Tissue Specificity of X-Chromosome Inactivation Patterns

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The analysis of X-chromosome inactivation patterns has been used in a number of clinical situations such as the identification of carrier status in X-linked genetic disorders and the establishment of the monoclonal origin of tumors. Interpretation of the result obtained requires comparison with the constitutive pattern for the individual, and for hematopoietic malignancies, skin biopsies or cultured fibroblasts have often been used as the control tissue because normal cells of the same lineage as the malignancy are not generally available. However, this assumes that patterns in the different tissues are constitutionally the same. We have therefore compared X-chromosome inactivation patterns from peripheral blood (granulocytes, E- cells, and T cells), skin, and muscle from 20 hematologically normal females, and colonic mucosa from 9 individuals. In 11 patients (55%), the results obtained were similar for all tissues of an individual, but in 9 patients, significant differences were observed between tissues. The most consistent feature was a skewing in peripheral blood (>75% expression of one allele) but not skin and/or muscle. These studies suggest that skin cannot be used as a control tissue for the interpretation of X-chromosome inactivation patterns in hematopoietic cells.

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In 1961, the hypothesis of X-chromosome inactivation was first proposed by Lyon1 to explain the mosaic phenotype of female mice heterozygous for certain sex-linked mutants, with evidence that in each cell one X-chromosome was genetically inactivated and its origin, ie, either paternal or maternal, varied in different cells of the same animal. Since then, application of this information has been used in a number of situations, eg, in the detection of carrier status for X-linked genetic disorders and, more extensively, in the investigation of the monoclonal origin of a variety of tumors.5,7

Studies have shown that X-chromosome inactivation occurs at an early stage of embryogenesis, in humans certainly by the late blastocyst stage, and is not a single event but rather a sequence of events. In mammals, inactivation first occurs in the trophoectoderm then in the primitive endoderm, and in these extraembryonic tissues the paternal X-chromosome is preferentially inactivated. Inactivation then occurs in the cells of the embryonic ectoderm, ie, the inner cell mass, the cells that go on to form the adult somatic cells of the female. This event is random, in most cases affects either the paternal or maternal X-chromosome, and is stably inherited by daughter progeny of that cell. More recent research has shed some light on the molecular mechanism by which the process of inactivation occurs and there is good evidence to suggest that methylation may be responsible for the inactivation imprint and its maintenance, although not for initiation of the process nor the spreading along the chromosome.9,10

The actual timing of random inactivation in the inner cell mass has remained uncertain, partly because of the lack of suitable markers and the difficulty in obtaining sufficient tissue for analysis. In the mouse embryo, only one X chromosome is active in somatic cells of the epiblast at the onset of gastrulation, and it was thought that inactivation is probably complete in all cells by 5.5 days postcoitus (DPC).11 However, a recent report using visual assessment of the X-chromosome activity in individual cells has clearly demonstrated that X-inactivation does not occur simultaneously in all cells of the gastrulating mouse embryo but proceeds gradually in subpopulations of cells of the embryonic ectoderm and mesoderm.12 Although random X-chromosome inactivation was completed in most ectodermal and mesodermal tissues by 9.5 DPC, delayed inactivation was noted in a number of tissues, including hindgut. These results have important implications for the interpretation of X-chromosome inactivation patterns in disease states as the number of cells present at the time of X-chromosome inactivation and/or the number of cells that eventually contribute to a particular organ will influence the variance in patterns obtained.13 The larger the progenitor cell pool size, either when inactivation occurs or when the cells become committed to a particular differentiation pathway, then the greater the probability that most values for X-chromosome inactivation patterns will be close to the expected mean of 1 for random inactivation, ie, with 50% expression of each allele. Conversely, if the pool size is small, a much wider distribution of patterns would be expected, with a significant proportion of values falling at the extremes of the distribution.

Differential methylation of active and inactive X chromosomes forms the basis of analysis of X-chromosome inactivation patterns using DNA probes because methylation of CpG-rich regions at the 5' end of X-chromosome genes appears to be tightly regulated, whereas methylation at other regions of the X-chromosome is not.14-16 Maternal and paternal alleles can be distinguished either by polymorphisms in specific restriction endonuclease sites, eg, in the phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyl transferase (HPRT) genes, or by the variable number of tandem repeat (VNTR) sequences, eg, the 26-bp repeat sequence in the DXS255 locus recognized by the probe M27/β. The relative expression of the two alleles in a population of cells can then be quantified by digestion with methylation-sensitive restriction enzymes that will digest only the unmethylated alleles.15,17 A number of studies have compared X-chromosome inactivation patterns in various tissues and from this derived an estimate of the progenitor cell pool size.
in some cases equating this with the time of overall X-chromosome inactivation.\textsuperscript{18,19} As a consequence, tissues of different lineages have sometimes been used as controls to interpret results from patient’s samples, e.g., in analysis of a number of neoplastic and X-linked blood disorders, skin samples have been used for comparison with the blood patterns obtained.\textsuperscript{2,3} This assumes that patterns in the two tissues are constitutionally the same and therefore the results are directly comparable. However, studies using the M27β probe have found different X-chromosome inactivation patterns in different tissues.\textsuperscript{2,22} It has not been clear whether this was restricted to analyses with this probe only. In this study, we have therefore analyzed multiple tissues from hematologically normal individuals using PGK, M27β, and HPRT.

**Materials and Methods**

**Patients.** In total, 28 females were studied with informed consent. Tissue samples of heparinized peripheral blood, skin, and muscle were obtained from 20 patients undergoing gastric surgery for a variety of disorders. Normal colonic tissue was available in 9 of these patients. Peripheral blood only was collected from a further 3 patients and from 3 members of the laboratory staff. All individuals were randomly selected, had a normal full blood count, and a normal white blood cell differential. The age range was 18 to 96 years (median, 68 years). In addition, peripheral blood was obtained from the fathers of five PGK heterozygotes in remission from acute myeloid leukemia (AML) who had previously been shown to have a skewed Lyonization pattern in both granulocytes and T lymphocytes.\textsuperscript{23}

**Sample preparation.** Peripheral blood was separated into granulocytes and mononuclear fractions using density gradient centrifugation according to the manufacturer’s specifications (Nycoderm, Oslo, Norway). Granulocytes were recovered from the heavier fraction by hypotonic lysis of the red blood cells. T lymphocytes were purified by E-rosetting of the mononuclear cells using aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocytes.\textsuperscript{24} The remaining cells were pooled as the E-negative (E-) fraction. For skin specimens, adipose cells and the keratinized stratum corneum were removed, leaving the dermal and epidermal layers. Muscle samples were rinsed thoroughly in normal saline to wash away any loose blood cells and superficial connective tissue was removed. The muscularis and adventitial layers were dissected away from normal colonic specimens, leaving the mucosa and submucosa. For the five paternal peripheral blood samples, total white blood cell populations were collected. High molecular weight DNA was prepared from all samples by proteinase K/detergent digestion, phenol-chloroform extraction, and ethanol precipitation.\textsuperscript{25}

**Heterozygosity screening and X-chromosome inactivation patterns.** DNA from individuals was screened for heterozygosity of the PGK gene and 12 were informative for this probe. A further 13 females could be analyzed using M27β. One patient was only heterozygous for HPRT and 2 patients were not informative for any of the three probes. One patient was studied with both PGK and M27β.

**X-chromosome inactivation patterns were obtained from granulocytes, E- cells, and T lymphocytes of 25 individuals** (Table 1, patients no. 2 through 26). There was a wide variation in the ratio of expression of the two alleles, and an imbalanced inactivation pattern with greater than 75% expression of one allele was found in 10 individuals (40%). There was no close correlation between values obtained from granulocytes and E- cells (correlation coefficient \( r = 0.99 \)). Comparison of the values obtained from granulocytes and T lymphocytes also showed a good correlation (\( r = 0.97 \); Fig 1A). In 24 cases the difference in allele expression between the granulocytes and T cells for any one individual varied between 0% and 13% (median, 2%; mean, 4%). In 1 patient (Table 1, patient no. 22), there was 26% difference between the two values, with 58% expression of the lower allele found in granulocytes and 84% in T cells. There was no apparent reason for this difference, with no overt evidence of a clonal expansion of her lymphoid cells. However, it must be noted that she was the oldest patient in the series at 96 years of age.

**X-chromosome inactivation patterns were obtained from skin and muscle of 20 patients** (Table 1, patients no. 1 through 20). For the skin samples, most patients showed a balanced pattern of allele expression and only 4 individuals...
(20%) showed greater than 75% expression of one allele (Table 1, patients no. 2, 11, 13, and 18). Comparison of skin patterns with values obtained from granulocytes gave variable results ($r = .74$; Fig 1B). There was a wide difference between ratios obtained from the two tissues of any one individual, e.g., there was no difference in the ratios obtained from granulocytes and skin in patient no. 11 (Table 1), whereas there was 51% difference in allele expression between the same two tissues in patient no. 6. In 12 patients (60%), the pattern found in skin was very similar to that from granulocytes, with less than 15% difference in the values obtained. This was not restricted to patients with balanced X-chromosome inactivation patterns and included the 4 patients with skewed Lyonization patterns (patients no. 2, 11, 13, and 18). In 7 patients there was greater than 20% difference between allele expression in the two samples and in 3 of these the granulocytes showed an extremely skewed pattern, whereas the skin was nearly balanced (patients no. 6, 10, and 14). Similar results were obtained with the muscle samples; most patients showed a balanced Lyonization pattern, but 5 patients (25%) showed greater than 75% expression of one allele. The correlation between granulocyte and muscle values was only 0.63 (Fig 1C), with a difference in expression of the same allele varying between 0% and 59% in different patients. In 13 patients, there was no difference (<15%) between the values obtained for the two samples,
including 3 with skewed patterns in all samples studied (patients no. 2, 11, and 13). The difference in expression was greater than 20% in 7 patients; in 2 of these the granulocytes had an extremely skewed pattern, but muscle was balanced (patients no. 6 and 14). Skin and muscle did not show a tight correlation \((r = .79)\).

Of the X-chromosome inactivation patterns obtained from nine colonic mucosa samples, only one (11%) showed greater than 75% expression of one allele (Table 1, patient no. 2), with similar results found in the patient’s peripheral blood, skin, and muscle. The correlation was not tight between values obtained for granulocytes and colon \((r = .69)\) or skin and colon \((r = .71)\); however, there was a good correlation between muscle and colon \((r = .94)\).

There was considerable variability in the different tissue patterns obtained between individuals. In 11 patients (55%), the values from peripheral blood granulocytes, E- cells, T lymphocytes, skin, and muscle could be taken as the same, ie, with less than 15% difference between expression of the alleles (Fig 2A). Five patients showed a similar pattern in skin and muscle, but this differed from peripheral blood (patients no. 3, 6, 8, 12, and 14; Fig 2B). In 2 patients, results from skin samples differed markedly from both peripheral blood and muscle, which were similar to each other (patients no. 7 and 10; Fig 2C). Another patient had similar values in peripheral blood and skin, but the muscle value was completely different (patient no. 5), and in a further patient peripheral blood, skin, and muscle were all different from each other (patient no. 20; Fig 2D). Overall, the frequency of skewing was higher in granulocytes and T lymphocytes (10 of 25, 40%) than in skin (4 of 20, 20%), muscle (5 of 20, 25%) or colon (1 of 9, 11%). Analysis of patients with skewing in peripheral blood but not skin showed that this was not limited to use of one probe only but was observed with PGK (patients no. 5 and 10), MZ77β (patients no. 6, 14, and 20), and HPRT (patient no. 3 with a borderline skewed value for granulocytes but a balanced pattern for skin). Patient no. 5 was analyzed using both PGK and MZ77β and the differences found between the various tissues were similar for both probes. It would have been desirable to also study patient no. 10, who showed a marked discrepancy between the patterns for blood cells and skin, with MZ77β, but unfortunately she was not informative for this probe.

Expression of parental alleles in females with a skewed Lyonization pattern. To identify whether females with a skewed Lyonization pattern show preferential inactivation of the paternal or maternal allele, blood was analyzed from the fathers of five PGK heterozygotes in remission from AML and previously shown to have a skewed Lyonization pattern in all myeloid and lymphoid cells, indicative of a constitutively skewed polyclonal pattern.25 For PGK, the active allele is unmethylated and therefore the allele remaining after methylation-sensitive digestion is inactive. In 3 patients, the predominant allele seen in the X-chromosome inactivation pattern was the same as the paternal allele, ie, the paternal allele had been inactivated, and in 2 patients the maternal allele had been inactivated.

DISCUSSION

The results presented here suggest that in a significant proportion of females their X-chromosome inactivation pat-


terns vary from tissue to tissue, with good correlation between granulocytes, E- cells, and T lymphocytes, but not between other tissues (Table 2). In 11 of 20 (55%) patients, there was little or no difference in the relative expression of the two alleles found in the tissues studied, eg, in Table 1 patients no. 11 and 15, 0% and 6% difference, respectively, were observed overall between values obtained for peripheral blood granulocytes, T cells, skin, and muscle. However, in other patients, there was a wide variation between the
found to operate in female carriers of certain X-linked disor-

ders, eg, Lesch-Nyhan syndrome, Wiskott-Aldrich syndrome, agammaglobulinemia, and severe combined immunodeficiency in which tissue-specific nonrandom X-

Table 2. Correlation Coefficients for X-Chromosome Inactivation Patterns From Multiple Tissues

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<td>Granulocytes</td>
<td>T lymphocytes</td>
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different tissues, eg, for patients no. 6 and 20 (Table 1) there was 59% and 54% overall difference observed, respectively. Of the 9 patients that showed differences between tissues, the most consistent feature was a skewing in the peripheral blood (with greater than 75% expression of one allele) but not in skin and/or muscle; this was observed in 7 patients. In the remaining 2 patients, all values fell within the balanced range but skin in 1 patient (patient no. 7) and skin and muscle in the other (patient no. 8) differed from peripheral blood. No patients were found in which a skewed pattern was observed in skin and/or muscle but not in peripheral blood. The patient with a skewed pattern in colon also had skewing in her other tissues studied (patient no. 2), whereas 3 other patients with a skewed pattern in peripheral blood (but not skin or muscle) had a balanced colon pattern. The discrepancies between different tissues were seen with all three probes and are in accord with our previous findings of a close correlation between results using these probes in doubly informative individuals.

A high proportion of individuals (10 of 25, 40%) had a skewed Lyonization pattern with greater than 75% expression of one allele in peripheral blood granulocytes, E- cells, and T cells, and in 5 patients this skewing was restricted to the peripheral blood alone. This has been reported previously in a number of studies of blood cells from hematologically normal females, including phenotypically monoclonal patterns in healthy individuals caused by the expression of one allele only.

The proportion of skewing observed in this study is greater than the 21% found in our previous studies. This may simply reflect the small sample size. Alternatively, most of the patients used in this study are elderly due to the nature of their surgery (median, 68 years), and it has been suggested that clonal predominance may increase with age. However, this is unlikely to affect both myeloid and lymphoid cells in all individuals as observed with the present patients, and in 5 patients their skin and/or muscle as well. From analysis of peripheral blood from 100 hematologically normal individuals, including 25 reported here (the patient with a discrepancy between granulocytes and T cells has been excluded), there was no correlation between age and the level of allele expression (Fig 3).

There are several possible explanations that might account for this skewing in peripheral blood. Firstly, it is possible that somatic cell selection occurs after random X-chromosome inactivation has been established and as a consequence of a selective pressure on blood cells. This mechanism has been found to operate in female carriers of certain X-linked disor-

Fig 3. Correlation of X-chromosome inactivation patterns with age for 100 hematologically normal females. (O) Patients reported in the current study. The dotted line demarcates the arbitrary level used to denote a skewed pattern, ie, with greater than 75% expression of one allele.
time of X-chromosome inactivation. For a random process, the proportional expression of the two alleles is expected to follow a Gaussian distribution about a mean of 50% expression for each allele, but this will depend on the cell pool size at the time of inactivation, and a broader distribution would be expected with a smaller cell pool size. McLaren has suggested that there are a number of stages in the process, but that the critical determinant for the X-chromosome inactivation pattern of any tissue is the point at which cells become restricted to that tissue and cell mixing ceases. Patterns will therefore vary from tissue to tissue according to the number of cells from which they are derived and their spatial relationship to one another. If the inactivation process takes place gradually over a period of time, as has been demonstrated by Tan et al, this will also influence the pattern obtained, e.g., inactivation of the progenitor blood cell pool at an earlier development time (i.e., fewer cells involved) would lead to considerably more skewing in blood cells than in gut cells, which are apparently one of the last cell populations to be inactivated. Estimates for the progenitor cell pool size for human hematopoietic cells have ranged from 7 to 16, 19, 26, 31. Because E-cells and T lymphocytes arise from the same hematopoietic cell as myeloid cells, they would be expected to have the same X-chromosome inactivation pattern and this is confirmed by the high degree of correlation obtained between these cell populations in the present study (r = .99 and .96, respectively).

One of the major diagnostic applications of X-chromosome inactivation patterns has been in the demonstration of clonal populations in tumors, differentiating between a reactive and neoplastic process. Because skewing is known to occur, the pattern from a tissue sample must be interpreted with reference to normal tissue of the same lineage before a clonal population can be demonstrated. For solid tumors, this is not a problem because normal tissue is usually available for comparison. However, this presents a problem for hematologic malignancies because blood or bone marrow predating the transforming event is rarely available for analysis and alternative tissues have been used, predominantly skin biopsies or fibroblast cultures. The results reported here suggest that such controls are not appropriate because in 6 of 11 (55%) patients with skewed X-chromosome inactivation patterns in blood, the skin pattern was balanced. Hair follicles are a potential source of control DNA but this is not advisable because considerable patchiness in the tissue distribution has been noted in studies using G6PD isoenzymes. Buccal cells are another alternative control tissue. We have found that buccal washings are frequently contaminated with large numbers of neutrophils (unpublished observations) and buccal scrapes rarely generate sufficient DNA for analysis using Southern blotting. Because certain X-linked and differentially methylated genes that are highly polymorphic can now be studied on small samples using polymerase chain reaction technology, it will be possible to examine buccal scrapings as a potential control tissue for the analysis of hematologic disorders.

As T cells and myeloid cells are derived from a common embryonic stem cell, T cells would appear to be the best choice of control tissue in myeloid malignancies. For example, in the studies of myelodysplastic syndromes by Abrahamson et al and van Kamp et al, the granulocytes were shown to be clonal and the T cells polyclonal. However, there are limitations to the use of T cells. Interpretation is straightforward when a polyclonal pattern is seen in the T cells, but when an apparently clonal pattern is observed in such cells, this could be due to involvement of the T cells in the malignant process or to extreme constitutive Lyonization. These two entities cannot be rigorously distinguished without additional clonal markers and in such individuals a definitive statement about clonality cannot be made.

REFERENCES


28. Gale RE, Linch DC: Investigation of methylation at Hha I sites using the hypervariable probe M27β allows improved clonal analysis in myeloid leukemia and demonstrates differences in methylation between leukemic and remission samples. Leukemia 8:190, 1994


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