Evidence of the Monoclonal Composition of Human Endometrial Epithelial Glands and Mosaic Pattern of Clonal Distribution in Luminal Epithelium

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The endometrium is a highly regenerative tissue that plays a crucial role in implantation. We examined the clonal constitution of glandular cells as well as the luminal epithelium of this unique tissue. Using collagenase-based digestion techniques with microscopic manipulation, we isolated individual human endometrial glands and examined their clonality using a polymerase chain reaction-based assay for nonrandom X chromosome inactivation with an X-linked androgen receptor gene. Most of the glands analyzed were composed of monoclonal populations of epithelial cells and one of the glands exhibited a loss of heterogeneity in the androgen receptor gene. In addition, adjacent glands within a 1-mm² area shared clonality, suggesting that clonality of the luminal epithelium is regionally defined. The clonality of endometrium was further confirmed in a study of female mice that harbor the green fluorescent protein gene on either the maternal or paternal X chromosome. Fluorescent microscopy of uterine sections revealed that individual endometrial glands consisted completely of either fluorescent or nonfluorescent cells and that the surface epithelium exhibited a clear boundary between these cell types. These findings suggest that single or multiple stem cells with uniform clonality exist on the bottom of each endometrial gland and genetic alterations occurring in such cells may play a critical role in endometrial carcinogenesis. The possible association between area-specific X inactivation of the endometrial surface and the endometrial receptivity of embryo implantation remains to be clarified. (*Am J Pathol* 2003, 163:295–301)

The human endometrium is a unique tissue characterized by constant and rapid cell proliferation, differentiation, and breakdown in a menstrual cycle. After the shedding by menstruation, the endometrium dramatically proliferates and completely regenerates within 2 weeks. Regeneration of the endometrium incessantly repeats through

out the reproductive years. Such a highly regenerative feature suggests the presence of stem cells in the endometrial glands; otherwise, the proliferative potential would become exhausted after several dozen menstrual cycles. However, we have no information about the endometrial stem cells.

The colonic crypts, one of the most regenerative somatic tissues, have been known to consist of monoclonal cell composition and form patches composed of multiple crypts with uniform clonality.^{1,2} Although the exact number and phenotypic characteristics of stem cell in a crypt are unknown, the clonal analysis of crypts has contributed to elucidating stem cell dynamics.

The most consistently informative marker of clonal tissues is X chromosome inactivation. During embryogenesis of the female, either the paternal or the maternal X chromosome is randomly and permanently inactivated.^{3–7} As a consequence, normal tissues are composed of cellular mosaics with various X chromosome inactivation patterns. Therefore, a uniform pattern of X chromosome inactivation in a specific cell population indicates clonality.

The clonality of normal endometrial glands has not been previously analyzed despite regenerative activity equal to the crypts, perhaps because collecting sufficient DNA for clonality analysis from a single endometrial gland is difficult. Most studies analyzing the clonality of tumors and their precursors used special techniques, such as selective ultraviolet radiation fractionation or microdissection, to extract DNAs from desired regions on paraffin tissue sections.^{8–11} However, these methods are not appropriate for the analysis of the single gland because it does not occupy a large area of a tissue section and therefore sufficient DNA cannot be obtained for the clonality analysis. The size of a lesion is undoubtedly a limiting factor in the determination of clonality from a thin

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tissue section. We therefore established a collagenase-based method to isolate intact individual endometrial glands from the stromal tissues of surgically removed specimens.¹² By analyzing the X chromosome inactivation patterns of DNA extracted from individual endometrial glands without the contamination of stromal tissues or other glands, we examined the clonal constitution of glandular cells and the luminal epithelium of this unique tissue and discussed the presence of endometrial stem cells as well as endometrial carcinogenesis.

Materials and Methods

Isolation of Human Endometrial Glands

Human endometrial tissue samples were obtained from patients undergoing hysterectomy as a treatment for benign neoplasms other than those associated with endometrial diseases. Minced endometrial tissues were placed in Dulbecco's modified Eagle's medium containing 350 U/ml of deoxyribonuclease I (Takara, Ohstu, Japan) and 180 U/ml of collagenase type 3 (Washington Biochemical Corporation, Lakewood, NJ) in plastic dishes and gently shaken for 40 minutes at 37°C.¹² Individual glands on the bottom of the dishes were selected under a microscope, put into separate Eppendorf tubes, and incubated in extraction buffer consisting of 10 mmol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 0.3% sodium dodecyl sulfate, and 100 µg/ml proteinase K (Takara) at 56°C for 6 hours. The DNAs were then isolated by phenol/chloroform extraction and ethanol precipitation.

Clonality Analysis of Human Endometrial Glands

Of the DNA that was extracted from one endometrial gland, two thirds were digested with 10 U of *HhaI* (Takara) in 10 µl of the buffers recommended by the manufacturer. After a 12-hour incubation at 37°C, digested DNA was extracted with phenol/chloroform and precipitated with ethanol. This digested DNA and the remaining undigested DNA were used as templates for the polymerase chain reaction (PCR) to amplify CAG repeats in the androgen receptor (*AR*) gene. The PCR primers were as follows: primer 1, 5'-GCTGTGAAGGTT-GCTGTTCTCAT-3' and primer 2, 5'-TCCAGAATCTGT-TCCAGAGCGTGC-3', as described by Allen and colleagues.¹³ Template DNA was mixed in 50 µl of PCR buffer containing 4 ng/µl each of primers 1 and 2, 200 µmol/L of each dNTP, 0.025 U/µl of *Taq* polymerase (Nippon Gene, Toyama, Japan), 1.5 mmol/L MgCl₂, 20 mmol/L Tris-HCl (pH 8.0), 60 mmol/L KCl, 0.005% Tween 20, and 1 mmol/L ethylenediaminetetraacetic acid. One cycle of PCR consisted of 1 minute at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. After initial denaturation at 95°C for 5 minutes, the DNA underwent 30 cycles of PCR amplification. The amplification products were precipitated with ethanol, resuspended in 5 µl of water, resolved by electrophoresis on 4.5% polyacrylamide

gels, and visualized with SYBR Gold nucleic acid gel stain (FMC BioProducts, Rockland, ME).

Fluorescent Microscopic Analysis of the Endometrium Resected from the Female Mice Heterozygous for Green Fluorescent Protein

The uterus of the female transgenic mouse that harbors a green fluorescent protein (*GFP*) gene on either the maternal or the paternal X chromosome,¹⁴ was removed after death and endometrial tissue sections were prepared, fixed, and examined under a fluorescence microscope.

Results

The clonality of the endometrial epithelial cells was examined by PCR amplification of the CAG repeats in the androgen receptor (*AR*) gene. The X chromosome-linked androgen receptor gene has a polymorphic short tandem repeat of the trinucleotide [CAG]_n, and individuals are heterozygous for the length of CAG repeats in ~90% of the general population.^{8,13} Methylation of the *HhaI* endonuclease sites near the CAG repeats correlates with X chromosome inactivation.^{5,13} The enzyme *HhaI* can cut the unmethylated sites on an active X chromosome but cannot cut the methylated sites on an inactive X chromosome. Therefore, digesting DNA samples with *HhaI*, then amplifying the CAG repeats by PCR, generates homogeneous products derived from either the paternal or the maternal inactive X chromosome. Because cells in a clonal population have a uniform X chromosome inactivation pattern, the amplification products of the CAG repeats in the *AR* gene migrate as a single band.

Human endometrial samples were collected from 10 patients undergoing hysterectomy to treat benign neoplasms other than those associated with endometrial diseases. These tissues were minced and digested in a collagenase solution to isolate the endometrial glands from the stromal cells (Figure 1). The individual endometrial glands were then collected by microscopic manipulation and placed into separate Eppendorf tubes. The DNA was extracted from each gland and digested with *HhaI*; the resulting fragments then underwent PCR amplification of the CAG repeats in the *AR* gene. The sizes of the amplification products differed between the two alleles in each of seven patients, making those seven samples appropriate for clonality analysis and excluding the remaining three patients from the study. Representative results from two of these seven patients are shown in Figure 2. The DNA recovered from almost all of the glands from each patient yielded two distinct PCR bands in the absence of *HhaI* digestion. However, after digestion with *HhaI*, the DNA yielded only a single band. These findings indicated that the cellular composition of the glands was monoclonal. To exclude the possibility that a small, undetectable fraction of cells with different clonality was contained within a gland, one gland from patient 2 was further divided by microscopic manipulation into

Figure 1. Isolated human endometrial glands. Minced endometrial tissues were digested with collagenase to isolate the endometrial glands from the stromal cells. Individual glands were microscopically selected for clonality analysis and placed in a solution that maintained their integrity.

three sections (Figure 2; A, B, and C, gland 7) and the clonality of each fraction was determined. The clonalities of the three fractions were the same, confirming that each endometrial gland is composed of epithelial cells with the same clonality. We found an exceptional band pattern of the PCR products from gland 4 of patient 2. The DNA from this gland showed a single band before *HhaI* digestion, indicating a deletion of one of the *AR* alleles.

We then examined whether the monoclonal composition is limited to a single gland or extends to the surrounding glands. An endometrial surface area of 25 mm² was surgically extracted from patient 2 and subdivided into 25 pieces, each of 1 mm². Each piece was minced and digested in a collagenase solution. Ten single glands were isolated from each piece and their DNA was extracted for the analysis of clonality. In 15 of 25 pieces, all

Figure 2. Clonality analysis of human endometrial glands. **a:** The CAG repeats of the androgen receptor gene on the inactive X chromosome allele were amplified by the PCR method. The AR-a and AR-b primers target two X chromosome-linked androgen receptor alleles containing variable numbers of CAG repeats flanked by *HhaI* sites methylated on inactive X chromosomes and unmethylated on active X chromosomes. The *HhaI* digestion cut activated X chromosomes, leaving only those repeated sequences from inactive X chromosomes for amplification. **b:** Endometrial glands were separated from stromal cells and individually isolated. The DNA was then extracted from each gland and a portion of it was digested with *HhaI*, followed by PCR to amplify the CAG repeats in the androgen receptor gene. Without *HhaI* digestion, the DNA extracted from the glands of patients 1 and 2 (except from gland 4 of patient 2) produced two distinct bands derived from either paternal or maternal alleles, indicating their suitability for clonality analysis. After digestion with *HhaI*, the same DNA yielded single PCR products, suggesting that epithelial cells comprising each gland are monoclonal. One gland (gland 7) from patient 2 was further divided into three sections (**A**, **B**, and **C**) and the clonality of each section was examined. The three sections exhibited the same clonality. Samples of DNA from each patient's white blood cells (WBC) were used as controls. Two distinct bands were detected in WBC samples before and after *HhaI* digestion, suggesting a polyclonal composition.

Figure 3. Mapping endometrial X chromosome inactivation. Endometrium, 25 mm² in area, was collected from patient 2 and divided into 25 pieces, each 1 mm². After the pieces were digested with collagenase, 10 glands were individually selected from each area and clonality was examined. Once we confirmed that each gland was composed of a monoclonal cell population (data not shown), then the paternal or maternal origin of the inactive X chromosomes was determined on the basis of analyses of samples from the parents of patient 2. Fifteen of 25 areas showed uniform clonality (100% paternal origin is represented as P and 100% maternal origin is represented as M; M/P indicates glands with mixtures of paternal and maternal inactive X chromosomes).

10 glands isolated from each piece exhibited the same clonality (either of paternal or maternal origin) (Figure 3). These findings suggest that the surrounding glands located within a 1-mm² area share clonality and that the monoclonal composition of endometrial glands is regionally defined. The remaining 10 pieces contained glands that exhibited clonality of mixed parental origins. Whether these pieces contained abutting areas of monoclonal groups or one field with both clones integrated remains unclear.

The clonality of endometrial glands was further examined using a mouse bred to harbor a *GFP* gene on either the maternal or the paternal X chromosome.¹⁴ Mouse cells that have the transgene on the active X chromosome are fluorescent, whereas those that have the transgene on the inactive X chromosome are not.^{15,16} Therefore, these green mice are useful for analyzing the clonality of organs *in vivo*. We took out the uteri of green mice and prepared their histological sections to observe the fluorescence of each endometrial gland. Under fluorescence microscopy, we counted a total of 100 endometrial gland in randomly selected fields and found that individual glands of this mouse consisted of either fluorescent or nonfluorescent cells in similar ratios (Figure 4, b and e). These findings demonstrated the monoclonal composition of glandular cells. We also found that the epithelium lining of the endometrium had a clear boundary between the fluorescent and nonfluorescent cell layers (Figure 4, d and e). This finding indicated that epithelial cells with different clonalities converge at this boundary (Figure 4e).

Discussion

This is the first report demonstrating the monoclonal composition of epithelial cells in human endometrial glands. Monoclonal growth is a hallmark of cancer cells, whereas polyclonal growth is exhibited by normal cells. Our data showed that human endometrial gland is one of the exceptions to this theory.

Because the human endometrium is a continuously renewing tissue, its cells must be capable of long-term self-maintenance with the properties of stem cells.¹⁷ During menstruation, epithelial cells in the functional layer of an endometrial gland are shed, whereas those in the basal layer remain intact (Figure 5). The anatomical continuity of each gland is interrupted because the lining cells that bridge glands are lost. Thereafter, cells in the basal layers proliferate and expand to regenerate the gland and lining surface epithelium during the next menstrual cycle. It is thus likely that stem cells with the same clonality are located in the basal layers in each gland (Figure 5).

The fact that endometrial glands are composed of monoclonal cells provides important insight into molecular aspects of endometrial carcinogenesis (Figure 6). Endometrial cancers are unique tumors that arise from tissues that peel off and regenerate repeatedly every month. Genetic alterations that induce endometrial cancers would likely be found in nonshedding cells in the basal layers of the endometrium, because most epithelial cells comprising endometrial glands are shed in every menstruation cycle. Stem cells in the basal layers might be the primary source of genetic alterations (Figure 6). The findings of gland 4 from patient 2 support this hypothesis. The DNA extracted from this gland yielded a single PCR band regardless of *HhaI* digestion, indicating that one allele of *AR* is deleted in this gland. The quantity of DNA isolated from a single gland was sufficient for accurate analysis; we extracted ~0.2 to 0.5 µg of DNA from a single gland with our methods, which was sufficient for PCR analysis. We surmise that this allelic loss occurred in all of the cells comprising this gland because the amplification of intact alleles in any of the cells would result in two bands. The uniformity of allelic loss in all of the cells comprising a gland with normal phenotype, indicates that such genetic alteration occurred at the level of the stem cells, which might be passed onto daughter cells through the subsequent clonal expansion, supporting the stem cell-hit theory in endometrial carcinogenesis (Figure 6). A similar concept has been proposed involving colonic crypts.^{18–20}

How many stem cells are present in the single gland? Our findings that all of the glandular cells in gland 4 from patient 2 have common genetic alterations might be simply explained by the presence of a single stem cell. However, according to the established concept in colonic crypts, multiple stem cells are present at the bottom of a crypt, called the niche.^{18,19,21} Although each stem cell has the capacity to divide in this area, the total number of stem cells is limited. Eventually, divided stem cells are randomly pushed out of this area. This random selection may provide a chance to a certain stem cell

Figure 4. Clonality analysis of endometrial glands from a female hemizygous transgenic mouse carrying an X-linked transgene *GFP* encoding the green fluorescent protein. Uterine sections were fixed and examined by microscopy. **a:** Histological view of endometrium, displaying endometrial glands in cross-section. **b:** Fluorescent microscope examination shows endometrial glands composed of either fluorescent (A) or nonfluorescent (B) cells. **c:** Histological view of endometrium, displaying surface-lining cells in vertical section. **d:** Fluorescent microscope examination shows a distinct boundary between fluorescent and nonfluorescent epithelial cells (C) in the surface lining. **e:** Schematic diagram of the three-dimensional endometrial structure. Sections from **b** and **d** are shown.

Figure 5. Proposed role of stem cells in endometrial glands. During menstruation, functional layers of endometrium are shed, leaving basal layers intact. During the next menstrual cycle, basal layers must provide epithelial cells to regenerate functional layers. Each gland consists of monoclonal cell populations; thus stem cells with same clonality in the basal layer may produce descendant cells. The common clonality in neighboring glands indicates that primitive cells originate during development and thereafter become stem cells in individual glands.

lineage originating from one stem cell to occupy a niche. This theory might be applied to endometrial glands. Therefore, the presence of multiple stem cells can also explain our findings of gland 4 from patient 2. In the crypts, the concepts of niche and genetic alterations in stem cells are critical to clearly explain the colonic carcinogenesis.¹⁹ When a genetic alteration hits a stem cell in a niche, this stem cell lineage will occupy the niche with certain probability, according to the theory described above. After additional genetic alterations are accumulated in similar ways, such crypts are expected to eventually grow as neoplasia. Further analysis is needed to clarify the number of stem cells and the existence of niches in endometrial glands.

Figure 6. Proposed model of stem cell hit theory in endometrial carcinogenesis. Genetic alterations occurring in stem cells in the basal layer are passed on to all of the cells in the gland through clonal expansion. Cells in the functional layer are shed by menstruation, while stem cells with the genetic alterations remain intact. These cells clonally expand again in the next menstrual cycle to regenerate a gland. Thus, once genetic alterations hit stem cells, they are passed on throughout numerous menstrual cycles, making possible the accumulation of additional genetic alterations that may lead to the development of endometrial cancers.

Although endometrial cancers, like other tumor types, have been shown to be composed of monoclonal cells,⁸ few studies have analyzed the clonality of endometrial cancer precursors and normal endometrium.⁹⁻¹¹ These studies have demonstrated that atypical hyperplasia exhibited monoclonal composition, while most hyperplasia without atypia and normal endometrium exhibited polyclonal compositions. Although our study did not extend to the clonality of hyperplasia, our data showed that a normal endometrial gland is composed of monoclonal cells, seemingly contradicting previous studies. According to our theory of monoclonal composition in a gland, hyperplastic cells in a gland should also grow in a monoclonal pattern. In general, hyperplasia is well known to arise at multiple foci in endometrium. Even though hyperplastic cells grow in a monoclonal pattern in each focus, a clonality analysis of a cluster of hyperplasia lesions containing multiple foci may generate results of a polyclonal pattern. Similarly, analysis of normal endometrium in these studies might not strictly target individual glands. Biopsy samples invariably contain multiple glands, which would give results as a polyclonal pattern. Other studies have used selective ultraviolet fractionation methods to isolate the lesions of interest.^{9,10} Although these studies have not mentioned how many regions of normal endometrial glands were collected from one tissue section, multiple regions might have been collected to obtain the sufficient amounts of DNA for clonality analysis, which would make the results incomparable with those from a single gland. Therefore, refined analysis of a tissue section may revise the results of clonality in these lesions, although the problems of limited DNA quantity remain unresolved in these methods.

Our data showed that glands in adjacent areas shared clonality; this is the first report indicating a mosaic structure of clonal distribution in lining epithelium. This suggests the presence of more primitive cells that develop into stem cells in each gland during the human development (Figure 5). Endometrium is also a main component for the successful process of pregnancy, especially for implantation. It is known that trophinin, a cell adhesion molecule that is expressed on the surface of luminal epithelium and mediates an initial attachment of the embryo into endometrium at the time of implantation, is linked to the X chromosome.^{22,23} We showed that the X chromosome inactivation pattern on the endometrial surface is regionally defined, representing a mosaic-like structure. It is thus assumed that abnormalities of X-linked genes such as trophinin may reduce the endometrial receptivity toward embryo implantation, causing infertility because of implantation disorders. The association between area-specific X inactivation of the endometrial surface and the endometrial receptivity requires additional research for clarification.

Finally we showed that genetic alterations that may lead to cancer progression occur in all cells of a gland that is morphologically normal. This is an especially important finding with regard to the molecular screening of endometrial cancers. A modality for detecting genetic changes in a gland with a normal phenotype is now needed;²⁴ the single gland analysis on biopsy

samples of endometrium will provide a great advantage for this purpose.

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