Heterogeneous X Inactivation in Trophoblastic Cells of Human Full-Term Female Placentas


Summary

In female mammalian cells, one of the two X chromosomes is inactivated to compensate for gene-dose effects, which would be otherwise doubled compared with that in male cells. In somatic lineages in mice, the inactive X chromosome can be of either paternal or maternal origin, whereas the paternal X chromosome is specifically inactivated in placental tissue. In human somatic cells, X inactivation is mainly random, but both random and preferential paternal X inactivation have been reported in placental tissue. To shed more light on this issue, we used PCR to study the methylation status of the polymorphic androgen-receptor gene in full-term human female placentas. The sites investigated are specifically methylated on the inactive X chromosome. No methylation was found in microdissected stromal tissue, whether from placenta or umbilical cord. Of nine placentas for which two closely apposed samples were studied, X inactivation was preferentially maternal in three, was preferentially paternal in one, and was heterogeneous in the remaining five. Detailed investigation of two additional placentas demonstrated regions with balanced (1:1 ratio) preferentially maternal and preferentially paternal X inactivation. No differences in ratio were observed in samples microdissected to separate trophoblast and stromal tissues. We conclude that methylation of the androgen receptor in human full-term placentas is specific for trophoblastic cells and that the X chromosome can be of either paternal or maternal origin.

Introduction

Normal female mammalian development requires dosage compensation for some genes on the X chromosome. This takes place through X inactivation, also known as Lyonization (Lyon 1992). In somatic cells of both female mice and female humans, X inactivation is usually random, resulting in about the same number of cells with the paternal or the maternal X chromosome inactivated (for review, see Lyon 1992; also see Tan et al. 1993). In mice, the final pattern of X inactivation is influenced by genetic and epigenetic factors (for review, see Belmont 1996): including variation at the X-chromosome controlling element (Xce) (Cattanach et al. 1970; for review, see Migeon 1998). In addition, because X-inactivation patterns are established in a relatively small number of progenitor cells, stochastic effects on X inactivation are to be expected. In extraembryonic tissues of female mice, the paternal X chromosome is specifically inactivated (Takagi and Sasaki 1975; West et al. 1977; Harper et al. 1982; Tan et al. 1993). In human female placentas, however, both random and specific paternal X inactivation have been found. Most of these studies are based on enzyme analyses of individuals informative for a polymorphism in the glucose-6-phosphate dehydrogenase (G6PD) gene, which is expressed only from the active X chromosome (Disteche 1995). Of six published investigations, three report preferential inactivation of the paternal X chromosome (Ropers et al. 1978; Harrison and Warburton 1986; Harrison 1989), whereas the other three report random X inactivation (Migeon and Do 1979; Migeon et al. 1985; Mohandas et al. 1989). More recently, PCR-based methods were used to analyze the methylation status of two HpaII sites in the androgen-receptor gene and, hence, the X-inactivation pattern in fetal placentas (Goto et al. 1997). A polymorphic CAG repeat within the amplified fragment allowed distinction between the parental alleles (Allen et al. 1992). This study showed methylation (i.e., inactivation) preferentially on the paternal allele in cultured trophoblastic cells. In contrast, mesodermal cells exhibited either a 1:1 ratio or complete demethylation. The results of this...
study could have been skewed because of the limited number of informative cases (n = 2), as well as by the selection of cells during culture. However, the finding of either random or completely absence X inactivation in mesodermal cells of the human placenta is in agreement with an earlier publication of findings based on G6PD analysis (Harrison 1989). Moreover, one study based on G6PD investigations suggested preferential maternal X inactivation in some fetal placentas (Migeon and Do 1979), although this finding was not specifically discussed.

To study the pattern of X inactivation in human full-term placentas in more detail, we investigated the methylation status of two HpaII sites and two HhaI sites in the androgen-receptor gene at multiple nonadjacent distinct sites within a single placenta, in principle as reported before (Goto et al. 1997). Microdissection of frozen tissue sections allowed selective investigation of the methylation status of both trophoblastic and stromal cells. We find that methylation of the androgen receptor is specific for trophoblastic cells. Within a single placenta, distinct patches appear to have inactivated either the maternal or paternal X chromosome preferentially, indicating that a heterogeneous pattern of X inactivation exists in human female placentas.

Material and Methods

Tissue Collection and Processing

Placentas were collected by the Department of Gynecology and Obstetrics, Zuiderziekenhuis (Rotterdam), and were processed directly after delivery at full term. Only normal placentas resulting from normal pregnancy and delivery of a healthy child were included in this study. In all cases, samples of maternal blood from spontaneous bleeding during delivery was collected in a heparin-containing tube. The placenta and the umbilical cord were washed with PBS to remove contaminating constriction to remove fetal blood. High-molecular-weight DNA from maternal blood was isolated according to standard procedures (proteinase K–SDS treatment followed by phenol-chloroform extraction and ethanol precipitation [Maniatis et al. 1982]). Subsequently, three fragments of umbilical cord were taken from the site of constriction and were thoroughly washed three times in fresh PBS. One sample was fixed in formalin overnight and was embedded in paraffin, whereas the other two were snap-frozen in liquid nitrogen and were stored at -80°C until being used. Of the two male and nine female placentas (cases 1–11), four samples from one region within one cotyledon were dissected and thoroughly washed as described above. The samples were each ~0.5 cm² in size. One sample was fixed in formalin and embedded in paraffin, and the others were snap-frozen. In principle, the same protocol was used on two more placentas (cases 12 and 13), with the exception that, instead of one placenta specimen, nine nonadjacent regions (including all cotyledons) were collected, numbered “1”–“9.” From each site, four samples were collected and were designated “A1,” “A2,” “B1,” and “B2” (fig. 5A). After the samples were rinsed, they were divided into two groups; one was fixed in formalin and embedded in paraffin, and the other was snap-frozen. In addition, the corresponding umbilical cords were sliced into six fragments of similar size. From these fragments, two samples were taken, and, after they were rinsed, one sample was fixed in formalin and embedded in paraffin, and the other was snap-frozen. Formalin-fixed paraffin-embedded samples were cut into 4-µm sections and were stained with hematoxylin and eosin, for histological examination. In addition, for each frozen sample from which DNA was isolated, additional 4-µm sections were made, fixed with acetone, and stained with hematoxylin and eosin, for histological examination.

Microdissection

Snap-frozen tissue sections were cut by a Reichert-Jung 2800 Frigocut E cryostat. One 5-µm and three 20-µm serial sections were cut and placed on an ethanol-cleaned glass slide. Sections were stained for enzymatic alkaline phosphatase reactivity as described elsewhere (Mosselman et al. 1996) and air dried. The 5-µm section was used for histological examination by light microscopy after hematoxylin and eosin staining. The areas of specific histological interest were selected by means of an inverted microscope (Axiovert 10; Carl Zeiss) and were microdissected by a modified Pasteur pipette with an ultrathin pointed tip. The pipette was prepared by brief flaming and gentle pulling of glass piping. The microdissected tissues were immediately placed in a prechilled Eppendorf tube and were dissolved in TNE (10 mM Tris, 400 mM NaCl, 2 mM EDTA, pH 8.2). Proteinase K (10 mg/ml) and SDS (10%) were added, and the sample was incubated overnight at 37°C. DNA was then extracted with phenol/chloroform/isoamylalcohol (25:24:1) (Merck) and was precipitated by use of glycogen (10 mg/ml) (Boehringer) as the carrier. Pellets were dissolved in 10 µl of TE (Tris 10 mM, EDTA 0.1 mM, pH 8.0). The amount of DNA extracted was estimated by comparison with a series of DNA samples with known concentration, on a 1% agarose gel.

Androgen-Receptor Methylation

All experiments were done in duplicate, essentially as described by Allen et al. (1992). For each DNA sample from one isolation of the nonmicrodissected samples, three reactions were done, as follows. Two micrograms of DNA was digested at 37°C overnight, with either 10 U of Rsal alone, 10 U of Rsal and 20 U of HpaII, or
10 U of Rsal and 20 U of HhaI, in a total volume of 50 μl of digestion buffer (Pharmacia). The restriction endonucleases were purchased from Pharmacia LKB Biotechnology. Rsal was used to reduce the size of the genomic DNA fragments to facilitate HpaII digestion and HhaI digestion. The samples were then purified by phenol/chloroform extraction and ethanol precipitation, and the pellet was dissolved in 10 μl of H2O. The same protocol was used for the microdissected samples, except that 50 ng of DNA was digested, and one tenth of the digested material was subsequently used for amplification. 5’ End-labeling of the PCR products was done by use of 10 pmol of primer AR-a (5’-CCGAGGACGT-TTCCAGAATC-3’) [Mutter et al. 1995] with T4-poly-nucleotide kinase (New England Biolabs) and [γ-32P]-ATP (ICN), according to standard procedures. PCR was conducted in a total volume of 20 μl that contained 1 μl of solubilized DNA (∼150 ng), 1 × Taq DNA polymerase buffer with 1.0 mM MgCl2, 0.1 mM of each dNTP, 25 pmol of primer AR-b (5’-TCGATGGCCT-GGGGAGAA-3’), 15 pmol of unlabeled primer AR-a, 10 pmol of end-labeled primer AR-a, and 1 U of Taq DNA polymerase (Promega). The reactions were done in a PTC-200 Thermal Cycler (MJ Research), as follows: denaturation at 94°C for 5 min; 28 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 6 min. Labeled amplified DNA was mixed with 5 μl of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Four microliters of each sample was denatured at 95°C for 3 min and were loaded on a 6% 0.4-mm polyacrylamide gel. Electrophoresis was at 60 W for ~3 h. Kodak BioMax MR-1 film was used for autoradiography of the gel after the latter was dried. The ratio between the alleles was analyzed quantitatively by an Alpha Imager® 200 Digital Imaging & Analysis System (Alpha Innotech). In the absence of a PCR product after amplification, control for the presence of DNA after HpaII digestion or HhaI digestion and purification was accomplished by amplification of an H19-specific region (exon 5) of 331 bp without HpaII sites and HhaI sites, by means of primers HN11 (5’-GGCAGCTCCATC-TTCATGGGCCAC-3’) and HN12 (5’-GGCCCTGCACA-GGGACTTGC-3’) (Looijenga et al. 1997; Verkerk et al. 1997).

Results

To verify the usefulness, for our purpose, of methylation analysis of the androgen receptor, we analyzed two full-term male placentas (cases 1 and 2). Whereas amplification products representing one of the maternal alleles were observed in the Rsal-digested DNA samples isolated from the placenta, no products were found after digestion with either HpaII or HhaI (see fig. 1). The presence of DNA in these samples was verified by amplification of an H19-specific fragment lacking either of these restriction sites (not shown).

We subsequently applied the protocol to tissue sections of nine full-term female placentas (cases 3–11). Two closely apposed tissue samples (A and B) within one cotyledon, the functional unit of placenta, were studied. The areas from which DNA was isolated were ∼0.5 cm in diameter. The amount of DNA within a single experiment corresponded to that found in ∼17,000 cells. Because, for each sample, the percentage of methylation of HpaII sites and HhaI sites was similar (schematically illustrated in fig. 3), we will refer only to the HpaII data. We were able to identify the parental origin of the alleles in all cases except case 6, which lacked maternal DNA.

Figure 1  PCR analysis of X inactivation in two full-term male (i.e., XY) placentas (cases 1 and 2). One allele was detected in these samples after Rsal digestion only, confirming the presence of one X chromosome. Also as expected, two alleles were found in the matched maternal (i.e., XX) blood samples after Rsal digestion and amplification, representing the presence of two X chromosomes. Analysis of the methylation status of both the HpaII sites and the HhaI sites within this particular fragment of the androgen-receptor gene showed complete demethylation (i.e., absence of amplification products) in the placental samples. This confirms that the only X chromosome present is active. The presence of DNA after digestion with either HpaII or HhaI in these cases was verified by amplification of an H19-specific fragment lacking either of these restriction sites (not shown).
of sufficient quality. The upper allele was maternal in all informative cases, except for case 11, in which it was paternal. The results of the methylation analysis of the placentas are shown schematically in figure 4A. The percentage of the upper allele found after RsaI digestion only (50% is expected and represents a 1:1 ratio) is compared with the percentage of methylation of the upper allele after digestion with RsaI and HpaII. In the A series (see fig. 4A), X inactivation was found in nearly a 1:1 ratio in case 7, with preferential paternal inactivation in cases 8 and 10, preferential maternal inactivation in cases 4, 5, and 9, and almost complete maternal inactivation in cases 3 and 11. Most (78%) of the results from the B series (see fig. 4A) were in agreement with the results from the A series, although two placentas showed a clear difference. In particular, either paternal or maternal X inactivation was found in case 3, and preferentially maternal or preferentially paternal X inactivation was detected in case 9. Although the parental origin of case 6 could not be determined, in both samples the inactive X chromosome was from the same parent. The results for cases 3, 7, and 11 are shown in figure 4B. The validity of this finding for placental samples is supported by the balanced pattern of X inactivation found in matched samples of umbilical cord (see fig. 4A). Of note is the balanced pattern found in microdissected regions of smooth muscle and epithelial cells from the umbilical cord (not shown), explaining the balanced ratio found in umbilical cord as total sample, despite the absence of methylation in the stromal component (see above). The lower allele is preferentially detected after RsaI digestion only, in most umbilical cord samples (median 45, range 41–50) and all placental samples (median 47, range 43–48). Although this is most likely explained by amplification selection, the method remains informative for our purpose. Together, these data suggest that, at least to a certain extent, X inactivation can be

Figure 2  Representative example of results of histological analysis of a frozen tissue section of a human full-term female placenta (case 9) before (panel A) and after (panel B) microdissection of stromal (S) and trophoblastic (T) tissue. The trophoblastic cells were identified, prior to microdissection, by an enzymatic staining for alkaline phosphatase reactivity. C, Methylation analysis of microdissected stromal cells taken from two sites (I and II). All samples (one region from site I and two regions from site II) showed absence of methylation after digestion with HpaII, while DNA was present, as demonstrated by amplification of an H19-specific fragment lacking either of these restriction sites. Amplification products of the androgen receptor were also observed when undigested DNA was studied (not shown). D, Methylation analysis of microdissected trophoblastic tissue: three regions from site I and two regions from site II. Note that the ratio between the maternal (M) and paternal (P) alleles is similar, ~65:35 for site A and ~30:70 for site B, as in the original samples (see fig. 4A and text).

Figure 3  Schematic representation of correlation between methylation status of HpaII recognition sites and HhaI recognition sites, in the androgen-receptor gene, as determined in the samples included in this study. Only the percentages found for the upper allele are shown. Because of the linear relationship between the findings, only the data of the HpaII analysis are discussed.
heterogeneous within the trophoblast of a single human full-term placenta.

To study in more detail this phenomenon of heterogeneous X inactivation, we investigated two additional full-term female placentas (cases 12 and 13). Four samples (A1, A2, B1, and B2) were taken from nine (cases 1–9) distinct regions (including all individual cotyledons). The alleles of the androgen receptor of case 12 could not be separated sufficiently to allow accurate measurement. In spite of this, a heterogeneous pattern of X inactivation was found (not shown), varying from preferential inactivation of the X chromosome from one parent (maternal DNA of appropriate quality was not available) to preferential inactivation of the X chromosome derived from the other parent. Six of the nine sites within this placenta showed such heterogeneity. The sites from which the samples for analysis of case 13 were taken are illustrated schematically in figure 5A, the results are shown schematically in figure 5B, and representative examples are illustrated in figure 5C. For case 13, the upper allele was found to be of maternal origin (see fig. 5C). On average, the four samples taken within a site are more similar than samples taken from different sites (see fig. 5B). All four samples taken from each of six of the nine sites showed a similar pattern of X inactivation. This varied from an almost balanced pattern (site 5) to preferentially maternal (sites 3, 4, and 6) to preferentially paternal (site 8) to almost absolutely paternal (site 9) X inactivation. A more heterogeneous pattern was found for the other three sites, varying from almost completely maternal (in one sample from site 1, although the other samples showed a balanced pattern) to preferentially paternal (site 2) to balanced (site 7) X inactivation. These data demonstrate that the inactive X chromosome in full-term human female placentas can be of either maternal or paternal origin. Overall, the pattern of X inactivation seems to be independent of location, although some clustering might be present (e.g., at sites 3 and 6 and sites 7–9). Different patterns were observed in two of the three cotyledons for which two separate sites were investigated. Again, a ratio of ~1:1 was detected for the matched umbilical cord samples tested, of which six per case were studied (see case 13 in fig. 5B and C).

Subsequently, trophoblastic tissue from placentas 7 and 9 was isolated by microdissection of frozen tissue sections from the samples previously studied (see fig. 2A and B). The trophoblastic cells were identified prior to microdissection, by enzymatic staining for alkaline phosphatase activity. Placentas 7 and 9 were specifically selected for this purpose because the samples taken from placenta 7 showed a balanced ratio whereas placenta 9 included one sample with preferential inactivation of the maternal X chromosome and another sample with preferential inactivation of the paternal X chromosome (see fig. 4A). All three microdissected samples (all from site I) from placenta 7 (not shown) and five microdissected samples (three from site I and two from site II) from
Figure 5  A, Schematic representation of geography of sites (1–9) from which samples of placenta 13 were taken for analysis. Note that all cotyledons were included and that four samples per site (A1, A2, B1, and B2) were tested. B, Schematic representation of results of methylation analysis of HpaII sites in umbilical cord samples (U) and placental samples (1–9) of case 13 (as described in the legend to fig. 4A). Each of the four samples is denoted only in terms of its site of origin, not in terms of its specific sublocalization. C, Representative examples of results with regard to methylation analysis of samples of case 13, obtained and represented as described in the legend to figure 4B. Note both the balanced ratio between the alleles in the umbilical cord samples and the heterogeneity in the different placental samples (e.g., between samples within a particular site [e.g., 1A2 and 1B2] and between sites [1–3 and 9]).

Discussion

To date, the pattern of X inactivation in human full-term placentas has not been determined, although both random and preferential inactivation of the paternal X chromosome have been described. Here we have shown that human full-term female placentas can exhibit inactivation of both the paternal and the maternal X chromosome. This reflects the pattern in trophoblastic cells, because the stromal cells lacked methylation of the androgen receptor, as has been reported by others (Goto et al. 1997). Our results from the nonmicrodissected samples are therefore not influenced by the presence of contaminating stromal tissue. This is supported by the histological observation that all placental samples included in this study showed a similar contribution of stromal tissue, including those that had either absolute paternal or absolute maternal X inactivation. In addition, the possibility that results could be skewed because of the limited number of clonal cells under investigation can be disregarded, because the results from the microdissected samples of DNA (equivalent to the DNA in ~850 cells) are similar to those obtained from the matched total samples of DNA (equivalent to the DNA in ~17,000 cells). Moreover, no discrepancies between the duplicate experiments were observed (not shown). Our data show that the pattern of X inactivation in the trophoblastic cells of full-term female placentas is similar to that found in the fur of tortoiseshell cats, thus differing from the pattern found in most somatic tissues, including mouse and human liver (Ricciuti et al. 1976; Mrozek et al. 1991). This coarser pattern explains the conflicting results in the literature. The differences be-
tween samples and sites within a single full-term female placenta might be explained by clonal expansion of a limited number of trophoblastic progenitor cells with either an inactive maternal X chromosome or an inactive paternal X chromosome. Although this idea has to be proved by analysis of a large number of samples taken from an informative placenta, it is in accordance with the generally accepted view of the trophoblast formation from a limited number of cells functioning as progenitors. In fact, the trophoblast is formed at approximately day 3 postcoitus in mice and at approximately day 4 postcoitus in humans; during this period of development in mice, X inactivation occurs specifically in the trophoblast (whereas it occurs at approximately days 5–6 in somatic cells) (Takagi and Sasaki 1975; Tan et al. 1993). In this model, it is likely that the timing of X inactivation during development of female mice and female humans is approximately the same, except that, in humans, both the paternal X chromosome and the maternal X chromosome can become inactivated in somatic—as well as in placental—tissues. It has been suggested that, in mice, the strength of the Xce allele determines which of the two X chromosomes present in female cells will be inactivated (Cattanach et al. 1970), which might result in a skewed pattern of X inactivation. More recently, a similar phenomenon has been suggested to occur in humans (Naumova et al. 1998). This process might explain our data found in full-term placentas, if it is assumed that, in the most heterogeneous cases (i.e., those which show either sites with paternal X inactivation only or sites with maternal X inactivation only) XCE alleles of similar strength are present. In addition, placentas showing preferential inactivation of either the paternal or the maternal X chromosome would be the result of the presence of XCE alleles with different strengths. Although this model is of interest and deserves to be studied in more detail, it does not explain why the heterogeneity in X inactivation, as observed in the present study, is restricted to trophoblastic tissue—that is, is not found in the matched samples of the umbilical cord. This indicates that, if an XCE-like mechanism does exist, it is likely to be under the control of tissue-specific factors.

The heterogeneity of X inactivation in human full-term placentas is consistent with the phenotypic differences reported between mice with an extra copy of the X chromosome and humans with an extra copy of the X chromosome. In the mouse, the presence of an extra X chromosome of maternal origin results in an almost complete absence of placental tissue and is incompatible with normal development (Takagi and Sasaki 1975). Unlike humans, mice are unable to inactivate the maternal X chromosome in extraembryonal lineages. In humans, supernumerary X chromosomes of either maternal or paternal origin is not lethal but lead to Klinefelter syndrome (Jacobs et al. 1988). This difference between mice and humans might be explained by the X-inactivation pattern that normally occurs in the human placenta, as has been demonstrated in the present study. Consistent with this model are the results reported by Plenge et al. (1997), who recently identified a mutation in the promoter of the XIST gene in families with a skewed pattern of X inactivation. Female carriers of the mutation were found to preferentially inactivate the affected chromosome in somatic tissue. In all informative cases, this X chromosome was inherited from the mother. It is likely that the maternal X chromosome is also preferentially inactivated in the extraembryonal lineages of individuals who show no overt phenotype. Recently, however, a functional difference between the maternal and paternal human X chromosomes has been suggested (Skuse et al. 1997); consistent differences in social adjustment of Turner syndrome patients have been linked to the parental origin of the only X chromosome present. It is conceivable that the maternal and paternal X chromosomes are functionally different but randomly inactivated. Therefore, it would be of interest to study the human homologue of Esx1, which has been found to be an X chromosome–imprinted regulator of early mouse fetal and placental development (Li and Behringer 1998).

Acknowledgments

This work was supported by the Dutch Cancer Society (Koningin Wilhelmina Fonds grant DDHK 94-867). We acknowledge the department of Gynaecology and Obstetrics of the Zuidereekhuis (Rotterdam) for its collaboration in the collection of samples. We thank Drs. H. F. Willard (Case Western Reserve University, Cleveland) and C. Mummery (Netherlands Institute for Developmental Biology, Hubrecht Laboratory, Utrecht) for critically reading the manuscript.

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