Contiguous Patches of Normal Human Mammary Epithelium Derived from a Single Stem Cell: Implications for Breast Carcinogenesis

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ABSTRACT

Tissue clonality can be assessed in females by analyzing the methylation status of polymorphic DNA markers on X-linked genes because extensive de novo methylation of one allele at the preimplantation stage is associated with its permanent inactivation. We applied X chromosome inactivation toward understanding human breast morphogenesis by examining the nonmalignant breast epithelium from two reduction mammaplasties and a mastectomy. We found that entire lobules and large ducts of normal breast tissue have the same X chromosome inactivated, suggesting that they are derived from the same stem cell. The regions of inactivation of a particular X chromosome do not extend over an entire breast, so that ducts and lobules with opposite chromosomes inactivated are present within a single breast. Potential relevance of these observations for malignant transformation is discussed.

INTRODUCTION

Analysis of X chromosome inactivation has been used to determine the clonality of several kinds of tumors in female patients, including leukemias and colorectal, bladder, and ovarian tumors (1—4). These studies rely on the fact that X chromosome inactivation occurs at the preimplantation stage of development, resulting in the permanent marking of cellular progeny by random inactivation of either the paternal or maternal X chromosome. Tissue clonality can be assessed by analyzing the methylation status of polymorphic DNA markers on X-linked genes because extensive de novo methylation is associated with their permanent inactivation.

Although some of the above studies have shown that tumors are clonal, less attention has been paid to the composition of normal tissues from the standpoint of clonality. Early studies showed the existence of large monoclonal patches of normal cells ranging in size from 0.2 to 1.0 mm² in hair follicles and 1 cm² in gastric epithelium (5—7); we have recently demonstrated the presence of 1-cm² patches containing approximately 2 × 10⁶ cells in female bladder urothelium (8). However, no detailed studies on the clonality of breast epithelium have been published.

We applied X chromosome inactivation to understanding human breast morphogenesis by examining the nonmalignant breast epithelium from two reduction mammaplasties and a mastectomy. In a previous study of X chromosome inactivation in the human breast, Noguchi et al. (9) concluded that normal breast tissue was polyclonal in origin with random distribution of cells having either allele on the X chromosome hypermethylated and, hence, inactivated. However, because they did not isolate epithelium specifically, the microdissected normal breast tissue used in their study contained both stromal and epithelial components. Because mammary epithelium and stroma might have arisen from different stem cells, we reasoned that the clonality of normal breast epithelium required reevaluation. After careful microdissection to isolate mammary epithym, we were able to demonstrate that normal ducts and lobules are usually clonal.

MATERIALS AND METHODS

DNA Extraction. Epithelial cells were carefully microdissected from 4—5 hematoxylin and eosin-stained, 4-μm-thick serial sections. DNA was extracted with proteinase K as described previously (8), except that the boiling steps were omitted to maintain the DNA in double-stranded form for subsequent enzymatic analysis. Proteinase K was quenched with 1 mN final concentration of phenylmethylsulfonyl fluoride.

Assay for X Chromosome Inactivation. Exon 1 of the human androgen receptor gene has three HhaI and two HpaII sites within 100 bp 5’ to a polymorphic CAG repeat region. The CAG repeat polymorphisms were used to distinguish between the two X chromosomes alleles in informative females. Because methylation of the HhaI and HpaII restriction sites was associated with lack of gene expression on the inactive X chromosome (10), cleavage with the methylation-sensitive restriction enzymes HhaI and HpaII was used to distinguish between the active and inactive genes. Before PCR amplification, DNA was digested with HhaI and HpaII. This restriction enzyme digestion caused the restriction sites on the unmethylated active X chromosome to be degraded, whereas the sites on the inactive X chromosome remained intact.

After digestion, samples were amplified by two PCR reactions using nested primers specific for exon 1 of the androgen receptor gene. The two sets of oligonucleotide primers were designed to flank both of the methylation-sensitive restriction enzyme sites, together with the CAG repeat. Therefore, PCR amplification yielded a product only from the uncleaved (inactive) X chromosome. Therefore, only one band was present in the gel. In contrast, PCR amplification of DNA extracted from cells in which either allele was randomly inactivated, as well as PCR amplification of an undigested DNA control, yielded products from both the active and inactive X chromosome.

X chromosome inactivation analysis using the above scheme was performed as described (8). The DNA solution from each microdissected region was divided into two aliquots and subjected to either double digestion at 1—2 units each of HhaI and HpaII or mock digestion with the restriction enzyme buffer for 90 min at 37°C. The primary PCR was performed with outside primers 1A and 1B (1A, 5’-GTGCGCAGAAGTGATCAGAGAA-3’; 1B, 5’-TCTGGAGCC- CAACTCTTCTC-3’) in a standard reaction volume of 25 μl, and consisted of 17 cycles at 94°C for 1 mm, 55°C for 1 mm, and 72°C for 1.5 mm. The secondary PCR was performed with inside primers 2A and 2B (2A, 5’- AGAGGGCGGACGGCACACTC-3’; 2B, 5’-GCTGTGAAGGUGCT AGAGGGCGGACGGCACACTC-3’) in a standard reaction volume of 25 μl, and consisted of 17 cycles at 94°C for 1 mm, 55°C for 1 mm, and 72°C for 1.5 mm. The secondary PCR was performed with outside primers 1A and 2B (2A, 5’- AGAGGGCGGACGGCACACTC-3’) in a standard reaction volume of 25 μl, and consisted of 17 cycles at 94°C for 1 mm, 55°C for 1 mm, and 72°C for 1.5 mm. For both PCR reactions, the initial denaturation and final elongation steps were lengthened to 2 and 3 mm, respectively. The radioactively labeled secondary PCR products were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel and subjected to autoradiography.

RESULTS AND DISCUSSION

We carefully microdissected single normal lobules or large ducts from individual hematoxylin and eosin-stained 4-μm sections (Fig. 1). The same histological areas from four sequential slides were microdissected and pooled to obtain sufficient cells for an X chromosome inactivation study. In both of the normal reduction mammaplasties tested and in the nonmalignant breast tissue peripheral to a carcinoma, discrete patches of cells with a single inactivated X chro-
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In Fig. 2, each allele is represented by a main band and several weaker bands. These weaker bands arise because of slippage of the DNA strand being synthesized along its template with a CAG repeat.

The size of the microdissected regions with a single X chromosome inactivated ranged from 0.08 to 0.45 mm². These dimensions represent a lower limit on the size of the clonal patch in the mammary gland. The patches with a single X chromosome inactivated could be significantly larger because, in one reduction mammaplasty (case 2) and in the peripheral mastectomy tissue (case 3), we found only one hypermethylated allele in all of the dissected regions within a tissue block (Table 1). Multiple blocks representing different regions of the breast were available for case 2, and they differed in inactivated allele. Some of the microdissected regions showed equal retention of each allele (Fig. 2b, Lanes 11 and 12; Table 1), suggesting that some ducts and/or lobules may be polyclonal or that contaminating stroma contributed to the polyclonal pattern.

In summary, these data establish that mammary epithelium in the human breast is organized into discrete regions in which all cells have the same inactive X chromosome. Hence, finding that a tumor has a single inactivated X chromosome indicates only that the initial events leading to the tumor occurred at some time in development after X chromosome inactivation. These events could have occurred before maturity of the breast, so that many epithelial cells, all containing the same inactive X chromosome and initiating genetic changes, could progress to malignancy and contribute to a tumor that is seen at the time of clinical presentation. The regions of the tumor that result from each of these epithelial cells would then be expected to have a different suite of genetic abnormalities representing their independent evolution into a tumor after the initiating events.

Breast cancer is a poorly understood disease. There is circumstantial evidence in the literature that some breast cancers may result from multiple pathways within a localized region of the breast. Recently, O’Connell et al. (11) examined invasive cancers and associated preinvasive and premalignant components such as ductal carcinoma in situ and atypical hyperplasia for loss of heterozygosity at various autosomal loci. There were some cases that showed patterns of loss of heterozygosity consistent with the conclusion that the premalignant and invasive components resulted from two independent pathways of malignant progression. Additionally, we have seen a case in which the
ductal carcinoma in situ and invasive components had lost opposite alleles of chromosome 3p, suggesting a similar conclusion.3

If breast cancers can develop from a large region of initiated cells, one might predict that the normal-appearing epithelial cells in the vicinity of the tumor may have genetic abnormalities. Discovery of these may give an indication of which genetic changes are critical early in breast tumorigenesis. Thus, an interesting experimental strategy would be to analyze apparently normal tissue in the vicinity of the tumor that was homogeneous for X chromosome inactivation and pulmonary tuberculosis and in women who were younger than age 10 at the time of exposure to atomic bomb irradiation. These predifferentiation stem cells may be uniquely sensitive to other agents in addition to radiation.

REFERENCES


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