fig. 2, A and C). hPAP staining of reconstituted mammary glands from WAP-TGFα/R26-hPAP single-marker control recipient mice showed hPAP expression of variable intensity in epithelial cells and HANs (Fig. 2, B and D). The strength of hPAP staining varied within an individual gland and between glands. We also observed hPAP histochemical staining of the nontransgenic stroma (adipocytes) immediately adjacent to R26-hPAP mammary epithelium in reconstituted glands. We concluded that in this system, the hCK18 transgenic lineage was the more precise and consistent marker strain. Therefore, we performed our initial analyses on chimeric mammary glands using hCK18 immunohistochemistry only and then in selected cases confirmed that the hCK18-negative epithelium was marked by the R26-hPAP transgene.

Generation and Characterization of Chimeric Mammary Epithelium. To determine the clonal composition of mammary ducts and alveoli in reconstituted mammary glands, hCK18 immunostained and R26-hPAP/WAP-TGFα ↔ hCK18/WAP-TGFα chimeric mammary glands from day 18 pregnant and virgin mice were prepared as described above for single-marker control glands and then examined microscopically. The predominant staining pattern observed consisted of relatively large brown or nonbrown patches with variable mixing of the two lineages (Fig. 2, E and F). For example, on a single tissue section, the following alveolar staining patterns could be identified: (a) all brown cells except for one nonbrown cell; (b) one-half of the alveolus brown and the opposite half nonbrown; and (c) variable numbers and patterns of brown cells alternating with variable numbers of nonbrown cells (Fig. 2F). Chimeric mammary ducts displayed similarly variable patterns of genotype mixing (Fig. 2E). Several hCK18-immunostained tissue sections were costained with hPAP histochemistry (Fig. 2, G and H), demonstrating that nonbrown cells stained blue, indicative of R26-hPAP marker transgene expression.

Histological Examination of Hyperplastic Alveolar Nodules. To assess the clonal composition of HANs, we examined immunohistochemically stained sections from nonpregnant, parous WAP-TGFα/hCK18 ↔ WAP-TGFα/R26-hPAP chimeric mammary glands. We identified seven reconstituted glands that were informative, containing both hCK18-positive and hCK18-negative epithelial cells and multiple HANs (Table 1 and Fig. 3). Individual HANs from these glands were scored as to whether they were: (a) all hCK18 negative; (b) all hCK18 positive; (c) chimeric at the cell level (i.e., within a single alveolar cross-section, some cells were hCK18 positive and some were hCK18 negative); (d) chimeric at the alveolar level (i.e., some alveolar cross-sections were all hCK18 positive, and some alveolar cross-sections were all hCK18 negative); or (e) chimeric at both the cell and alveolar level. From a total of 134 HANs in these seven chimeric mammary glands, 62 were all hCK18 positive (Fig. 3B), 20 were all hCK18 negative (Fig. 3C), and 52 were mixed hCK18 positive/hCK18 negative (39%; Table 1). Among mixed HANs, 37 were chimeric at the cell level (Fig. 3E), 2 were chimeric at the alveolar level, and 13 were chimeric at both the cell and alveolar levels (Fig. 3, D and F). Finally, to determine if the occurrence of chimerism was influenced by HAN size, we counted the number of alveolar lumens (an indicator of relative HAN size) for each chimeric HAN described in Table 1. Chimerism was observed in HANs of all sizes (Table 2).

Because this analysis is based on staining of a single histological section from each chimeric gland, a portion of any HAN that is out of the plane of section may have been chimeric, even if the examined cross-section appeared monoclonal. Furthermore, HANs containing a pure population of hCK18-positive or hCK18-negative cells may be polyclonal, but this could not be ascertained if the different clones expressed the same marker. Therefore, this experimental approach underestimates the true frequency of polyclonal HANs.

DISCUSSION

Our data indicate that many epithelial hyperplasias in WAP-TGFα transgenic mouse mammary glands are polyclonal. We also show that normal mammary ducts and alveoli generated by mammary epithelial cell transplantation are polyclonal. Furthermore, we show that similar patterns of marker (genotype) mixing can be observed in pregnant and hyperplastic alveoli. These findings suggest that HAN polyclonality reflects persistence of the polyclonal architecture of ducts and alveoli that exists in recipient mice before the onset of disease. This outcome indicates that TGFα-induced HAN formation can involve the coordinated growth of groups of cells within the context of the local mammary environment, rather than being exclusively a monoclonal event initiated by a single cell with altered growth potential.

Our findings are consistent with the observations of Mintz and Slemmer (44). In that early study of mouse mammary tumor virus-induced HANs in chimeric mice, the genotype composition of each HAN was inferred from its transplantability into recipients of both chimera donor strains. However, Cardiff et al. (36) reached a different conclusion when they observed monoclonality in hyperplastic outgrowths produced from transplantation of single mouse mammary tumor virus-induced HANs into gland-free fat pads. Because the latter analysis was not performed directly on the HANs but rather on their descendant outgrowths, the possibility of emergence of a clonal dominant outgrowth from an originally polyclonal HAN must be considered.

Our observation of polyclonal mammary ducts and alveoli in transplantation chimeras is consistent with the reported polyclonal com-
Table 1. HAN clonality within mammary glands of WAP-TGFα/hPAP → WAP-TGFα/hCK18 transplantation chimeras

<table>
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<tr>
<th>Chimera</th>
<th>All hCK18-negative</th>
<th>All hCK18-positive</th>
<th>Chimeric (cell level)</th>
<th>Chimeric (alveolar level)</th>
<th>Total chimeric</th>
<th>Total examined</th>
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<td>28%</td>
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<td>10%</td>
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* The numbers of pure hCK18-positive, pure hCK18-negative, and chimeric HANs were determined from a single random sagittal section from each of seven reconstituted chimeric mammary fat pads. The composition of each HAN was assessed by immunohistochemical staining for hCK18 immunoreactivity as described in “Materials and Methods.” For selected samples, hPAP histochemistry was performed on hCK18-immunostained tissue sections to confirm that hCK18-negative cells were of R26-hPAP origin.
position of the mammary gland in normal mice, based on \textit{in situ} histochemical detection of polymorphisms in the enzyme glucose-6-phosphate dehydrogenase (45). The genotype-specific staining patterns of mouse mammary glands in that study and ours suggest that alveoli can originate from more than one cell; however, this may not be true for human breast development. Tsai \textit{et al.} (46) used X chromosome inactivation analysis and laser capture microdissection of isolated epithelium and concluded that individual lobules and ducts in normal human breast are monoclonal. Recently, Novelli \textit{et al.} (47) determined that in human breast the terminal ducts and lobules are monophenotypic based on \textit{in situ} glucose-6-phosphate dehydrogenase histochemistry. However, they concluded that the large X-inactivation patch size confounded assessment of clonality.

The primary evidence suggesting polyclonality of both premalignant human breast lesions and carcinomas has been cytogenetic (17, 18, 26, 48, 49), with almost 50% of breast carcinomas and benign breast tumors containing multiple clones lacking common cytogenetic abnormalities (17, 26, 49). However, whether such divergent clones represent a polyclonal origin \textit{versus} polyclonal evolution after the neoplastic phenotype was established is undetermined (18).

Studies in other tissues also have suggested a polyclonal development for some epithelial neoplasms. Novelli \textit{et al.} (50) showed that the majority of the adenomas in a human XO/XY mosaic familial adenomatous polyposis (FAP) patient were polyclonal. Polyclonal adenomas also were found in chimeric \textit{Min} mice in which both components of the chimera carried the \textit{Min} mutation and one component also carried a \textit{lacZ} marker, allowing the clonality of the resulting intestinal adenomas to be assessed (12). \textit{Min} mice and familial adenomatous polyposis patients both carry mutations in the adenomatous polyposis coli gene. Using a similar experimental approach using mouse embryo aggregation chimeras expressing different H2 haplotypes, Winton \textit{et al.} (13) showed that 20% of chemically induced skin papillomas and antecedent foci of epidermal hyperplasia were polyclonal.

Merritt \textit{et al.} (12) speculated on the origin of mixed intestinal adenomas. Possible explanations for mixed lesions in chimeric mouse tissues include: random collision between lesions, somatic mosaicism for the marker, epigenetic silencing of marker expression, and interaction between multiple clones. Similar to those workers, we have found evidence against the first three of these hypotheses: (a) chimeric HANs were found in fat pads with low HAN multiplicity, arguing against random collision events; and (b) chimeric alveoli

| Table 2 Number of alveoli in chimeric HANs of WAP-TGFα/hPAP ↔ WAP-TGFα/hCK18 transplantation chimeras |
|-----------------------------------------------|-------|-------|-------|-------|
| No. of alveoli/HAN | ≤5    | 6–10  | 11–30 | >30   |
| No. of chimeric HANs | 12    | 14    | 14    | 12    |

* The numbers of alveolar lumens in chimeric HANs were determined from a single random sagittal section of each complete, reconstituted, chimeric mammary fat pad. The composition of each HAN was assessed by immunohistochemical staining for hCK18 immunoreactivity as described in “Materials and Methods.” A total of 52 chimeric HANs were examined.
never were found in nonpregnant or pregnant hCK18 or R26-hPAP transgenic mice or mammary glands reconstituted with only WAP-TGFα/hPAP or WAP-TGFα/hCK18 cells. These observations suggest that somatic mosaicism or epigenetic silencing of markers is not the cause of chimerism, because marker gene expression persisted in all cells in these mice. We believe that the presence of chimeric HANs suggests that they arise via interaction between multiple clones within ductal and alveolar units under the influence of the local microenvironment.

Overall, our findings have significance regarding fundamental mechanisms underlying mammary carcinogenesis:

(a) The presence of polyclonality in small, putatively preneoplastic lesions conflicts with the dogma of a cell autonomous origin for neoplasia, in which a neoplasm originates via a series of changes initiated in a single cell. Specifically, these data suggest that focal lesions conflicts with the dogma of a cell autonomous origin for ductal and alveolar units under the influence of the local microenvironment.

(b) The development of polyclonal preneoplastic lesions supports clonal evolution, rather than only clonal origin, as an important feature of mammary tumor progression.

(c) Our findings emphasize the importance of non-cell autonomous interactions during mammary tumor progression and complement recent suggestions that interactions between stromal and epithelial cells may provide critical signals that accompany development of neoplasia.

(d) The suggestion that preneoplastic mammary lesions are polyclonal may be important to understanding the potential behavior of individual premalignant breast lesions in women and to developing rational medical strategies for their management. By identifying the factors regulating multicellular interactions in these early lesions, we may find ways to halt their progression to invasive cancer.

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