Androgen-Dependent Hereditary Mouse Keratoconus: Linkage to an MHC Region

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PURPOSE. To better understand the pathogenesis of hereditary keratoconus, an inbred line of spontaneous mutant mice with keratoconus-affected corneas (SKC mice) was established and studied with a multidisciplinary approach.

METHODS. Using a mutant mouse with corneas having a keratoconical appearance as the progenitor, an inbred line of SKC mouse was established by repeated sibling mating. Morphology, cell growth, apoptosis and protein expression of SKC mouse corneas were examined. Castration of males and androgen treatment for females were conducted to determine any androgen dependency of the phenotype. Linkage analysis was conducted to reveal the responsible or predisposing gene of SKC mouse keratoconus.

RESULTS. Corneas of the SKC mouse resemble those of human eyes with keratoconus. Both are conical and show similar corneal changes, including apoptosis of keratocytes and increased expression of c-fos protein. The SKC mouse phenotype was transmitted in an autosomal recessive manner, although it was observed almost exclusively in males. Intriguingly, female mice showed the phenotype when injected with testosterone, whereas male incidence of the phenotype diminished drastically when mice were castrated. Linkage analysis localized a predisposition locus to an MHC region on mouse chromosome 17, which includes a locus for the gene for sex-limited protein (Slp).

CONCLUSIONS. SKC mouse keratoconus is a potential model for a subset of human keratoconus, which is a disease entity with heterogeneous pathogeneses. Alternatively, SKC mouse keratoconus could be a model for other human or mouse-specific keratopathies. (Invest Ophthalmol Vis Sci. 2002;43:51–57)

A number of hereditary corneal diseases are found in humans, and the molecular pathogenesis of some of these has been clarified. Notably, responsible genes have been isolated in various kinds of hereditary corneal dystrophy,1 a subset of human keratoconus has been shown to be inheritable,2 a recent epidemiologic study suggests autosomal recessive heredity with major gene determination,3 and a gene locus on human chromosome 21 has been linked in a large keratoconus-affected family.4 In contrast, although numerous strains of mutant mice with hereditary cataract have been reported, few cases of mutant mice with hereditary corneal diseases have been reported until now. Herein, we report spontaneous mutant mice that have corneas with a keratoconical appearance. Corneas of these mice (SKC mice) resemble corneas in human keratoconus in various aspects, such as macroscopic appearance, expression of a transcription factor, and apoptosis of stromal cells (keratocytes). Intriguingly, the conical change in corneas in the SKC mouse is androgen dependent. The phenotype is found almost exclusively among male mice but also appeared in females when they were injected with testosterone, and it did not appear in male mice when they were castrated. Linkage analysis of backcrosses between SKC and A/J mice linked a predisposition gene(s) to the D17Mit32 and D17Mit34 loci, which are located in the major histocompatibility complex (MHC) region, close to the gene for sex-limited protein (Slp).

METHODS

Mice

SKC mice were developed and kept in-house. BALB/c mice were purchased from a local vendor and used for control, because the genetic background of SKC mice is BALB/c, at least in part. MSM mice, an inbred Mishima strain of Japanese wild mouse Mus musculus molossinus, were the kind gift of Kazuo Moriwaki (RIKEN BioResearch Center). Treatment of all animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Some female SKC mice were injected intramuscularly with 2 mg testosterone (Mochida Pharmaceutical Co., Tokyo, Japan) at approximately 4 weeks of age, and some male SKC mice of approximately 4 weeks of age were castrated under ether anesthesia. For a sham operation, the same procedure was performed, except for the removal of testes.

Macroscopic Observation

Eyes were macroscopically observed and severity of keratoconus was rated. Corneas that were cloudy but showed no apparent deformity were rated clinical score (CS) 1, corneas that showed any deformity were rated CS 2, corneas that were ruptured or showed severe deformity were rated CS 3. Keratoscopic examination was conducted using a keratoscope (Sun Contact Lens Co., Ltd., Kyoto, Japan).

Light Microscopy

Eyes were immersed in 10% buffered formalin overnight, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections were cut, mounted on glass slides, and deparaffinized. For histologic observation, sections were stained with hematoxylin and eosin.

Transmission Electron Microscopy

Eyes were removed under ether anesthesia and fixed with 4% buffered glutaraldehyde and 4% OsO4. Tissues were then dehydrated with a graded series of ethanol and embedded in Epon 812. Thin sections were taken, stained with uranyl acetate and lead citrate, and examined.
Scanning Electron Microscopy

Eyes were fixed with 2% glutaraldehyde and with 4% OsO4. Tissues were then dehydrated with a graded concentration of ethanol and isoamylacetate. Critical-point dried tissues were sputter-coated with platinum and examined.

Immunohistochemistry

Sections were immersed in 10 mM citrate buffer (pH 7.4) and treated with an 800-W microwave at boiling temperature for 8 minutes. Sections were then treated with 0.3% hydrogen peroxide in methanol for 30 minutes. Successively, sections were treated with normal goat serum for 10 minutes, followed by a rabbit polyclonal antibody against c-fos (diluted to 1:20, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Sections were then immunostained, by using a kit (Histofine SAB-PO; Nichirei, Tokyo, Japan) and 0.05% 3,3'-diaminobenzidine tetrahydrochloride-0.05% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6), according to the manufacturer’s recommendation.

Cytochemical Detection of Apoptosis

Frozen sections were cut and fixed in 10% buffered formalin for 10 minutes and then in ethanol-acetic acid (2:1) solution at −20°C for 5 minutes. They were then examined cytochemically to detect apoptosis. Peroxidase-based TdT-mediated dUTP nick-end labeling (TUNEL) was performed using a peroxidase in situ apoptosis detection kit (Apop-Tag; Oncor, Gaithersburg, MD), according to the manufacturer’s instruction.

BrDU Incorporation

Mice were injected intraperitoneally with 100 mg/kg body weight of 5-bromo-2-deoxyuridine (BrDU; Sigma, St. Louis, MO). After 24 hours, eyes were immersed in 70% ethanol and fixed overnight. Sections were cut as described earlier, and the incorporated BrDU was immunohistochemically visualized, by using a kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s recommendation.

Primary Culture of Keratocytes

Eyeballs were extracted from four mice of each group under ether anesthesia. The eight excised corneas of each group were then transferred to a sterile culture dish containing phosphate-buffered saline and chopped into pieces of approximately 1 mm3, which were transferred to RPMI-1640 containing 10% fetal bovine serum. After one week of incubation, cells were disaggregated with 0.5% trypsin-0.2% EDTA (Life Technologies, Gaithersburg, MD). Cell numbers were counted (Model Z1 counter; Beckman-Coulter Electronics, Fullerton, CA), and approximately 1 × 106 cells were seeded in 6-cm dishes. Disagggregation, cell counting, and reseeding were thereafter repeated once a week and observed during a period of 4 weeks.

Linkage Analysis

In the first set of linkage analyses, we used wild-type mice, which evolutionarily separated from laboratory mice approximately 1 × 109 years ago1 and thus have more informative polymorphic markers than do laboratory mice. F1 hybrid mice (obtained by mating SKC and MSM mice) were reciprocally backcrossed with parental SKC mice and were tested for the keratoconus phenotype at least every 2 weeks. In another set of experiments, F1 mice (SKC and A/J mice) were bred and backcrossed with parental SKC mice. Backcrosses judged to have the phenotype at least two times consecutively on separate days were deemed keratoconus positive. Backcrosses that did not show development of keratoconus for at least 4 months after birth were designated phenotype negative. Genomic DNA was prepared from kidneys, according to standard protocols.9 Genotyping with simple sequence length polymorphism (SSLP), using microsatellite markers, was performed by PCR with commercially supplied primer pairs under conditions recommended by the supplier of the markers (Research Genetics, Huntsville, AL).7 Annealing temperature was 55°C or 55°C. Amplified DNAs were separated on either 2% agarose gel (NuSieve; FMC BioProducts, Rockland, ME; or Spredex El 300 gels; Elchrom Scientific, Inc., Jamaica Estates, NY).

RESULTS

Establishment of Inbred SKC Mice

In 1995 we noticed the appearance of keratoconus-like corneas in a male mouse among a closed colony kept in our animal facility. F1 hybrids of this progenitor were backcrossed to the progenitor. One third of male backcrosses showed the corneal phenotype, whereas no females did. Repeating sibling mating more than 20 times, we established an inbred strain of SKC mice.

SKC Mouse Keratoconus Compared with Human Keratoconus

SKC mouse keratoconus often starts as cloudiness of the corneas, with the corneas bulging gradually outward to form a conical shape, as shown by macroscopic, keratoscopic, and scanning electron microscopic observations (Figs. 1A, 1B).

Male SKC mouse cornea showed various degrees of macroscopic changes, divided into three levels (Fig. 1A, numbers in right lower corners), changes that could be objectified by keratoscopic observation (Fig. 1A, inset). The most severely affected (level 3) corneas showed disruption of the surface, whereas the most lightly affected (level 0) showed only cloudiness. The typical conical shape of corneas of SKC mice was evidenced by scanning electron microscopic observation (Fig. 1B). With few exceptions, corneas of female SKC mice appeared to be normal, whereas those of testosterone-injected females showed keratoconus-like changes (described later). Histologic examination revealed that the central area of the corneas in male SKC mice was often thin, because of the decreased thickness of the stroma, although these corneas were sometimes edematous and thick (Fig. 1C). In addition, expression of c-fos, which has been shown to be enhanced in human keratoconus,8 was increased in kerocyte nuclei of corneas in the male SKC mouse when compared with those in female SKC mice and BALB/c mice (Fig. 2A). Although immunostaining for c-fos was also observed in epithelial cells, it was not localized to nuclei and thus may have been nonspecific staining.

These results show that corneas of SKC mice resemble human keratoconus, although there are some distinctions between the two.

Growth and Death of SKC Mice Corneal Cells

Because apoptosis of keratocytes is a feature of human keratoconus,4 we investigated whether apoptosis is involved in corneas in SKC mice, using transmission electron microscopy and histocytology. Electron microscopic examination revealed that many keratocytes (but not other cells) of corneas of SKC mice showed apoptotic features, including cell shrinkage and chromatin condensation and fragmentation (Fig. 2B), whereas those of female SKC mice and BALB/c mice of both sexes did not (data not shown). Consistent with this finding, apoptosis was confirmed in keratocytes of male SKC mouse keratoconus corneas using the TUNEL technique, but not in corneas of BALB/c mice (Fig. 2C) or in female SKC mice (data not shown).

We next investigated mitotic activity in corneal cells by examining BrDU incorporation. Basal layer epithelial cells of BALB/c mice and female corneas of SKC mice incorporated BrDU at both peripheral and central regions, as is the case in rabbit corneal epithelium.10 However, the central region in
male corneas of SKC mice incorporated it very weakly (Fig. 2D), and mitotic activity in keratocytes of corneas of all examined mice was virtually nonexistent.

Next, we examined cell growth in vitro using a primary culture system: Consistent with the fact that the used culture medium was not supplemented with any growth factors required for growth of epithelial or endothelial cells, most of the observed cells were judged to be keratocytes, when using their fibroblastic feature as the criterion. Proliferation activity of these cells was roughly the same in female SKC mice and BALB/c mice of both sexes, but higher in male SKC mice (Fig. 2E).

Androgen Dependency

SKC mouse keratoconus was observed almost exclusively in sexually mature males. We have so far found only three females with the keratoconus phenotype. Despite this fact, the phenotype is inherited in an autosomal recessive manner, which led us to postulate that the phenotype is androgen dependent. To test this hypothesis, we examined the effect of androgen on the SKC phenotype by administering androgen to female SKC mice and by castrating male mice. A single injection of testosterone (2 mg/mouse) at approximately 4 weeks of age caused keratoconus in most females (Fig. 3), and the symptom was sustained during an observed period (Fig. 3C), although it was weaker than with male SKC mice. In contrast, keratoconus did not develop in the majority of castrated SKC mice, and symptoms in the minority in which it did were weaker and unsustained (Figs. 3D, 3E).

Linkage analysis mapped the predisposition gene to an MHC region. In the first set of experiments, using wild MSM mice, only a small percentage of male backcrosses between the (MSM x SKC) F₁ hybrids and SKC mice showed keratoconus (Figs. 3F, 3G), and no significant linkage was found, possibly due to interference of suppressive modifier genes (see later description). In the second set of experiments, using laboratory mice A/J, keratoconus developed in approximately 50% of male backcrosses between (A/J x SKC) F₁ hybrids and SKC mice, and SSLP analysis using these backcrosses linked the phenotype to an MHC region on mouse chromosome 17 (Table 1, Fig. 4). Although the relation between phenotype and genotype is statistically highly significant \((P = 2.0 \times 10^{-11})\), the percentage of genotype homozygosity was below 70% in phenotype-positive mice, whereas that in phenotype-negative mice was approximately 35% (Table 1). This concordance between phenotype and genotype suggests that the linked locus represents the modifier gene rather than the responsible gene. The highest lod score was 9.77 and was assigned to markers D17Mit32 and D17Mit34. Several genes have been mapped in the vicinity of loci for these markers, including genes for histocompatibility 2 (H2), compliment 4 (C4), tenascin-X (Tnx), steroid 21-hydroxylase gene (Cyp21a1), sex-related protein (Slp), and peripherin 2 (Prph2; Fig. 4).
FIGURE 2. Enhanced expression of c-fos and apoptosis in SKC mouse keratoconus corneas. (A) Expression of c-fos in epithelial cells and keratocytes was enhanced in male SKC when compared with that in female SKC mice and BALB/c mice. (B) Electron microscopic observation revealed apoptotic keratocytes in male SKC mice. (C) The TUNEL method detected apoptosis in keratocytes of male SKC mice but not in those of BALB/c mice. (D) BrdU incorporation was diminished in the central area of SKC mouse corneal epithelium when compared with female SKC mice and male BALB/c mice. (E) Growth of keratocyte-like cells in primary culture was faster in male SKC mice than in female SKC and BALB/c mice. Scale bars, (A, C, D) 100 μm; (B) 2 μm.
Keratoconus is a relatively common corneal disease in humans, in which corneas show progressive ectasia and thinness, with scar formation at the center. As discussed earlier, genetic factors play a significant causative role in keratoconus. Curiously, except for one case report of a rhesus monkey, keratoconus had not been reported in nonhuman mammals, and

**DISCUSSION**

Keratoconus is a relatively common corneal disease in humans, in which corneas show progressive ectasia and thinness, with scar formation at the center. As discussed earlier, genetic factors play a significant causative role in keratoconus. Curiously, except for one case report of a rhesus monkey, keratoconus had not been reported in nonhuman mammals, and...
the lack of animal models has hampered progress in keratoconus study. Rodent models for hereditary keratoconus would be particularly valuable for genetic and molecular research on keratoconus, and this prompted us to seek a murine model for hereditary keratoconus. SKC mouse keratoconus described herein shares some macroscopic and histologic features with human keratoconus. 

Human keratoconus has been shown to enhance expression of some proteins, such as the collagenases, and transcription factors, such as c-Fos. Immunochemical study revealed the increased expression of c-fos in corneas of SKC mice, particularly in their keratocytes. Furthermore, as in human keratoconus, apoptosis of keratocytes was observed in corneas of SKC mice. These parallel aspects led us to conclude that SKC mouse keratoconus is a potential animal model for human keratoconus.

Linkage analysis located the predisposition locus in an MHC region, where several non-MHC genes are localized: Tnx, Prph2, Cyp21a1, and Slp. Deletion of Tnx was found in a patient with Ehlers-Danlos (ED) syndrome. However, this patient was not reported to have keratoconus, although ED syndrome is associated with keratoconus on rare occasions. In addition, SKC mice did not show symptoms similar to those of ED syndrome. Prph2 is a gene responsible for the retinal degeneration slow (rds) mutation, but retinal degeneration was not observed in the SKC mice. In contrast to these two genes, Tnx and Prph2, which probably are not involved in the pathogenesis of SKC mouse keratoconus, the two other genes, Cyp21a1 and Slp, may be relevant to the pathogenesis in terms of its androgen dependency.

One of the striking findings in this study is that SKC keratoconus appears almost exclusively in sexually matured males. Females exhibited keratoconus only exceptionally, but occurrence increased when androgen was injected. We considered that Cyp21 and/or Slp might play a role in this androgen dependency. Cyp21a1 encodes steroid 21-hydroxylase (21-OHase), whose deficiency causes congenital adrenal hyperplasia and hyperandrogenemia, and we previously reported the occurrence of androgen receptor in keratocytes, epithelial cells, and endothelial cells of mouse corneas. These results led us to investigate whether a different androgen level, one due to polymorphism of 21-OHase, might play a part in the SKC phenotype. However, we did not find any difference in serum testosterone levels between backcrossed mice, with or without keratoconus (data not shown), and we concluded that Cyp21 is probably not involved in the androgen dependency of keratoconus in SKC mice. The second gene, Slp, encodes sex-limited protein, which is expressed in tissues of adult males and is androgen-inducible in females. It is interesting to note that this protein has a protective role in mouse spontaneous lupus erythematosus, one of the autoimmune diseases that are often linked to the MHC region. Although there is no clear evidence as yet that autoimmunity is involved in the pathogenesis or exacerbation mechanism of SKC mouse keratoconus, some keratoconus in corneas in SKC mice, particularly in advanced cases, show lymphocyte and capillary infiltration (data not shown), which is consistent with the possibility of autoimmunity. We are currently exploring this possibility by examining the occurrence of autoantibody in SKC mouse serum.

An association between human keratoconus and human MHC, the histocompatibility haplotype, has been reported in some studies. Furthermore, frequent occurrence of autoantibody against corneal extract has been found in patients with keratoconus. Although human keratoconus is seen in both sexes, it appears usually only after puberty, suggesting
that sex hormones are involved in its pathogenesis. The higher prevalence in males (male-to-female ratio 1.5–2.0:1) reported in several epidemiologic studies supports this hypothesis.24,26,28,29 Thus, we see a distinct possibility that SKC mouse keratoconus represents a particular subset of human keratoconus, which is a disease entity with heterogeneous pathogene-

ses.30 However, there are some distinctions between SKC mouse keratoconus and the human, most notably, the fact that corneas in the SKC mouse often show cloudiness, which is unusual in keratoconus in the human. Therefore, SKC mouse keratoconus may represent some other human keratopathy or may be a mouse-specific keratopathy. In any case, it provides an excellent opportunity for a clearer understanding of corneal biology and pathology.

References