X-inactivation pattern in the epididymis of sex-reversed mice heterozygous for testicular feminization

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SUMMARY

Female mice heterozygous for testicular feminization were sex-reversed by means of the autosomal sex reversal mutation (Sxr). Due to X-inactivation, the blastemata for male sex organs in these animals are composed of a mixture of cells, carrying either the wildtype X chromosome or the X chromosome affected with Tfm in an active state. Thus, the two types of cells are sensitive to androgens or insensitive to androgens, respectively. This mosaic could be demonstrated in the epididymis on a cellular level. Segments of undifferentiated Tfm cells were found alternating with normally differentiated wild-type cells. The ultrastructural appearance of the mosaic is described.

INTRODUCTION

In placental mammals, genetic females are natural mosaics in respect to X-linked allelic genes, due to random inactivation of one of the two X-chromosomes in each cell (Lyon, 1961, 1972). This holds true also in the case of the X-linked mutation for testicular feminization (Tfm) in the mouse, which was described by Lyon & Hawkes (1970). Only genetic males carrying the Tfm locus on their X-chromosome are affected (X^{Tfm}/Y). These mice possess no internal sex organs except for intra-abdominal testes and a short blind-ending vagina. Externally, the female phenotype is expressed. The deficiency can be explained by androgen insensitivity of the sex organ anlagen (Ohno & Lyon, 1970). During embryogenesis, male sex organs are not induced, although endogenous testosterone is secreted by the testes. In addition, the female appearance of the X^{Tfm}/Y cannot be altered by exogenous testosterone administered to the mother, although female litter-mates become masculinized (Goldstein & Wilson, 1972). Tfm-heterozygotes (X^{Tfm}/X^+) are normal females and function as carriers in the propagation of the mutation. Naturally, they do not have testosterone-dependent organs. Nevertheless, because of random X-inactivation, the heterozygous females must be mosaics in terms of the androgen sensitivity or insensitivity of single cells or clones. Indeed, the mosaic can be

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demonstrated by the pattern of androgen-dependent enzyme induction in the kidney (Tettenborn, Dofuko & Ohno, 1971).

Against this background, it seemed potentially interesting to study animals heterozygous for Tfm and at the same time possessing male sex organs. According to the X-inactivation hypothesis, in such animals a varying percentage of cells making up the male sex organs would be insensitive to androgens, that is, insensitive to the very hormones which during foetal life function as inducers of these organs and, later on, maintain their functional state. Such animals should offer a good model for studying X-inactivation in internal organs. Furthermore, clonal analysis of the mosaic should provide new insights into the embryonic origin of the sex organs, and into the action of androgens.

In order to construct the postulated animals, the sex reversal mutation (Sxr) described by Cattanach, Pollard & Hawkes (1971) was introduced. The sex reversal mutation is inherited autosomal and causes genetic females to develop as phenotypic males. The mutation behaves like a dominant trait and hence is expressed in heterozygous females, while heterozygous males function as carriers. In sex-reversed genetic females (X/X (Sxr/+)), normal male sex organs develop. The only difference from X/Y males seems to be that the seminiferous tubules of their testes do not contain germ cells, probably due to the fact that X/X germ cells cannot survive in the testis environment.

From the combination of Tfm and Sxr, X^{Tfm}/X^{+} phenotypic males resulted. In the sex organs of these animals, major asymmetries were found in random distribution. Often whole organ structures were missing. The blastemata of the sex organs, indeed, seemed to be made up of a mixture of androgen-sensitive X^{+} cells and androgen-insensitive X^{Tfm} cells, and under the selective influence of endogenous testosterone characteristic malformations had developed. But in contrast to expectation, in the epididymides no gross asymmetries were seen. Surprisingly, the reason was that the mosaic pattern predicted for the embryonic Wolffian duct was preserved in the epididymis of the adult. Evidently, the X^{Tfm} cells had managed to survive in the epididymis of the heterozygote, though in X^{Tfm}/Y animals the Wolffian duct, being made up exclusively from X^{Tfm} cells, is doomed to regression.

The rationale of the genetic experiment and the basis for the development of a clonal mosaic in the epididymis are schematically outlined in Fig. 1 A, B. Besides providing a good example of X-inactivation, the finding provokes further questions, for example on the mechanism of survival or regression of the Wolffian duct cells. Therefore, the light- and electron-microscopic appearance of the mosaic present in the epididymis will be described here in detail, while the consequences of this mosaic on the development of the other internal and external sex organs will be analysed in a later paper.
Fig. 1. Expression of testicular feminization (Tfm) in the epididymis of the mouse. (A) In the hemizygous state. During embryogenesis in normal male embryos, testes develop from which testosterone is secreted. Testosterone stimulates the Wolffian duct rudiment to differentiate into an epididymis. In the female embryo, ovaries are formed, producing no testosterone. Therefore, the Wolffian duct is doomed to regression. In males affected by Tfm (XTfm/Y), though testosterone is produced, the Wolffian duct cannot respond to testosterone and hence regresses, as in the female (stippled arrow). (B) In the heterozygous state. Genetic females heterozygous for Tfm are sex-reversed by means of the autosomal Sxr mutation (XTfm/X+(Sxr/+)). Despite their female sex chromosome complement, testes develop in the embryos. Due to X-inactivation in the Wolffian duct cells, either the XTfm chromosome or the wildtype X+ chromosome is active. Under the influence of endogenous testosterone only the X+ cells are capable of differentiating into epididymis cells. The XTfm cells were expected to die off as in the XTfm/Y animal. But, instead, they were found incorporated into the adult epididymis as undifferentiated cells.
MATERIALS AND METHODS

Sex-reversed Tfm heterozygotes were obtained from the progeny of two pairs of mice carrying the Tfm as well as the Sxr mutation. Both stem pairs were kindly given to us by Dr S. Ohno, City of Hope, Duarte, California. The breeding schedule was as follows: male mice carrying Sxr were mated to females heterozygous for Tfm (X<sup>Tfm</sup>/X<sup>+ </sup>× X<sup>+ </sup>/Y(Sxr/+)). Two of the three X-chromosomes present in the mating were tagged by the coat colour markers blotchy and tabby. Thus, in the offspring the sex chromosome complement could be determined from the variegated heterozygous or the hemizygous phenotype. Half of the genetic females derived from the mating were converted into phenotypic males by the paternal Sxr. From these in turn, one-half carried the X<sup>Tfm</sup> of the mother, thus being genetically X<sup>Tfm</sup>/X<sup>+</sup>(Sxr/+). Litter-mates affected by testicular feminization (X<sup>Tfm</sup>/Y) and sex-reversed females without Tfm (X<sup>+</sup>/X<sup>+</sup>(Sxr/+)) were taken for controls. The animals were killed for examination between the ages of 40 and 60 days.

Histology was performed on the epididymides of 21 sex-reversed Tfm heterozygotes. For electron microscopy, the lower part of the caput epididymis of 8 animals was fixed in 3% glutaraldehyde, post-fixed in osmic acid, embedded in Epon 812, sectioned on an LKB ultratome, stained with uranyl and lead acetate, and examined in a Zeiss EM 9A electron microscope.

LIGHT AND ELECTRON MICROSCOPE OBSERVATIONS

The epididymis constitutes a single convoluted duct destined for maturation and storage of sperm. Two main functions, residing in the main cell type, the principal cell, have been ascribed to the epididymis epithelium: resorption and secretion (Hoffer, Hamilton & Fawcett, 1972). Structural specializations associated with resorption are endocytic invaginations in the pits between the stereocilia, which detach in the form of 'coated vesicles', and are finally combined in large multivesicular bodies—a type of ingesting lysosome. Specializations indicating secretion are the extended rough endoplasmic reticulum and a huge Golgi complex, though the secretional product and the mode of secretion are not yet known. From autoradiographic studies (Clermont & Flannery, 1970), it has become clear that the epididymis epithelium is not of the renewal type, but rather consists of an expanding cell population with a slow increase after puberty.

Figure 2 shows a section from the epididymis of a sex-reversed Tfm heterozygote (X<sup>Tfm</sup>/X<sup>+</sup>(Sxr/+)). The epididymal duct is lined by segments of high columnar epithelium alternating with segments of flat cuboidal cells. In the flat segments, the stereocilia typical for epididymis epithelium are missing. For comparison, in Fig. 3, typically differentiated epithelium in the epididymis of a sex-reversed genetic female mouse (but without Tfm) is shown (X<sup>+</sup>/X<sup>+</sup>(Sxr/+)). The genetics
Fig. 2. Cellular mosaic due to X-inactivation in the epididymis epithelium of a sex-reversed mouse heterozygous for Tfm (X^{Tfm}/X^{+}(Sxr/+)). × 300.

Fig. 3. Epididymis of a sex-reversed control animal for comparison (X^{+}/X^{+}(Sxr/+)). × 300.

of such an animal suggest that in the Tfm heterozygote the flat segments represent androgen-insensitive X^{Tfm} cells, while the normally differentiated epithelium represents androgen-sensitive X^{+} cells.

Out of the 21 Tfm heterozygotes examined histologically, in 19 the mosaic pattern was found bilaterally and all over the epididymis, with a constant proportion of flat and high cells in each animal. Quantitative differences, however, were evident between individual animals. Though the number and size of different clones have not yet been evaluated exactly, the number of X^{Tfm} cells present in the epididymis seems to correspond to the development of abnormalities in the rest of the sex organs, as well as to the variegated phenotype produced by the X-linked coat colour genes. In 2 animals, both epididymides were made up of differentiated cells only. Since they also appeared to be otherwise normal males, the possibility of the Tfm mutation having been lost by crossing-over in the mother cannot be excluded.

The electron microscopic observations are summarized in Table 1. There are certain morphological features common to X^{+} and X^{Tfm} cells. The two cell types have similar basal lamina and surrounding connective tissue layers (Fig. 4). In respect to cell contacts, no differences between X^{+} to X^{+}, X^{Tfm} to X^{Tfm}, and X^{+} to X^{Tfm} cell connexions could be detected (Fig. 5). Basal cells, undifferentiated cells which do not extend to the lumen, were present in high columnar as
Table 1. Comparative electron microscope observations of X+ and X_Tfm epididymis cells

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<thead>
<tr>
<th></th>
<th>X+ cells</th>
<th>X_Tfm cells</th>
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<tr>
<td>Nucleus</td>
<td>Round or ovoid</td>
<td>Small, rather</td>
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<tr>
<td></td>
<td></td>
<td>condensed chromatin</td>
</tr>
<tr>
<td>Golgi complex</td>
<td>Elaborate</td>
<td>Small</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>Extended cisternae</td>
<td>Many free ribosomes</td>
</tr>
<tr>
<td>Stereocilia</td>
<td>Long and numerous</td>
<td>Few small protrusions</td>
</tr>
<tr>
<td>Coated vesicles</td>
<td>Abundant</td>
<td>Few</td>
</tr>
<tr>
<td>Multivesicular bodies</td>
<td>Present</td>
<td>Not present</td>
</tr>
<tr>
<td>Cell contact</td>
<td>Regularly developed</td>
<td>Regularly developed</td>
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well as in flat epithelial segments. Thus it can be said that the X_Tfm cells have, in common with the normal X+ cells, all the structural requirements necessary for full integration into the epithelium and organ structure.

Other features, such as height of the epithelium, and nuclear, mitochondrial and Golgi complex size, show clear quantitative differences between the two cell types, indicating an active, stimulated state in the X+ cell, and an inactive, dormant situation in the X_Tfm cell.

Quantitative differences seem to exist in the formation of stereocilia, and in the ultrastructural evidence of resorptive function. The latter is represented in the X_Tfm cells by some coated vesicles confined to the region immediately beneath the surface membrane; they do not extend further down into the cell, and no multivesicular bodies (the site for further processing of coated vesicles) are formed.

Qualitative differences exist in the elaboration of the endoplasmic reticulum.

Figures 4, 5

Fig. 4. Electron micrograph of the cellular mosaic, showing differentiated X+ cells at the left and undifferentiated X_Tfm cells at the right. The arrow indicates the borderline between the two cell types. × 3640.

Fig. 5. As Figure 4. The differentiated X+ cells (left side) contain coated vesicles in the apical cytoplasm and extended endoplasmic reticulum with few adherent ribosomes in the cytoplasm below. In the undifferentiated X_Tfm cells (right side) only a few coated vesicles are found, immediately beneath the surface membrane. The cytoplasm is filled with free ribosomes and short segments of rough endoplasmic reticulum. Both cell types are regularly connected by a zona occludens, a zona adherens and desmosomes. × 26600.
$X^+$ cells contain characteristic extended cisternae sparsely lined with ribosomes (Fig. 5). Perhaps in this special type of endoplasmic reticulum, which is intermediate between the rough and the smooth forms, a specialized product of the epididymis cell is made. The extended cisternae are missing in $X^{Tfm}$ cells. Instead, the $X^{Tfm}$ cytoplasm is filled with free ribosomes, characteristic of undifferentiated cells. Since extended cisternae always are found in $X^+$ cells, but never, not even in small numbers, in the $X^{Tfm}$ cells, this criterion turned out to be the most decisive for distinguishing the two cell types. Another structure sometimes present in $X^{Tfm}$ cells but not in $X^+$ cells are basally located large dense bodies or lysosomes. They tend to appear in groups of neighbouring flat cells, while other segments remain free of them. Similar dense bodies were described by Flickinger (1969) in the embryonic Wolffian duct of the rat.

**DISCUSSION**

Electron-microscopic study of the mosaic found in the epididymis of sex-reversed $Tfm$ heterozygotes has shown that expression of the $X^{Tfm}$ or the $X^+$ chromosome leads to the presence of two clearly defined cell types, respectively an undifferentiated and a normally differentiated epithelial cell. Intermediate forms do not exist. Thus, the mosaic in the epididymis lends itself to further studies of X-inactivation on a cellular and clonal basis.

From this study of the mosaic, at least two different mechanisms for the action of androgens during embryogenesis can be suggested. Androgens keep the Wolffian duct alive, and maintain its epithelial structure; both $X^{Tfm}$ and $X^+$ cells respond to this action. Secondly, androgens induce cytoidifferentiation; to this action $X^+$ cells are able to respond, but not $X^{Tfm}$ cells.

For an explanation of the fact that $X^{Tfm}$ cells can participate in the trophic effect of androgens, the following possibilities may be considered. (1) Metabolic co-operation by neighbouring $X^+$ cells. Since rather large $X^{Tfm}$ segments were observed, showing no signs of deterioration in their central portions, the trophic effect does not seem to be mediated by $X^+$ epithelial cells. But underlying $X^+$ mesenchymal cells may be responsible for the effect. In any case, metabolic co-operation (or, in a broader sense, interaction of the two cell types during embryogenesis) seems to be the most interesting aspect of the model - not only in the epididymis but also in the other sex organs. (2) The possible production of $Tfm$-independent androgenic hormones induced by the $Sxr$ mutation has to be kept in mind, so long as the mechanism of the sex reversal is not known. (3) The expression of $Tfm$ may be altered by a changed 'controlling element'.

This new genetic variant affecting expression of $Tfm$ in the mouse has been described by Ohno, Christian, Attardi & Kan (1973). In $X^{Tfm}/Y$ mice carrying a changed ‘controlling element’ on their $X^{Tfm}$ chromosome, an undifferentiated Wolffian duct rudiment is present which is missing in the original $X^{Tfm}/Y$. It is true that one of the two $X^{Tfm}$ chromosomes propagated in our stock also
X-inactivation pattern

allows for the presence of some Wolffian duct remnants in the corresponding
$X^{Tfm}/Y$, though this effect is much less pronounced than that found by Ohno’s
group. The other $X^{Tfm}$ chromosome produces the original $X^{Tfm}/Y$ phenotype.
Since in the sex-reversed $Tfm$ heterozygotes described here the mosaic in the
epididymis developed equally well from both $X^{Tfm}$ chromosomes, we do not
think that a changed ‘controlling element’ is the basis for the survival of the
$X^{Tfm}$ cells in the epididymis. But this possibility deserves further consideration.

As outlined above, $X^{Tfm}$ cells are selectively insensitive to induction and
maintenance of the differentiated state by androgens. One component of this
androgen effect is the overall stimulation of the cell, another the induction of
specialized cell functions. Since the specific type of endoplasmic reticulum, in
the form of extended cisternae, is found exclusively in $X^{+}$ cells but not in their
$X^{Tfm}$ counterparts, it seems to be induced de novo by androgens. In contrast,
the presence of some coated vesicles in $X^{Tfm}$ cells and their abundance in $X^{+}$
cells indicates that the resorptive function of the epididymis is under stimulatory
control only. Therefore, one may speculate that this type of resorption is a
primitive kidney function, already expressed in the Wolffian duct before it is
exposed to androgens.

This work was begun when U. D. was a research fellow of the Max Kade Foundation, N.Y.,
at City of Hope, Duarte, Ca. We thank Dr S. Ohno for inspiring the genetic experiment. A
preliminary account was given at the Seventh International Congress of the ISDB, 1973.

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(Received 27 November 1973, revised 25 January 1974)