Opaque Eyes Developed in Transgenic Mice With T-Cell Receptor δ Gene

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Purpose. During the generation of transgenic mice (TGs) introduced with mouse T-cell receptor δ (TCR δ) gene, the authors found a TG line with corneal opacity that coincided with the presence of the transgene. The authors investigated the pathogenesis and molecular mechanisms of this corneal opacity in this line.

Methods. The pathologic features and pathogenesis of the corneal opacity in TGs were examined histologically using transmission and scanning electron microscopy, as well as light microscopy. DNA and RNA blot analyses were performed to examine the copy number and the expression of the transgenes, respectively.

Results. Histologically, edema of the corneal epithelium and adhesion of the iris to the cornea were observed in adult TGs. In the developmental analysis, the authors first observed relative hypoplasia of the ciliary body on day 18 of gestation and dysgenesis of the anterior chamber angle from postnatal day 2. Corneal opacity was observed from postnatal day 8, coinciding with the histologic vesicular change of the epithelium. No inflammation was observed through its life. In the sublines that have different copy numbers of the transgene, the occurrence of the opacity depended on the copy number of the transgene. Expression of the transgene in the thymus was consistent with the number of the introduced transgene.

Conclusion. In a TCR δ TG line, the overexpression of transgenes coincided with abnormal development of the ocular anterior segment and the corneal opacity. Pathogenesis is described, and possible molecular mechanisms are discussed. Invest Ophthalmol Vis Sci. 1995;36:467–477.

Two distinct subpopulations of T cells, αβ and γδ T cells, have been identified based on the expressed antigen receptors. Although γδ T cells are present as a minor (1% to 5%) subpopulation in the peripheral blood and thymus of adult mice, they predominate (50% to 100%) within the epithelia, such as in the epidermis and the small intestine. During the last few years, some clues have been obtained pertaining to possible functions of γδ T cells. They may represent a primary and critical defense function within animals, and they are found at sites at which foreign invaders would be most likely to intrude first. More recently, a role of γδ T cells in autoimmunity also has been proposed.

We found that a transgenic mouse (TG) line Kassumime (KSM), which was introduced with the mouse rearranged T-cell receptor δ (TCR δ) gene, exhibited opaque eyes. This anomaly was transmitted to their offspring, and transgenes were always observed in these mice. In this article, we investigate the pathogenesis of opaque eyes and the relationships between the eye disorder and the transgene expression.

METHODS

Transgenic Animals

A TG line, KSM, was established during the generation of a series of T-cell receptor (TCR) transgenic mice. The structure of the transgene is shown in Figure 1.
The transgene, which was isolated from a γδ T-cell hybridoma, contained a productively rearranged V₁DJ₂C₈ gene known to be dominantly expressed in early fetal thymocytes and dendritic epidermal cells. The transgene also contained its promoter and enhancer regions for γδ T cell-specific expression. Tail DNA was screened by DNA blot analysis using a part of the transgene as a probe for TG analysis. We used 25 TGs, 49 eyes for clinical observation of the adult; 13 TGs, 25 eyes for histopathologic observation of the adult; and 44 TGs, 72 eyes for developmental analysis in this study. As controls, non-transgenic littermates (NTGs) were used. All mice were handled in accordance with Shimane Medical University guidelines for animal experiments, which comply with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ophthalmologic Examination

We used a slit lamp to determine the site of opacity. The rose bengal test and fluorescein staining were carried out to identify degeneration and ulceration of the corneal epithelium, respectively.

Light Microscopy

Specimens were fixed with 10% formalin solution and embedded in paraffin according to the conventional method. Serial sections were stained with hematoxylin–eosin or the periodic acid–Schiff (PAS) reaction. From systemic histologic analysis, pathologic findings were obtained in the eye but not in other TG tissues, including the lacrimal gland, thymus, intestine, and skin (data not shown). Therefore, only findings in the eye are described in the Results section.
Transmission Electron Microscopy
The excised eyes were immediately put in 4% glutaraldehyde in 0.15 M phosphate buffer, pH 7.3, at 4°C. Specimens were postfixed with 1% buffered osmium tetroxide, dehydrated in a series of graded ethanol, and embedded in epoxy resin (Taab 812). Ultra-thin 70-nm sections were examined with a transmission electron microscope (TEM) (JEM 200CX; JEOL, Akishima, Japan) at 80 kV.

Scanning Electron Microscopy
The specimens were fixed and postfixed in a manner similar to transmission electron microscopy. These specimens were stained with 1% tannic acid solution,
followed by 1% buffered osmium tetroxide. After dehydration by a series of ethanol, they were critical point dried with liquid CO₂. They were sputter coated with gold and examined with a scanning electron microscope (SEM) (S-800; Hitachi, Tokyo, Japan) at 15 kV.

DNA and RNA Isolation and Analyses

Standard techniques of molecular biology, including DNA and RNA preparations, blotting, and hybridization, were performed according to the method of Sambrook et al. In DNA blot analysis, each genomic DNA was digested with EcoRI and hybridized with the radio-labeled DNA probe shown in Figure 1. The signal intensity of DNA blot was quantified by using a laser scanning image analyzer (GS-250, Bio-Rad, Tokyo, Japan). In RNA blot analysis, total RNA (10 µg each lane) was hybridized with the same probe used in DNA blot analysis and was rescreened with β-actin mRNA as a control.

RESULTS

Pathologic Features of the Opaque Eye in Adult TGs

All mice bearing the transgene, except some sublines (see Genetic Analysis of the KSM Line), showed "ground glass-like" opaque eyes. The opacity was already observed when the eyelids opened on postnatal days 13 and 14. The opacity in some TGs was observed in the peripheral region of the eye, whereas others showed the opacity on the whole surface (Fig. 2). No TG showed opacity limited to the center of the eye. Slit lamp observation revealed that the opacity was in the cornea, not in the lens. The anterior chamber tended to be shallow (data not shown).

With the rose bengal test, partial degeneration on the corneal epithelium was shown as positively stained spots in the opaque area. On the other hand, ulcers were not detected on the corneas with fluorescein staining (data not shown).

Light microscopically and electron microscopically, we examined the opaque eyes of adult TGs. The corneal epithelium was thinner compared with that of NTGs (Figs. 3A, 3B), and the epithelium became vesicular (Fig. 3B). TEM sections of the opaque eyes demonstrated the decrease in the number of the epithelial layers and large intercellular spaces of the corneal epithelial cells, but desmosomes were preserved (Figs. 3C, 3D). These findings demonstrate the edematous state of the corneal epithelium.

In the corneal endothelium of TGs, large, variously shaped cells were found by SEM observation (Figs. 3E, 3F). In addition, the packing density of the endothelial cells was significantly lower in TGs than in NTGs (data not shown). In one case, the retrocorneal membrane was identified as a result of the overproduction of the basement membrane (Fig. 3G). These findings suggest that the endothelial cells were damaged and that survived cells produced excess basement membrane.

Adhesion of the peripheral part of the iris to the cornea was also observed in all TG eyes (Figs. 3H, 3I). The processes of the ciliary body in TGs tended to be smaller than those in NTGs (data not shown). In one case, the retrocorneal membrane was identified as a result of the overproduction of the basement membrane (Fig. 3G). These findings suggest that the endothelial cells were damaged and that survived cells produced excess basement membrane.

Remarkable changes that suggest elevated intraocular pressure were not observed in the posterior segment. Although the affected areas of the cornea were varied among individuals and within individual eyes, the affected areas exhibited pathologic changes basically along the same line. The severity of pathologic features of the cornea and external opacity was almost parallel.

These findings, together with the nature of the transgene, led us to consider that certain inflammatory events around the ocular anterior segment before eyelid...
openings might be involved in the pathogenesis of these changes, although no infiltration or accumulation of the inflammatory cells was observed in the eye of adult TGs.

Developmental Analysis Revealed Abnormal Morphogenesis But Not Inflammation in the Eye

We next analyzed TGs during the prenatal and early postnatal period. The summary of developmental analysis was shown in Table 1. Unexpectedly, abnormal infiltration or accumulation of inflammatory cells were not observed through the development in TG eyes. Instead, some developmental or morphogenetic anomalies were identified in the ocular anterior segment, such as the ciliary body, trabecular meshwork, cornea, and iris.

Until embryonic day 16, no histologic difference was observed in the eye between TGs and NTGs. From
FIGURE 4. Histologic observations through development. (A) The anterior chamber segment in NTG on embryonic day 18 (×120, hematoxylin-eosin stain). (B) The anterior chamber segment in TG on embryonic day 18. The processes of the ciliary body and the fold between the iris and ciliary body (arrowhead) were smaller than those of NTG (×120, hematoxylin-eosin stain). (C) Hematoxylin-eosin stained section of the anterior chamber angle in NTG on postnatal day 6 (×120). (D) Hematoxylin-eosin stained section of anterior chamber angle in TG on postnatal day 6 (×120). (E, F) Schemes of (C, D), respectively. The bold double arrow in the scheme indicates the length of the trabecular meshwork, which was longer than that of the NTG. High insertion of the iris was observed. The ciliary body was hypoplastic. C = cornea; I = iris; CB = ciliary body; R = retina. (G) The cornea in NTG on postnatal day 8. The number of epithelial layers was two or three and the basal cells became larger in the perpendicular direction (×460, hematoxylin-eosin stain). (H) The cornea in TG on postnatal day 8. The epithelium was thinner than that of NTG. The basal cells remained flat (×460, hematoxylin-eosin stain). (I) The peripheral part of the cornea in TG on postnatal day 8. The vesicular change started to be observed (arrowheads) (×120, hematoxylin-eosin stain). (J) The cornea in TG on postnatal day 21. The extremely vesicular change was observed in all areas from the periphery to the center of the corneal epithelium (×80, hematoxylin-eosin stain).

TABLE 1. Pathologic Features of Kasumine Mice During Eye Development

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NTG = nontransgenic; TG = transgenic; NF = tissue has not formed.
T-Cell Receptor δ Transgenic Mice With Opaque Eyes

FIGURE 5. Abbreviated pedigree (A) and DNA blot analysis (B). (A) All TGs except for three mice (arrowheads, 1 to 3) had opaque eyes. All TGs with opaque eyes carried five to six copies of transgene per diploid. Three TGs without opacity carried a lower copy number of the transgene (LTG-2, one copy; LTG-1 and -3, two copies; LTG, low copy subline) (Fig. 5B).

In Figure 6, the abbreviated pedigree of the offspring of LTG-2 and -3 and the results of DNA blot analysis are shown. All hemizygous offspring derived from LTG-2 and -3 did not have the opaque eyes. The homozygous offspring derived from LTG-2 (two copies) did not have the opaque eyes, whereas all homozygous offspring derived from LTG-3 (four copies) had the opacity. Thus, a clear correlation is found between the copy number of the transgene per diploid but not the zygosity and development of eye opacity, and there appears to be a threshold between two and four copies.

Transgene Expression in a Copy Number-Dependent Fashion

We next examined the expression of the transgene in TGs by RNA blot analysis using the same probe as in DNA blot analysis. In TGs, expression of the transgene was demonstrated in the thymus but not in the eye, liver, or brain on embryonic day 18 and in adults (Fig. 7). Comparing the thymus on embryonic day 18, postnatal day 8, and in adults, the highest level of the transgene expression was detected on embryonic day 18, and the level of expression appeared to decrease during development after birth (Fig. 7).

We further investigated the relationship between the level of the transgene expression and its copy number in the thymus of several sublines on embryonic day 18. The expression of the transgene was detected in the thymus of all TGs. The level of transgene expression in TG sublines was much higher than the endogenous TCR δ gene in NTGs and was proportional to the introduced copy number (Fig. 8).

DISCUSSION

In this study, we examined a TCR δ transgenic mouse line that develops corneal opacity to elucidate its pathogenesis and possible contribution to transgene expression. Histologic observations of adult TGs suggested that corneal opacity might result from inflammatory events in the past. However, developmental analyses demonstrated that there were no abnormal inflammatory cells in the eye in the prenatal and early postnatal periods. Instead, dysgenesis of the iridocorneal angle structures, followed by corneal edema, was observed in the prenatal and early postnatal days. Genetic, DNA, and RNA blot analyses showed that the development of pathologic features corresponded to the level of transgene expression, which correlated to the copy number of the introduced transgenes.
FIGURE 6. The abbreviated pedigree and DNA blot analysis of the offspring of LTG-2 and -3. All hemizygous offspring derived from LTG-2 and -3 (one or two copies of the transgene in total) (lanes A, C–J, N, P, R–U) did not show the opacity. The homozygous offspring derived from LTG-2 (two copies) (lane B) did not have the opacity, whereas all homozygous offspring derived from LTG-3 (four copies) (lanes M, O, Q) had the opacity. ■, homozygous male mouse; ●, homozygous female mouse.

FIGURE 7. RNA blot analysis in the liver, brain, thymus, and eye. In TGs, expression of the transgene was demonstrated in the thymus but not in the eye, liver, or brain on embryonic day 18 and in adults. Among the thymus on embryonic day 18, postnatal day 8, and in adults, the highest level of the transgene expression was detected on embryonic day 18, and the level of expression appeared to decrease during development after birth.
Mechanisms for Corneal Opacity

In TG eyes, some abnormal features were identified during the development of the ciliary body, iris, and cornea. Possible relationships among these pathologic findings are shown in Figure 9. The first characteristic feature during development of TGs was hypoplasia of the ciliary body. During eye development, it has been suggested that the ciliary body plays important roles in the formation of the iridocorneal angle and cornea through components in the aqueous humor. Hypoplasia of the ciliary body may, therefore, interfere with development of the anterior chamber segment. A longer trabecular meshwork could disturb the outflow of aqueous humor. As a result, high intraocular pressure may damage the corneal endothelium and induce epithelial edema. This edema appears to be the direct cause of the eye opacity in the KSM line. Changes in stromal collagen fibril size and arrangement and in Descemet's membrane were not evident. These findings also suggest that it is unlikely that the stromal dysgenesis or endothelial dysfunction is the primary cause of the corneal opacity in the KSM line.

Possible Relationship Between Transgene and Corneal Opacity

As the cause of KSM line phenotype, there are two possibilities. One is the "gain of function" by expression of the introduced TCR δ gene, and another is...

Hypoplasia of the ciliary body
(decrease of humor production)

Dysgenesis of corneal epithelium
flat corneal epithelial cells fewer layers of epithelium

Dysgenesis of iridocorneal angle
high insertion of iris lower trabecular meshwork
(disturb out-flow of humor)
(increase of intracocular pressure)

Damaged endothelial cells
decrease of corneal endothelial cells large and variously shaped endothelial cells

Corneal edema
vesicular corneal epithelium dilatation of intercellular space

Peripheral corneal opacity

FIGURE 9. Possible mechanism for corneal opacity in the KSM line.

the "loss of function" by insertion mutation of an endogenous gene. However, based on the genetic analyses, the second possibility is unlikely because the development of the eye anomaly depended on the total copy number of the transgene but not the zygosity of the transgene insertion allele. It is, therefore, likely that opaque eyes in the KSM line are due to gain of function by the expression of the introduced TCR δ gene. Moreover, RNA blot analysis demonstrated that the level of the transgene expression was parallel with the copy number of the transgene. These results further suggested that overexpression of TCR δ gene above a certain threshold (expression from two to four copies per diploid) led to corneal opacity in mice.

The introduced TCR δ gene appears to be expressed under the appropriate regulation of its promoter because expression of the transgene is demonstrated in the thymus, where γδ T cells normally populate, whereas no expression was detected in other tissues in which γδ T cells do not normally distribute. The expression pattern of the transgene during thymus development in the KSM line also coincides with that of the endogenous TCR γδ genes. These findings suggest that the transgene is expressed only in γδ T cells in the KSM line.

The ciliary body and the iris are the candidate sites of the primary pathologic changes in the KSM line. A recent report has shown the possible relation-ship between the ciliary body and T-cell immunity. At this time, however, it remains unclear how overexpression of the introduced TCR δ gene, supposedly limited to γδ T cells, can lead to noninflammatory, developmental abnormalities in these tissues.

On the other hand, ectopic expression of the transgene below the detectable level on RNA blot analysis in the eye cannot be ruled out as the cause of pathogenesis. Because the transgene was overexpressed, albeit coincidentally, with the endogenous TCR δ gene, the regulation is not completely normal. Further, the expression level of the endogenous TCR δ gene was low in RNA blot analysis even in the thymus, which is a homogenous tissue with the highest ratio of γδ T cells to total cells. Therefore, it is possible that localized ectopic expression of the transgene at a functional level was undetectable by conventional blot analysis but that it contributed to the pathogenesis. In situ observations to examine this possibility are being conducted.

Key Words
corneal opacity, T-cell receptor δ, transgenic mice, anterior chamber segment, gain of function

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T-Cell Receptor δ Transgenic Mice With Opaque Eyes

T-Cell Receptor δ Transgenic Mice With Opaque Eyes