Karyologic studies on cells from rabbit cornea and other tissues grown in vitro

P. Sarkar,* P. K. Basu, and Irene Miller

Individual layers of the cornea as well as lung tissues of rabbit were cultured separately in vitro. The endothelial and stromal cells of the cornea maintained their distinctive tissue culture morphology in serial subcultures for a period of over 20 months, and in the case of one endothelial line, even in passages after thawing from storage at -79° C. These cell lines proved to be very suitable material for chromosome study. Normal karyotype of rabbit has been studied from some of these lines derived from the corneal and lung tissues. One cell line derived from the corneal endothelium was stored at -79° C. for over 3 months and subsequently thawed and subsequent passages after thawing showed heteroploid transformation of chromosomes, and subsequent passages, after thawing showed heteroploid transformation of chromosomes. The possible application of karyologic studies of corneal cells in ophthalmic research is discussed.

Recent advances in the field of mammalian cytology, particularly the use of tissue culture techniques for the study of chromosomes, may be profitably applied in ophthalmologic research. The karyology of the corneal cells would be of considerable interest if the number and morphology of the chromosomes could be utilized as a tool for the study of the fate of corneal grafts as well as the pathogenesis of abnormal cellular growths resulting from certain corneal lesions. Furthermore, routine chromosomal studies would be essential in maintaining cell lines in vitro for virologic and other investigations on the corneal tissue.

From the Department of Ophthalmology, Faculty of Medicine, University of Toronto, Toronto, Ontario.

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*Department of Botany, University of Toronto.

Karyologic studies of the ocular tissues have been very limited. Mitotic figures have been studied in histologic preparations of the corneal and lens tissues.\textsuperscript{1-5} Radioisotopes have been used recently to study the deoxyribosenucleic acid (DNA) synthesis in the lens epithelium and corneal cells.\textsuperscript{6,7} The sex chromatin, which is a heterochromatic mass found in the female nucleus, has been identified in ocular tissues.\textsuperscript{8} Basu and co-workers\textsuperscript{9} have studied the fate of corneal grafts in cats with the sex chromatin as a biologic cell marker. However, no detailed account of the chromosomes in ocular tissues, particularly of the cornea, could be found in the literature. So far as we are aware, no attempt has been made to study the chromosomes in the stromal and endothelial cells of the adult mammalian cornea.

The present investigation was undertaken to study the karyology of normal corneal tissues of mammalian species, and also to establish cell lines in vitro from the...
different layers of the cornea. A number of species have been used in our laboratory for the supply of corneal tissues, namely, rabbit, mouse, Chinese hamster, and cat. However, the present report is confined to the karyologic studies of cells from the adult rabbit cornea cultivated in vitro. The main observations relate to: (a) the morphology of cells grown in vitro from the different layers of the cornea, (b) the normal karyotype of rabbit as studied in cells from short-term cultures of the corneal and lung tissues, and (c) the heteroploid transformation in one cell line derived from the corneal endothelium.

Materials and methods

For the tissue culture and subsequent cyto逻辑 examination of corneal cells, the eyes of male and female adult albino rabbits were used. The animals were sacrificed and the eyes removed immediately and submerged for 10 minutes in Hanks' buffered saline solution which contained 500 units of penicillin and 250 units of streptomycin per milliliter. For the comparison of the karyotype, tissues from the lung of the same animal were also cultured.

The procedure employed for the separation of the individual layers of the cornea for culturing in vitro was essentially that of Stocker and co-workers. The technique used for growing a tissue explant and the subsequent subculturing of the cells from the primary outgrowth was as follows: Tissue fragments were planted in a Carrel flask containing a smear of chicken plasma and clotted with chick embryo extract. Two milliliters of Eagle's basic medium with 10 per cent sheep serum, and 100 units each of penicillin and streptomycin per milliliter were used for culturing the cells.

When there was adequate cellular outgrowth, tissue explants were removed from the flask and the cellular outgrowths exposed to a 0.25 per cent trypsin in phosphate buffered saline solution (pH 7.0) for 10 to 15 minutes at room temperature.

The cell suspension thus obtained was centrifuged at 1,100 r.p.m. for 3 minutes and the supernatant removed. The cells were then resuspended in growth medium and transferred to new culture containers. The cultures were maintained in an incubator at 37°C. with passages at weekly intervals.

For the study of chromosomes, following trypsinization of a subculture, the cells were transferred to a 100 mm. Petri dish which contained two 75 by 25 mm. slides in 15 ml. of tissue culture medium. The Petri dish was incubated in a humidified atmosphere containing 5 per cent CO₂. In about a week there was sufficient cellular growth on the slides. Ten micrograms of colchicine per milliliter of medium were added to each Petri dish 10 to 16 hours before the slides were removed. The cells attached to the slides were then fixed and stained by the air-drying method of Rothfels and Siminovitch.

Samples from one of the cell lines of endothelial derivation were frozen for storage after the seventh passage. For this purpose the cells were trypsinized and suspended in a culture medium containing 15 per cent glycerin. Two milliliters of this cell suspension were introduced in a 10 ml. ampule and "shell-frozen" in a mixture of dry ice and acetone, and stored at -79°C. After storage for 3½ months, one such sample was thawed rapidly in a water bath at 37°C. The thawed cells were found to be viable and successful cultures could be initiated from them. They were subcultured in 8 ounce bottles by trypsinization at weekly intervals for a period of over 1 year. This is the line referred to later as the established endothelial line. Chromosome studies were made on cells of this line at the fourteenth and later passages after thawing.

Results

The morphologic patterns of the cellular outgrowths from the epithelium, stroma, and endothelium of the rabbit cornea were recognizably distinct from one another (Figs. 1-3).

The primary epithelial outgrowth could be preserved for approximately 10 days. Our attempts to subculture the epithelial cells of the rabbit cornea have so far been unsuccessful.

The stromal and endothelial cells maintained their respective in vitro morphology after many passages (Figs. 4 and 5), and in the established endothelial line, even after thawing and subsequent subculturing (Fig. 6).

The normal karyotypes of male and female rabbits were determined from cells in short-term cultures (at passage 2) derived from the stroma and endothelium of the cornea as well as from fibroblasts of the lung (Figs. 7-10). The karyotype studied from the cells of the cornea and

*Karyotype, the characteristic chromosome complement of any individual or group of related organisms.
Figs. 1-3. Primary cellular outgrowths from corneal tissues of rabbit cultured in vitro: Fig. 1, epithelial; Fig. 2, stromal; Fig. 3, endothelial. (×75.)

Figs. 4-6. Endothelial cells from rabbit cornea cultured in vitro: Fig. 4, at passage 2; Fig. 5, at passage 22; Fig. 6, at passage 7 after thawing from storage at -79° C. (×195.)

of the lung from the same animal was identical. In our experience, the corneal tissues were very suitable material for chromosome studies.

The number of chromosomes in the rabbit is 44, including the X and Y in the male and the two X's in the female. Fig. 10 shows paired chromosomes from a typical nucleus of a male. It can be seen that although every chromosome cannot be unequivocally identified the chromosomes fall into distinct groups as regards total length and arm-ratio. The Y chromosome in the male is similar to the two smallest
Figs. 7-9. Diploid chromosome complement of rabbit in cells cultivated in vitro: Fig. 7, from stromal cells of cornea (male); Fig. 8, from lung cells (male); Fig. 9, from stromal cells of cornea (female). (x1,200.)

Fig. 10. Karyotype of male rabbit. Camera lucida drawing. (x2,600.)
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Fig. 11. Distribution of chromosome numbers in cells of the altered line derived from corneal endothelium of rabbit. (One cell with 124 chromosomes is not included in the histogram.)

Autosomes. The X is a medium-sized chromosome with arm-ratio of approximately 1 to 3. The chromosomes numbered 18 and 19 (Fig. 10) are distinct from the rest in being almost telocentric.*

In the established endothelial cell line accurate chromosome counts were made after the fourteenth passage following thawing. Thirty-nine well-spread colchicine metaphases were studied. The cells showed a range of chromosome numbers from 48 to 124, of which about 85 per cent were at a hypertriploid† level with the mode around 70 (Fig. 11). No cells with the diploid number of chromosomes were found. Apart from this change in chromosome number, these cells also showed alterations in chromosome structure, the most conspicuous being the presence of dicentric‡ chromosomes. About 20 per cent of the cells had dicentric chromosomes and one cell had three of them (Fig. 12). Furthermore, there was a reduction in the number of telocentric chromosomes in these aneuploid§ cells as compared to the diploid karyotype. The two pairs of small, telocentric chromosomes characteristic of rabbit (Fig. 10) were either absent or represented by only one or two such chromosomes in the altered cells.

A second batch of slides was prepared from the same endothelial line about 4 months after the first chromosome study was made. These preparations showed chromosome numbers grouped close to 70. Cells with chromosome numbers in the lower range of 40’s and 50’s, as well as the occasional cells with very high chromosome numbers (more than 100), seemed to have been completely eliminated. Fig. 13 shows a metaphase with 70 chromosomes in one of the cells from the second set of preparations.

It is noteworthy that in spite of the heteroploid* transformation of the chromosomes the morphology of these cells remained unaltered as compared with that of the primary outgrowth and serial subcultures of the endothelium (Figs. 3-6).

Discussion

In the primary culture, the epithelial, stromal, and endothelial cells of the cornea were distinct from one another in their morphologic characteristics.

Because of our failure to subculture

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*Telocentric chromosome, a chromosome with a terminal centromere (or kinetochore).
†Hypertriploid cell, a cell with a chromosome number slightly greater than three times the haploid (gametic) number.
‡Dicentric chromosome, a chromosome with two centromeres (or kinetochores).
§Aneuploid cell, a cell not containing an exact multiple of the haploid (gametic) number of chromosomes, one or more chromosomes being represented more or less times than the rest.

*Heteroploid cell, a cell with a chromosome number which is not an exact multiple of the haploid (gametic) number.
epithelial cells, we were unable to compare the morphology of the subcultured epithelial cells with that of the primary outgrowth from the explant. However, it was possible to subculture the stromal and the endothelial cells; they retained their distinctive morphology in serial passages. Endothelial cells remained viable during storage for 3½ months at -79°C.

Our observations on the chromosomal changes in the established endothelial cell line are in agreement with the usual experience of tissue culture workers handling mammalian cells. Almost all such cultures, once they become established, show unbalanced numbers of chromosomes, a phenomenon which has been termed heteroploid transformation.

This particular endothelial cell line was not checked for chromosomal abnormalities during the early period of its growth. Hence, it was impossible to determine the number of passages after which the altered cells began to appear and eventually replace cells with normal chromosome complement.

In spite of very careful handling, there have been cases of contamination of one cell line with another in various laboratories. In our case, two possible sources of contamination, viz., live cells in chicken embryo extract used for clotting the original explant, and in sheep serum used in the growth medium can safely be excluded because of the very different chromosome morphology of these two species. In our altered line, the karyotypes, particularly in the nuclei with chromosome numbers between 48 and 50 of which there were a few in the first batch of slides, were very similar to the normal karyotype of rabbit. Furthermore, at the time of the chromosome study the cells showed morphologic features characteristic of the endothelial cells. Thus, it may be assumed that the cell line we are carrying is genuinely of rabbit derivation and did not arise from contaminating cells of other species.

An interesting aspect of heteroploid transformation may be discussed here. Almost everyone studying established cell lines derived from normal tissues encounters heteroploidy. It is now speculated whether once a cell population becomes heteroploid it acquires malignant properties since neoplastic cells are usually heteroploid.
Ability to produce tumors upon inoculation into suitable hosts is a widely used method of estimating neoplastic properties. In recent experiments, Rothfels and associates induced tumors by inoculating strains of heteroploid mouse cells in C3H mice. We intend to undertake transplantation experiments using transformed cell lines derived from corneal tissues to test their tumor-forming potencies in the eye and brain of experimental animals.

Unrestricted cellular growths may be assumed to be due to a breakdown of the balance between the growth potential of a cell population and its environment. The normal aqueous humor is known to have an inhibitory action on cellular growth. However, under certain circumstances, corneal cells will grow in the anterior chamber in the form of epithelial downgrowth or fibrotic membranes in cases of abnormal corneal wound healing. Conceivably, this could be due to an adaptive genetic transformation of these cells in a new environment. If so, such pathogenesis could be better understood if it were possible to study the cytology of these cells.

Recent researches tend to show that a large proportion of the undamaged cell population of a corneal graft is able to survive without being replaced by the host cells. The chromosome constitution of the surviving cells of the graft is not known. It is not impossible that in the process of establishing itself, the grafted tissue would undergo genetic changes as a means of adaptation to the new environment, analogous to the behavior of a cell population in tissue culture. If any such alteration can be recognized at the chromosomal level, a karyotypic study of the grafted tissues would offer important clues regarding the biologic individuality of the transplants.

We are hopeful that, with karyologic studies of the cells from grafted tissues, it will be possible to determine the fate of the auto-, homo-, and heterologous grafts more definitely.

Addendum

After sending this paper to the press we came across a publication by Melander on the chromosome complement of the rabbit. We also received a personal communication (to P. Sarkar) from Dr. Johanna Clausen of the University of Minnesota together with her unpublished data on the rabbit chromosomes. The karyotypes of the rabbit as worked out by these investigators are in close agreement and differ with that shown in Fig. 10 in the present publication only in having 3 pairs instead of 4 pairs of almost telocentric small chromosomes.

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