Am. J. Hum. Genet. 63:339–346, 1998

Commitment to X Inactivation Precedes the Twinning Event in Monochorionic MZ Twins

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Summary

To gain insight into the timing of twinning, we have examined a closely related event, X-chromosome inactivation, in female MZ twin pairs. X-inactivation patterns in peripheral blood and buccal mucosa were compared between monochorionic MZ (MC-MZ) and dichorionic MZ (DC-MZ) twins. Overall, the MC-MZ twins displayed highly similar X-inactivation patterns, whereas DC-MZ twins frequently differed in their X-inactivation patterns, when both tissues were tested. Previous experimental data suggest that commitment to X inactivation occurs when there are 10–20 cells in the embryo. Simulation of embryo splitting after commitment to X inactivation suggests that MC-MZ twinning occurs three or four rounds of replication after X inactivation, whereas a DC-MZ twinning event occurs earlier, before or around the time of X inactivation. Finally, the overall degree of skewing in the MZ twins was not significantly different from that observed in singletons. This indicates that X inactivation does not play a direct role in the twinning process, and it further suggests that extreme unequal splitting is not a common mechanism of twin formation.

Introduction

The etiology and molecular mechanisms involved in MZ twinning are obscure. MZ twinning events appear to occur very soon after fertilization, spanning a time frame of as much as a week or more after conception. Current estimates for the timing of splitting of the embryo are based entirely on an examination of the placental anatomy of MZ twins at birth. Thus, MZ twins are broadly classified into two groups: (1) monochorionic MZ (MC-MZ) twins, who develop in a single chorion and share the same placental blood supply, and (2) dichorionic MZ (DC-MZ) twins, who develop within two separate chorionic sacs. The DC-MZ twinning event is postulated to occur early (0–4 d after fertilization), before formation of the blastocyst (Derom et al. 1995). DC-MZ twins account for approximately one-third all MZ twins. The other two-thirds of MZ twins are MC-MZ twins and appear to result from later twinning events (probably >4 d after fertilization), after the chorion is formed. The great majority of MC-MZ twins are located in separate amniotic sacs; rarely, a very late embryonic split (probably >8 d after fertilization) results in monoamniotic MC-MZ twins (Derom et al. 1995).

Another means of timing these early MZ twinning events is to relate them to other processes that occur early in development. On the basis of an analysis of hematopoietic cells, X-chromosome inactivation previously has been estimated to take place when the embryo consists of ~10–20 cells (Puck et al. 1992), well within the time frame at which MZ twinning is thought to occur. We therefore chose to compare X-chromosome inactivation patterns in female MZ twins. Elsewhere, we have shown that female MZ-MC twins have very similar patterns of X-chromosome inactivation in peripheral blood (Trejo et al. 1994). This finding generally has been ascribed to the fact that, by virtue of a shared placental blood supply, the circulating hematopoietic elements are thoroughly mixed and exchanged among MC-MZ twin pairs. However, an alternative explanation for the similarity of X-inactivation patterns in MC-MZ twins is that the twinning event in these pairs occurs well after X-chromosome inactivation, resulting in highly correlated X-inactivation patterns in such MZ twins.

In this report we show that the X inactivation in female MZ twins follows the same trend in buccal mucosa (a noncirculating ectodermal tissue) as previously had

Received March 25, 1998; accepted for publication June 4, 1998; electronically published June 29, 1998.

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0002-9297/98/6302-0009$02.00

been observed in peripheral blood—namely, that X-inactivation patterns are highly similar in MC-MZ twin pairs. This is in contrast to the large differences that frequently are observed among DC-MZ twin pairs. Furthermore, on the basis of an estimate of embryo cell number at X inactivation, we have performed simulation studies that suggest that MC-MZ twinning most often occurs at least three or four cell divisions after the start of X inactivation. In contrast, DC-MZ twinning appears to be a much earlier event, which precedes or is closely related, in time, to the onset of X inactivation. This difference, in timing, between DC-MZ twinning and MC-MZ twinning will have to be taken into account when hypotheses about the etiology of MZ twinning are developed. In addition, the distinction between these two types of twinning events may be important for interpretation of epidemiological studies of MZ-twin concordance rates.

Subjects and Methods

Subjects

Cord/peripheral blood samples and buccal swabs were collected from 33 female MZ twins (20 MC-MZ and 13 DC-MZ), with a mean age of 8 years (range of 0–24 years), at the Centrum voor Menselijke Erfelijkheid, Katholieke Universiteit, in Leuven, Belgium. A detailed anatomic analysis of the placenta and fetal membranes was performed in order to classify twin pairs as either MC-MZ or DC-MZ. Cord/peripheral blood samples were collected in EDTA, and buccal mucosa cells were obtained by brushing of a large surface area inside both cheeks by means of sample brushes (Cytobrush Plus; Medscand). DNA was extracted from both tissue types by means of the InVisorb Genomic DNA kit (ID Labs Biotechnology). In a similar manner, DNA was extracted from peripheral blood cells and buccal mucosa swabs from 21 normal female volunteers (mean age 38 years [range 21–57 years]).

Androgen Receptor (AR)–Specific Hpa*II/PCR Assay for X Inactivation*

X-inactivation patterns were determined by an *Hpa*II/PCR assay for the X-linked AR gene (Allen et al. 1992). In normal females, the AR gene is methylated on the inactive X chromosome and is undermethylated on the active X chromosome. Furthermore, as shown in figure 1, a highly polymorphic triplet repeat within the AR gene allows for the discrimination of each X chromosome in most female subjects.

Genomic DNA (~250 ng) was digested overnight with methylation-sensitive restriction enzyme *Hpa*II, according to the manufacturer's instructions (GIBCO-BRL). A mock sample was prepared in parallel, as a control, wherein *Hpa*II was omitted. After digestion, samples were boiled for 10 min to inactivate the *Hpa*II enzyme. PCR amplification of the AR gene was performed with 100 ng of DNA (either *Hpa*II digested or mock digested) and the standard 10 × PCR buffer (Perkin-Elmer), $MgCl_2$ at a final concentration of 2.5 mM, and AmpliTaq Gold (Perkin-Elmer). Primers AR-1 (5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′) and AR-2 (5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′) were chosen to flank the triplet repeat (CAG) and the *Hpa*II sites in the first exon of the AR gene (fig. 1). The AR-2 primer was radiolabeled with [P^{32}] ATP by means of T4 polynucleotide kinase (GIBCO-BRL). Three picomoles of the radiolabeled AR-2 was used, along with 7 pmol of unlabeled AR-2 and 10 pmol of AR-1, in a 50-μl PCR reaction. The PCR conditions were as follows: denaturation at 95°C for 12 min and 30 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. After cycling, an additional extension at 72°C for 10 min was used. Six microliters of the PCR products were run through standard sequencing polyacrylamide gels (National Diagnostics), to obtain optimal separations of the two AR alleles. The gels were dried and were exposed to the phosphorimaging screen and then were analyzed by means of a Phosphorimager (Molecular Dynamics). All samplings were performed in duplicate.

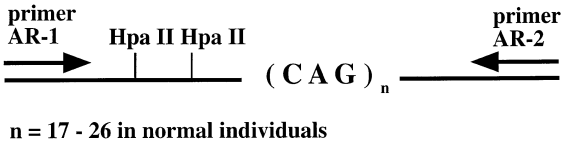

Figure 1 Schematic map of a portion of the first exon of the AR gene. The polymorphic CAG triplet repeat is flanked by *Hpa*II sites, and the locations of primers AR-1 and AR-2, used for the PCR assay, are indicated.

Calculation of Percentage Skewing of X Inactivation

Measurements of each band were obtained, in counts per minute (cpm), from the Phosphorimager. The relative intensity of the larger AR allele (upper band) with respect to the smaller AR allele (lower band) was calculated and expressed as a ratio: R = (cpm in upper band/cpm in lower band). The ratios in the mock-digested (R_M) and *Hpa*II-digested (R_H) samples were calculated separately and then were averaged for each of the two sets of duplicate samples. For each individual, a normalized ratio, $R_N = (R_H)/(R_M)$, was calculated to correct for occasional minor variation in efficiency of amplification of the two AR alleles. This normalized ratio was

used to determine the percentage of inactivation of the X chromosome bearing the larger (upper-band) AR allele: percentage of inactivation = $[R_N/(R_N+1)] \times 100$.

Statistical Methods

The number of cells, *N,* in the embryo/inner cell mass (ICM) at the time of commitment to inactivation was estimated by Monte Carlo simulation methods. In each simulation, we "constructed" 21 embryos (which corresponded to our actual sample size of singletons), with each embryo containing a fixed number of cells. The numbers of cells (*C*) used were 4, 5, 6, 8, 12, 16, and 24, and simulations were run separately for each of these seven scenarios, by means of the following algorithm:

Step 1: Using a fair coin, choose to inactivate with probability .5.
Step 2: Perform step 1 *C* times.
Step 3a: Calculate percentage of X inactivation, as (no. of cells inactivated/*C*).
Step 3b: Calculate percentage of skewing as absolute value of (% inactivation − 50.)
Step 4: Perform steps 1–3 21 times.
Step 5a: Calculate mean percentage of X inactivation.
Step 5b: Calculate mean skewness for the 21 embryos.
Step 6: Perform steps 1–5 500 times.
Step 7a: Calculate grand mean percentage of X inactivation and standard error of the mean (SEM).
Step 7b: Calculate grand mean skew and SEM.
Step 8: Perform steps 1–7 for each value of *C*.

Using a *t*-test, we then compared these population estimates with our observed data for 21 singletons, to see which scenarios were consistent with our observed data.

We also modeled various scenarios of twinning patterns (i.e., timing of twinning), to see which scenarios were most consistent with the observed data on MC-MZ and DZ-MZ twins. The scenarios used the values for *N* that had been determined in the first part of the statistical analysis, as described above.

In brief, the approach was to compute, for our observed sample, a measure of X inactivation similarly between co-twins. The same measure was computed for the simulated scenarios of co-twins and was compared with that for the observed twins.

Step 1: Using a fair coin, choose to inactivate a cell, with probability .5.
Step 2a: Perform step 1 *C* times.
Step 2b: Replicate *C, D* times.
Step 3: Randomly assign one-half of the cells to twin 1 and the other half to twin 2.
Step 4: Calculate percentage of X inactivation in each twin.
Step 5: Compute absolute difference (Δ) between X inactivation in twin 1 and that in twin 2.
Step 6: Perform steps 1–5 10 times.
Step 7: Calculate mean Δ for 10 twin pairs.
Step 8: Perform steps 6 and 7 500 times.
Step 9: Calculate grand mean Δ and SEM.

We then used the *t*-test to test the hypothesis that the observed mean absolute difference in our sample of DC-MZ twins was different from the theoretical mean for each of these scenarios. We then performed the same anaylsis for the MC-MZ twins.

Results

MZ Twins and Normal Female Singletons: Exhibition of a Similar Degree of Variation in X-Inactivation Patterns

By means of the AR-gene methylation-specific PCR assay, X-inactivation patterns of MZ twins were compared with those in a control population. Twenty-one normal female singletons were used as study subjects to test for X-inactivation patterns in buccal mucosa and/or peripheral blood. In these individuals, the range of inactivation of the upper AR allele was 15%–93% in peripheral blood and 23%–77% in buccal mucosa. In singletons, the mean values of the degree of skewing of X inactivation (measurement of X inactivation either <50% or >50%) in peripheral blood and in buccal mucosa of singletons were 18.46% (range 2.2%–42.8%) and 11.27% (range 2.9%–26.9%), respectively, as shown in figure 2.

On the whole, similar patterns of X inactivation were observed within both of these tissue types in these singletons, with the exception of one individual who displayed extremely skewed X inactivation in peripheral blood (~90% inactivation of the upper AR allele) but not in the buccal mucosa (~50% inactivation of the upper allele). In these singletons, no statistically significant difference was observed between the degree of skewing of X inactivation in peripheral blood and that in buccal mucosa (Wilcoxon two-sample–test Z = .1087, Kruskal-Wallis test $P = .1059$)

Also represented in figure 2 is the degree of skewing of X inactivation in cord/peripheral blood of DC-MZ twins (mean 15.6%) and in buccal mucosa of MC-MZ (mean 15.09%) and DC-MZ (mean 16.23%) twins. There were no significant differences between any of the three populations (Kruskal-Wallis test $P = .4035$). Similarly, no significant difference in skewing of X inactivation was observed between cord/peripheral blood from DC-MZ twins and peripheral blood from singletons (Mann Whitney test $P = .328$). The results for cord/peripheral blood from each of the MC-MZ twins individually are not shown in figure 2, since the blood supply

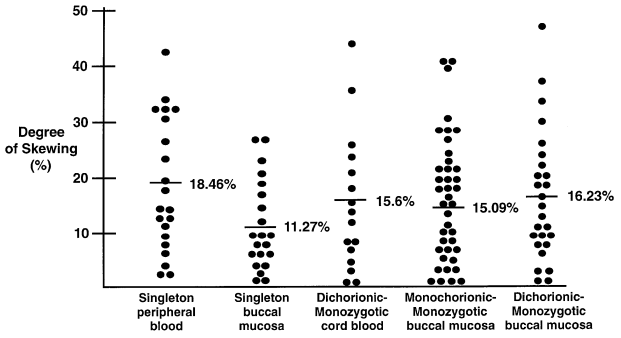

Figure 2 Degree of skewing of X inactivation in peripheral blood and buccal mucosa of female singletons, and comparison with that in cord/peripheral blood of DC-MZ twins and with that in buccal mucosa of MC-MZ and DC-MZ twins. Degree of skewing of X inactivation in peripheral blood and buccal mucosa of female singletons was found to be in the range of 2.2%–42.8% and 2.9%–27%, respectively. The degree of skewing of X inactivation was in the range of 0.02%–43.9% in cord/peripheral blood of DC-MZ and was 3.3%–40.1% and 0.6%–46.5% in buccal mucosa of MC-MZ and DC-MZ twins, respectively. Degree of skewing of X inactivation was calculated as the absolute difference between the percentage of inactivation of the upper AR allele and 50%. The mean values of the degree of skewing of X inactivation are indicated for each group of observations.

is shared in these twins; nevertheless, the results are similar, with a mean skewing of 17.13% in MC-MZ twins' cord/peripheral blood. Overall, the degree of skewing of X inactivation in MZ twins was not different from that observed in singletons.

Simulation of X Inactivation in Singletons, to Estimate Precursor-Cell Number at Which Commitment to X Inactivation Occurs

The process of X inactivation was simulated 500 times per individual in 21 singletons, under the assumption that there were 4, 5, 6, 8, 16, 32, or 64 cells in the ICM at the time of commitment to X inactivation; the results are shown in table 1. In this simulation, X inactivation at the 4– or 5–precursor-cell stage was most consistent with the mean skewing of X inactivation that we actually observed in peripheral blood (18.7% vs. 18.4%, respectively). The mean skewing predicted by simulated X inactivation at the 16–precursor-cell stage (9.8%) was closer to the mean skewing that we observed in the buccal mucosa of the singletons (11.2%). The mean skewing values predicted by simulation of X inactivation at ICM numbers of 24, 32, and 64 cells were lower than the observed values.

MC-MZ Twins: Highly Correlated X-Inactivation Patterns in Both Buccal Mucosa and Cord Blood

We analyzed X-inactivation patterns in both cord/peripheral blood and buccal mucosa of MC-MZ and DC-MZ twins. Figure 3*A* shows an example of highly similar X-inactivation patterns in buccal mucosa obtained from a pair of MC-MZ twins. Discordant X-inactivation patterns in the buccal mucosa of a pair of DC-MZ twins are shown in figure 3*B*.

The values of the absolute differences in percentage of inactivation of the upper AR allele are shown in figure 4. In the buccal mucosa, the mean difference between co-twins who are monochorionic is 5.9% (95% confidence interval [CI] = 2.9%–14.7%), whereas the mean difference between the twin pairs who are dichorionic is 14.3% (95% CI = 10.7%–39.4%). These differences are significant ($P = .0461$). As we have reported elsewhere (Trejo et al. 1994), X-inactivation patterns in cord/peripheral blood in MZ twins follow those observed in buccal mucosa. The mean absolute difference of X-inactivation patterns in cord/peripheral blood of DC-MZ co-twins was 23.1%, in contrast to a mean difference of 5.9% in MC-MZ pairs ($P = .0494$; data not shown).

To evaluate the degree to which the PCR assay contributed to the variation in the data recorded, we tested for reproducibility of the PCR assay, in both buccal mucosa and peripheral blood. The assay was repeated in 9 buccal mucosa samples and 10 peripheral blood samples isolated from the singletons. As is apparent from figure 4, the mean absolute differences of X inactivation recorded at time 1 versus that recorded at time 2 were 3.7% (range 1.3%–6.8%) and 4.3% (range 0.5%–7.6%), in buccal mucosa and peripheral blood, respectively. This indicates that the variation intrinsic to the assay does not explain the differences observed in DC-MZ twin pairs. Thus, a high degree of similarity in X-inactivation patterns, in both buccal mucosa and cord blood, was characteristic of the MC-MZ twin pairs.

Simulation Studies of Embryo Splitting after Each Round of Replication after X Inactivation: Estimation of Timing of Monochorionic Twinning

On the basis of the highly similar patterns of X inactivation in the buccal mucosa of MC-MZ twin pairs,

Table 1

Simulation of X Inactivation in 21 Female Singleton Embryos

ICM Number	Mean Skewing ± SEM (Range)[a] (%)
4	18.75 ± 3.55 (7.14–33.33)
5	18.77 ± 2.63 (11.90–27.14)
6	15.7 ± 2.94 (7.93–25.39)
8	13.57 ± 2.50 (6.54–20.24)
16	9.79 ± 1.71 (5.06–16.66)
24	8.06 ± 1.42 (2.97–14.08)
32	6.90 ± 1.19 (3.87–11.45
64	4.97 ± .83 (2.00–7.44)

[a] Away from 50:50 value.

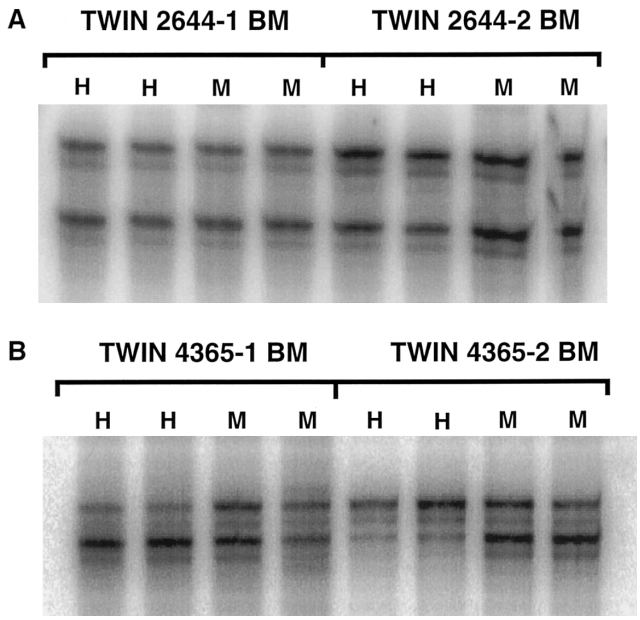

Figure 3 Comparison of X-inactivation patterns, in buccal mucosa (BM), among members of MC-MZ twins and DC-MZ twins. *A,* MC-MZ twins 2644-1 and 2644-2, who show similar patterns of X inactivation in buccal mucosa. The percentage of inactivation of the upper AR allele was 50% in buccal mucosa of twin 2644-1 and was 64% in that of twin 2644-2. *B,* DC-MZ twins 4365-1 and 4365-2, who show discordant patterns of X inactivation in buccal mucosa. The percentage of inactivation of the upper AR allele was 32% in buccal mucosa of twin 4365-1 and was 78% in that of twin 4365-2. M = mock-digested DNA; and H = *Hpa*II-digested DNA. All assays were performed in duplicate, as shown.

it can be inferred that the monochorionic splitting must occur after commitment to X inactivation. We wished to estimate how long after onset of X inactivation the splitting of the embryo must occur, if it is to yield the degree of similarity in X-inactivation patterns that we observed in MC-MZ twin pairs. For the purposes of this simulation, we assumed that embryo splitting results in an equal distribution of cells to each embryo. The four different simulations were performed under the assumptions that there are 8, 12, 16, or 24 cells in the ICM at the onset of X inactivation. We simulated the results of a set of 10 embryos that undergo splitting (1) one round of replication prior to onset of X inactivation (i.e., are totally independent of X inactivation), (2) concomitant with onset of X inactivation, and (3) as many as six replications after this. Figure 5 shows the results for one such simulation, for the case where there are 16 cells in the ICM at the onset of X inactivation. The figure plots the mean of the absolute difference, in X-inactivation patterns, between 10 twin pairs after 500 simulated events of twinning at different embryo-cell numbers, under the assumption that commitment to X inactivation occurs at the 16–precursor-cell stage in the ICM. The SEMs of these mean differences are shown by the thinner horizontal at the ends of the vertical bars in figure 5. Note that, for each round of replication after X inactivation, the mean difference, in X-chromosome skewing, between the co-twins decreases. This is because, the larger the number of cells at the time of splitting, the less likely that the pattern of X inactivation in each twin will differ from that which originally was present at the 16–precursor-cell stage—and, therefore, the more likely that the co-twins will be similar to one another. Also superimposed in figure 5 are the mean values of the absolute difference in X inactivation that we observed in buccal mucosa tissue from co-twins who are dichorionic (mean 14.3%) or monochorionic (mean 5.9%).

As seen in figure 5, when twinning occurs three or four rounds of replication after onset of X inactivation, the predicted mean difference matches closely with the mean difference that we observed in buccal mucosa of MC-MZ co-twins. This strongly indicates that MC-MZ twinning occurs after commitment to X inactivation, at a time when there are perhaps as many as 128–256 cells within the ICM.

In contrast, it is highly unlikely ($P < .0005$) that DC-MZ twinning occurs as late as two replications after onset of X inactivation; rather, the results indicate that DC-MZ twinning generally occurs before—or, at most, one replication after—the start of X inactivation. Similar conclusions resulted from simulations when there are 8, 12, or 24 cells in the ICM at the time of onset of X inactivation.

Discussion

The exact timing of MZ twinning has not been clearly defined. The current information on the timing of MZ

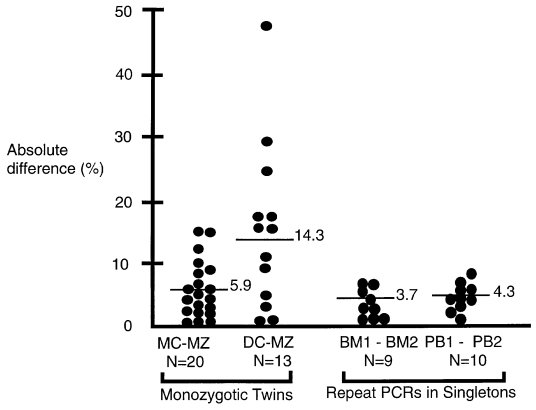

Figure 4 Absolute difference of X-inactivation patterns in buccal mucosa of MC-MZ and DC-MZ twin pairs, and comparison of repeat PCRs in buccal mucosa and peripheral blood from singletons, recorded at two times.

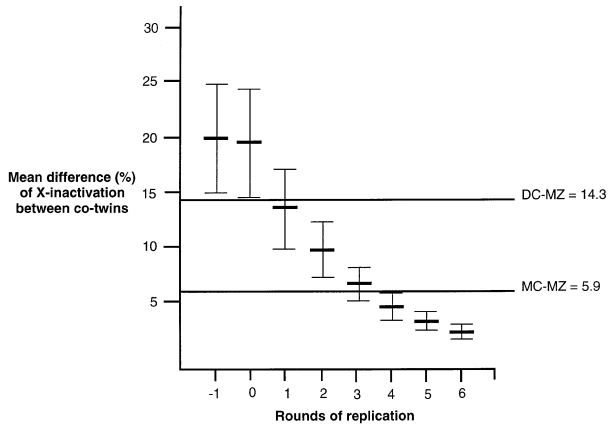

Figure 5 Simulation of MZ twinning, with respect to commitment to X inactivation. We simulated MZ twinning in 10 embryos 500 times, at one round of replication before commitment to X inactivation, at the time of commitment to X inactivation, and as many as six rounds of replication after X inactivation. For this simulation, we assumed that X inactivation occurs when there are 16 cells in the ICM. SEMs are indicated by the thinner horizontal bars.

splitting is based primarily on placentation anatomy. Thus, it has been postulated that dichorionic twinning occurs early, before blastocyst formation, whereas MC-MZ twinning occurs later. In this study, we chose to examine the pattern of X inactivation—a process closely associated temporally with twinning—in order to estimate the timing of early events involved in twinning. The central finding of our study is that MC-MZ twinning occurs after onset of X-chromosome inactivation.

Several groups have suggested that twinning may be influenced by the X-inactivation process, or vice-versa, by virtue of increasingly skewed X-inactivation patterns observed in MZ twins (Nance 1990; Bamforth et al. 1996; Goodship et al. 1996). However, we have observed no difference, in the degree of skewing, between MZ twins and female singletons when both buccal mucosa and peripheral blood of these groups are tested. These results are consistent with those of Watkiss et al. (1994), as well as with those of Jarvik and Motulsky (1997). Using a population-based estimate of X-linked color blindness as a consequence of skewed X inactivation, Jarvik et al. observed no evidence of a higher frequency of skewed X inactivation in their group of 374 MZ twins.

We simulated X inactivation in 21 female singleton embryos to identify the most likely time frame within which commitment to X inactivation takes place during early embryonic development. The simulation was performed by means of a very conservative approach and a very simplified model based on the assumptions that (1) variation in X inactivation is solely attributable to the stochastic nature of this process and (2) all cells in the embryo replicate equally. When these simplified assumptions are posited, our data suggest that commitment to X inactivation occurs when the ICM of the developing female human embryo contains $\leqslant 16$ cells—and possibly as few as 4 or 5 cells. This is lower than the predicted number of 10–20 precursor cells that was estimated by Puck et al. (1992) by means of maximum-likelihood analyses.

There is reason to believe that our results are an underestimate of the cell number at which onset of X inactivation occurs. In particular, we did not take into account other factors—such as preferential allelic usage, precursor pool size, or selection—that may contribute to skewed X-inactivation patterns in adult tissues. Preferential allelic usage may be controlled genetically, as Naumova et al. (1996) have shown in humans and as also is reflected by *xce* effects in mice (Rastan 1982). In our study, age may play a modest role in contributing to a higher frequency of skewing in MZ twins than in singletons, since the mean age of the normal female singletons is 38 years (range 21–57 years), whereas that of the MZ twins is 8.2 years (0–24 years). In peripheral blood, skewed X inactivation shows slight increases with aging (Busque et al. 1996), and this may be due to lymphocyte clonal expansion (Monteiro et al. 1995), stem-cell depletion, or selection pressures (Puck et al. 1990).

A number of investigators have analyzed *Xist* (X-inactivation–specific transcript) expression in developing embryos by studying the timing of molecular events involved in X inactivation (Panning et al. 1997; Sheardown et al. 1997). In the mouse, differential biallelic expression of *Xist* in the ICM can be observed by embryonic day 5.5, at a time when there are ~200 cells in the embryonic germ-cell layer (Hogan et al. 1994). Of course, differential expression of *Xist* must occur after commitment to X inactivation (Panning and Jaenisch 1998), and this undoubtedly results in overestimation of the number of cells present at the actual time of commitment to X inactivation. Our data and those of Puck et al. (1992), based on statistical analysis of skewing in somatic tissues, place commitment to inactivation at a much earlier time during embryo development.

We have been able to estimate the timing of twinning with respect to onset of X inactivation, by comparing X-inactivation patterns in MC-MZ and DC-MZ cotwins. Our earlier study of the cord blood of MC-MZ and DC-MZ twins showed that the MC-MZ twins exhibit very similar X-inactivation patterns whereas the DC-MZ twins frequently differ from each other. However, because of the complications, for analysis, that are attendant on shared blood supply during intrauterine development of MC-MZ twins, these results could be misleading. Therefore, in this study, we compared X-inactivation patterns among MC-MZ and DC-MZ twins, both in buccal mucosa (a tissue that is ectoder-

mally derived and is not exchanged between twins during development) and in cord/peripheral blood. MC-MZ co-twins displayed strikingly similar patterns of X inactivation in both types of tissue, whereas DC-MZ co-twins often differed. These data can be explained plausibly only by a timing difference, with respect to onset of X inactivation, in the DC versus MC twinning events.

The data indicate that MC-MZ twinning must occur subsequent to the start of X inactivation, with the most common MC twinning events taking place at approximately three or four replications after onset of X inactivation in the ICM. However, it is likely that MC-MZ twinning occurs over a continuous time frame after X inactivation. Indeed, one aspect of our results is consistent with this hypothesis. The absolute differences of X-inactivation patterns recorded in buccal mucosa of a subset of MC-MZ twins were in the range of 10%–15%, a value higher than the upper limit of PCR error (7%) recorded in repeat PCR samples from singletons (fig. 4). This may reflect splitting of these twins that is close in time to the start of X inactivation, leading to slightly less correlated X-inactivation patterns in these twin pairs. If so, we would predict that all of these pairs should be diamniotic MC-MZ pairs. Diamniotic MC-MZ twins are presumed to split earlier than monoamniotic MC-MZ twins. The corollary of this predicts that all monoamniotic MC-MZ twins should have X-inactivation patterns that are as similar as are the repeat assays in singletons, since these twins will have split very late in the time frame after X inactivation. Further analysis of X-inactivation patterns, in a larger population of MC-MZ twins belonging to the monoamniotic subtype, will allow us to confirm this hypothesis.

We should emphasize that the simulations in this study have been performed under the assumption that the embryo splitting that causes twinning results in a roughly equal distribution of cells to each co-twin. It has been proposed by some investigators that unequal splitting may commonly give rise to twins and that this may be a source of X-inactivation differences between co-twins (Nance 1990). Unequal splitting should also yield "patch size" differences among twins. In the literature, there is one report that may be consistent with this scenario; in that report, discordance for an X-linked trait—Duchenne muscular dystrophy (DMD)—was observed in a female twin pair (Richards et al. 1990). However, in this case, both twins exhibited extreme skewing in opposite directions, making accurate estimation of patch size difficult. In a subsequent report of another MZ twin pair, discordance for DMD was thought to have occurred in an MC-MZ twin pair (Lupski et al. 1991); however, the diagnosis of monochorionicity was based solely on the mother's recollection. It may be that unequal splitting occasionally accounts for differences in X-inactivation patterns in MC-MZ twins. However, it cannot be common, since it would lead to an overall increase in X-inactivation skewing in MC-MZ twins as a group. We have simulated unequal splitting (after X inactivation) at a 12–precursor-cell stage (25:75 split) and at a 24–precursor-cell stage (12.5:87.5 split). Both scenarios predict greater overall degrees of X-inactivation skewing than we have observed. To fully address this question, a comprehensive study of patch size would have to be performed in MZ twins and comparison with that in singletons would be required.

It is not clear whether the etiology of twinning is different at different times during embryonic development. The results of the present study suggest that, when a cutoff value of 15% for X-inactivation differences is used, it may be possible retrospectively to identify MZ twin pairs who were dichorionic and who therefore resulted from an early splitting event. This may aid in studies of MZ-twin etiology, or, alternatively, it may reveal differences, in disease concordance rates, among this subset of MZ twins; for example, the absence of both a shared placental blood supply and a shared immune system during intrauterine development may lower concordance rates for autoimmune diseases among the DC-MZ twin pairs. Our previous study of a small population of twins discordant for autoimmune diseases suggests that this is not the case (Trejo et al. 1994); however, a large population of twin pairs have to be studied in order to rule out modest effects on disease susceptibility.

Acknowledgments

We would like to thank Dr. Geoffrey Machin for his critical reading of the manuscript. We would also like to thank Dorothy Guzowski for synthesizing the oligonucleotide primers. Part of this research work was supported by NATO grant CRG 951364 and National Fund for Scientific Research (Belgium) grant G.3125.94.

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