A Scanning Electron Microscopic Study of Macrophages in the Lens Cavity of the Mouse Embryo.

Koji NISHITANI, Kazunobu SASAKI

Department of Anatomy, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-0192, JAPAN

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ABSTRACT. During early lens formation in mouse embryonic eyes, macrophages appear not only around the lens primordium but also inside the lens. At the lens vesicle formation stage, macrophages were randomly scattered within the cavity but, shortly after that stage, all of them specifically attached only to the inner surface of the anterior wall of the lens vesicle. At the primary lens fiber elongation stage, numerous small epithelial cell fragments were expelled from the lens epithelium into the cavity, and macrophages showed active phagocytosis and removed them from the cavity. In the early lens formation stage, macrophages move into the lens cavity, where they play an essential role in removing cell debris derived from the lens epithelium.

Key words ① **macrophage** ② **lens cavity** ③ **SEM** ④ **lens formation** ⑤ **lens epithelium**

Macrophages are known to play an important role in eye development in the mouse embryo[1)~6)]. By an immunohistochemical study using F4/80 monoclonal antibody, we previously showed that numerous macrophages appear and do not distribute uniformly in the lens cavity during early development of the mouse lens[7)]. To determine the precise localization of macrophages in the lens cavity, an SEM study is more informative than either immunohistochemical or TEM studies. The aim of our present SEM study was to confirm the localization, surface morphology and phagocytosis of macrophages in the lens cavity at the three-dimensional level.

MATERIALS AND METHODS

Animals

A total of 57 ICR mouse embryos were studied. An adult male and female were caged together overnight, and the next morning was taken as day 0 of pregnancy. Pregnant mice were killed by cervical dislocation at 10.5, 11, 11.5, 12, 12.5 and 13 days of gestation. At each gestational day, at least seven embryos were used for scanning electron microscopic observation.

Scanning Electron Microscopy

Fetal heads were midsaggitally cut with a razor blade, and then were immersed in phosphate buffered

西谷耕二，佐々木和信
e-mail:nishi@med.kawasaki-m.ac.jp

2.5% glutaraldehyde (pH7.4) at 4℃ for 2hrs. Two equally divided heads were embedded in a 1.5% agarose solution, which then was kept at 4℃ for coagulation. Subsequently, the fetal heads were cut saggitally through the centre of the eye anlage with ophthalmic scissors under a stereoscopic microscope. The sliced specimens were postfixed in phosphate-buffered 1% osmium (pH7.4) at 4℃ for 2hrs. After placement in 2% tannic acid, the fetal heads were re-immersed in 1% osmium at 4℃ for 1hr. After dehydration in graded ethanols, they were transported to t-butylalcohol for freeze-drying. The dried tissues were sputter-coated with osmium-Pt. The surface of the lens cavity was examined in a Field Emission Scanning EM (JSM-6340; JEOL) operated at an accelerating voltage of 15kV. To obtain the proportions of S- and F-type macrophages, a total of 103 macrophages in the lens cavities from nine fetuses between 11.5 and12.5 gestational days were observed.

Transmission Electron Microscopy

Tissues including the developing eyes of 12-day-embryos were immersed in a mixture of 4% paraformaldehyde and 5% glutaraldehyde (pH7.4) at 4℃ for 3hrs, and then they were postfixed in 1% osmium tetraoxide for 2hrs. After embedding in Epon 812, ultrathin sections were cut and impregnated with uranyl acetate and lead citrate to be observed in a JEM-2000EX electron microscope operating at 80kV.

These studies were approved by the Animal Research Committee of Kawasaki Medical School (No. 06-095) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

RESULTS

The lens placode as a lens primordium began to invaginate in mouse embryos at 10.5 gestational days. The lens vesicle began to form at 11 days, when the lens cavity became completely separated from the amniotic cavity. Due to the development of the primary lens fiber, the lens cavity gradually narrowed and became completely occluded at 13 days of gestation. During this process, numerous cell elements appeared in the lens cavity. Transmission electron microscopy showed the elements to be composed of two kinds of spherical bodies, large and small (Fig.1). The large spherical bodies, 10～15μm in diameter, were free macrophages, and the small spherical ones, 1～2μm in diameter, were fragments of lens epithelial cells of the anterior wall of the developing lens vesicle.

A. Localization of macrophages and epithelial cell fragments within the lens cavity

Lens Placode Invagination Stage

In the lens pit, as shown in Figure 2, numerous small spherical bodies of epithelial cell fragments were observed especially on the surface of epithelium surrounding the lens pore (Figs. 2a,b), and a few of them had been expelled into the lens pit. Epithelial cell fragments had either a smooth or a furrowed surface, on which small pits, about 0.2 μm in diameter, were occasionally seen (Fig.2c).

Lens Vesicle Formation Stage

The lens vesicle closed and detached from the surface ectoderm at the time the lens stalk disappeared.

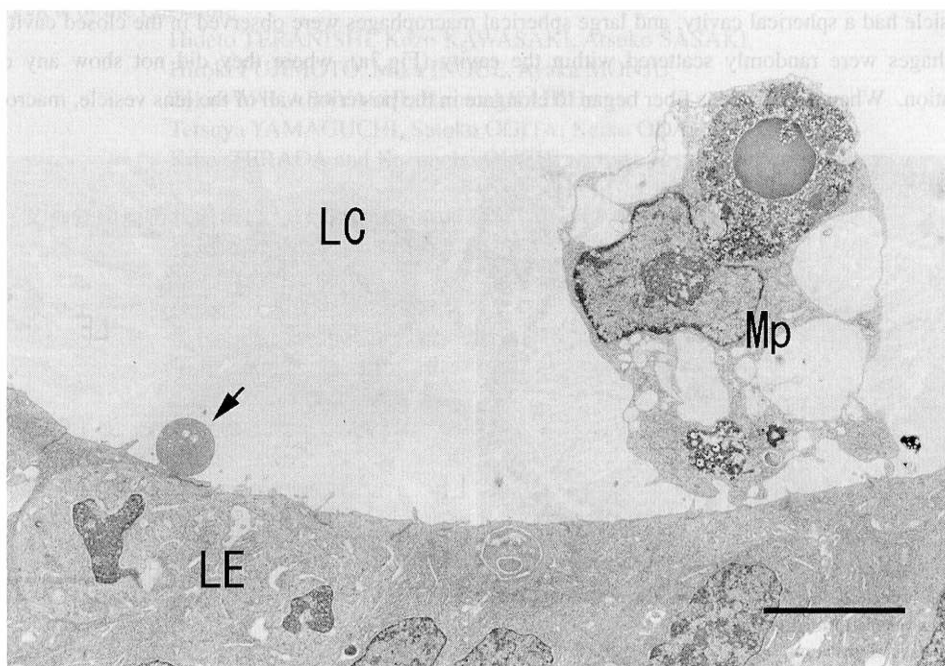

Fig. 1. Transmission electron micrograph of two kinds of spherical bodies in the lens cavity at 12 days of gestation.
Two kind of spherical bodies, large and small, are observed on the posterior surface of lens epithelium (LE) in the lens cavity (LC). The large spherical body is a macrophage (Mp) that contains numerous phagosomes in the cytoplasm. The small spherical body is an epithelial cell fragment (an arrow). Scale bar=5 μm.

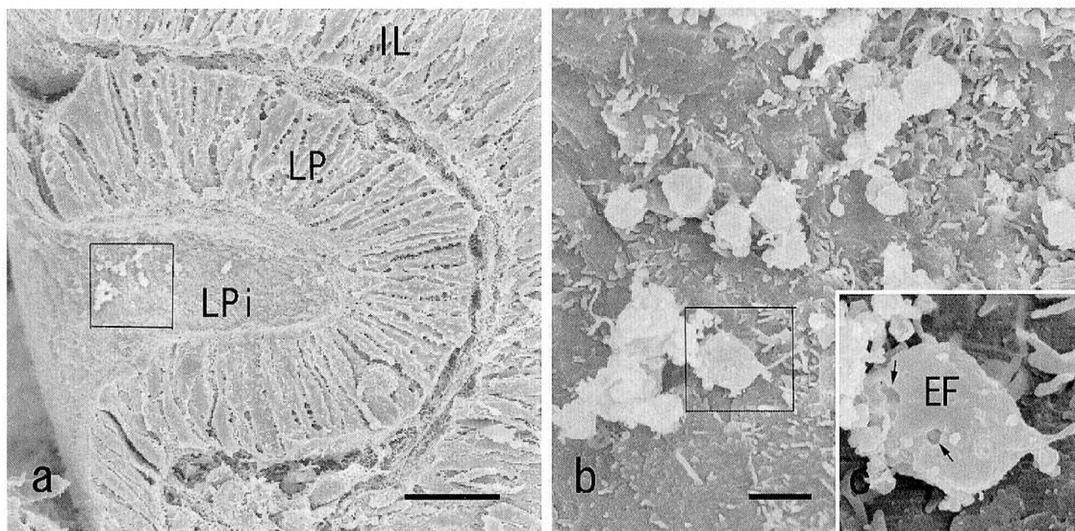

Fig. 2. Scanning electron micrographs of the lens placode invagination stage.

a) A low-power micrograph of the lens pit at 10.5 days of gestation.
The lens placode (LP) invaginates to form the lens pit (LPi).
IL: Inner layer of the optic cup; Scale bar=20 μm.

b) Surface of lens pit neck of framed area in a).
Numerous small round fragments, 1～2 μm in diameter, are seen on the surface. Scale bar=2 μm.

c) High-power micrograph of framed area in b).
Epithelial cell fragments (EF) are rather smooth with a few small pits (arrows) on the surface.

The vesicle had a spherical cavity, and large spherical macrophages were observed in the closed cavity. The macrophages were randomly scattered within the cavity (Fig.3a), where they did not show any definite localization. When primary lens fiber began to elongate in the posterior wall of the lens vesicle, macrophages

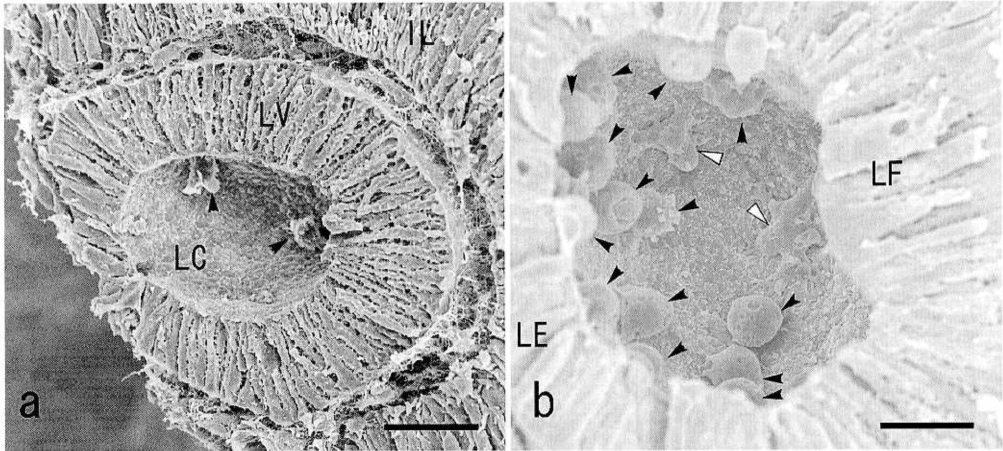

Fig. 3. Lens vesicle formation stage.

a) 11 days of gestation.
The lens vesicle (LV) forms a spherical lens cavity (LC). Macrophages (arrowheads) appear in the lens cavity.
IL: Inner layer of the optic cup; Scale bar=40 μm.

b) 11.5 days of gestation.
Numerous macrophages are seen on the inner surface of the anterior and lateral walls of the lens vesicle. Arrowheads show spherical macrophages, and white arrowheads, flat macrophages.
LE: Lens epithelium, LF: Lens fiber; Scale bar=20 μm.

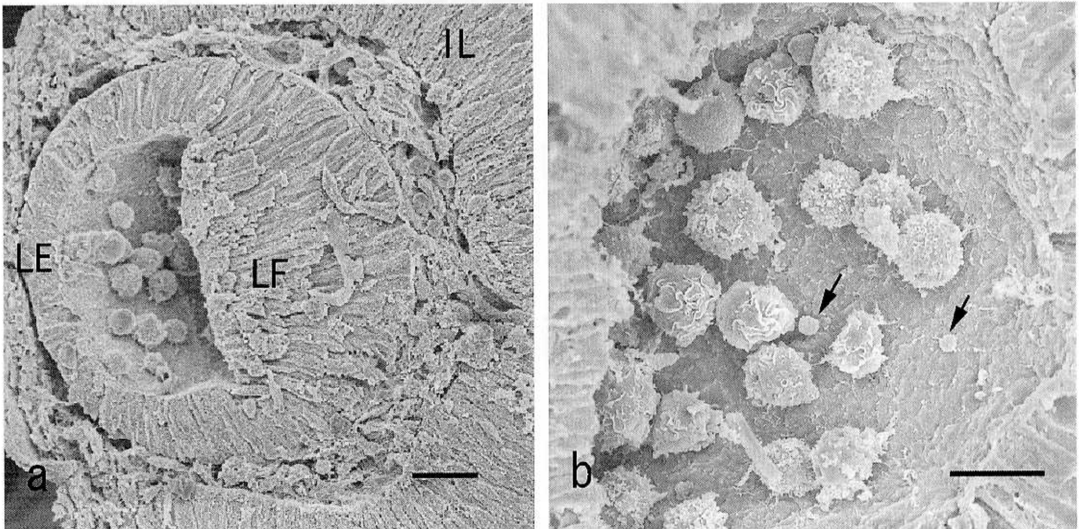

Fig. 4. Lens cavity at the early primary lens fiber elongation stage.

a) 12 days of gestation.
Due to primary lens fiber (LF) elongation, the lens cavity becomes concavo-convex in shape. Numerous macrophages attach to the inner surface of the anterior wall of the lens vesicle, i.e., lens epithelium (LE). Most of them are spherical in shape.
IL: Inner layer of the optic cup; Scale bar=25 μm.

b) Inner surface of the anterior wall of a frontal sectioned lens vesicle.
Two small epithelial cell fragments (arrows) are seen among numerous macrophages. Scale bar=10 μm.

appeared to move and attach to the inner surface of the anterior and lateral walls of the lens vesicle on which small round epithelial fragments appeared (Fig.3b).

Primary Lens Fiber Elongation Stage

Due to lens fiber development, the cavity became convexo-concave in shape. During this process, all the lens macrophages were specifically localized, being attached to the surface of the anterior wall of the lens vesicle (Figs.4a,b,5a), where epithelial cell fragments were scattered. When the cavity completely closed, no lens macrophages could be seen at all within the lens (Fig.5b).

B. Surface Morphology of Macrophages Within the Lens Cavity

Macrophages were mainly classified into two types, flat and spherical by scanning electron microscopy. At 11.5 days of gestation, the frequency of F-type macrophages was approximately 15%, with the remaining 85% being S-type macrophages. The F-type macrophages were approximately 30 μm in the long axis and 15 μm in the short axis, with irregular pseudopodia (Fig.6a). They appeared mainly at the lens vesicle formation stage and thereafter disappeared. The S-type macrophages were divided into three subgroups, S1, S2 and S3. The S1-type macrophages had a rather smooth cell surface with a few blebs and small pits (Fig.6b). The S2-type macrophages had numerous rufflings of their cell membrane (Fig.6c). The S3-type macrophages were characterized by spike-like processes on the cell surface (Fig.6d). The S3-type macrophages were often disc-shaped in the narrowing lens cavity. Although clear-cut classification of these

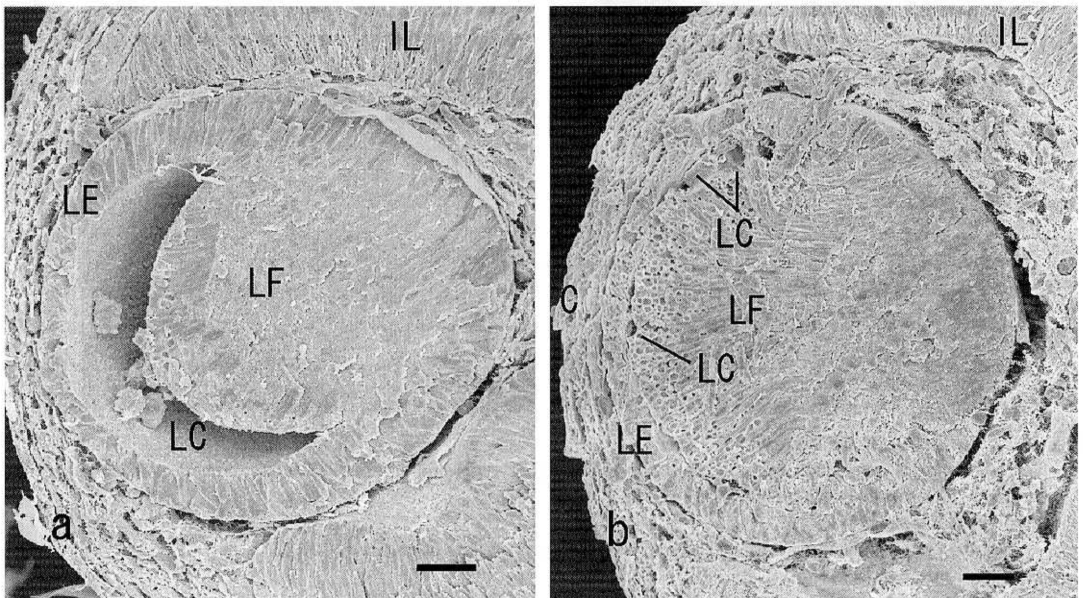

Fig. 5. Lens cavity at the late primary lens fiber elongation stage.
LC: Lens cavity, LE: Lens epithelium, LF: Primary lens fiber, IL: Inner layer of the optic cup
a) 12.5 days of gestation
The lens cavity becomes crescent in shape. A few macrophages are still present on the inner surface of the anterior wall. Scale bar=25 μm.
b) 13 days of gestation.
The lens cavity appears to be almost closed. The cavity does not contain any macrophages.
C: Cornea; Scale bar=25 μm.

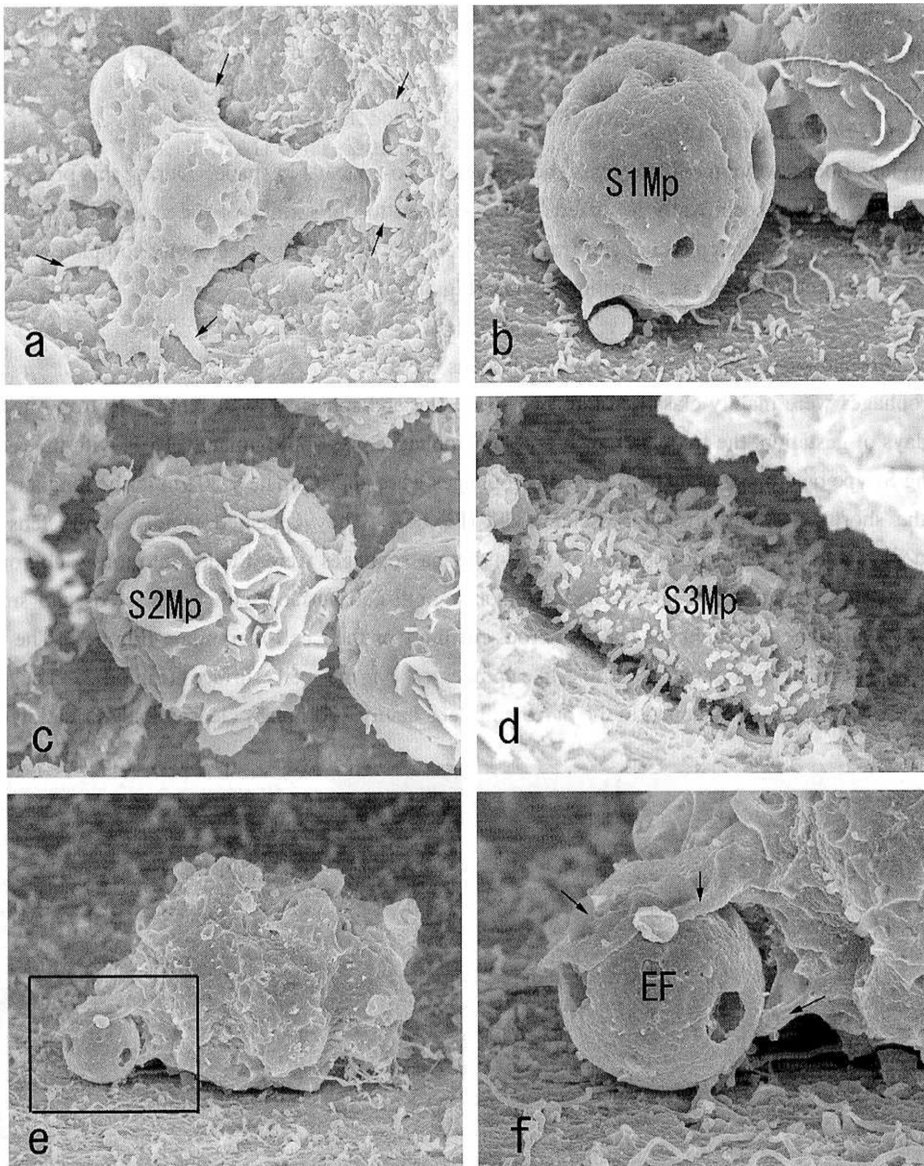

Fig. 6. Macrophages inside the lens cavity.

The macrophages are mainly classified into two types, flat (F-type) and spherical (S-type). Spherical macrophages are further divided into three subgroups, S1, S2 and S3.

a) F-type macrophage.
Irregular pseudopodia (arrows) are a characteristic feature. 11.5 days of gestation. 3000X

b) S1-type macrophage(S1Mp).
Their cell surface is smooth with a few blebs and small pits. 12 days of gestation. 3200X

c) S2-type macrophage(S2Mp).
Numerous rufflings on the surface are seen. 12 days of gestation. 3000X

d) S3-type macrophage(S3Mp).
The cell profile appears disc-like with numerous spike-like processes. 12.5 days of gestation. 3600X

e) An S1-type macrophage and an epithelial cell fragment.
A macrophage extends pseudopodia to an epithelial cell fragment. 12 days of gestation. 2900X

f) High-power micrograph of the framed area in e).
Arrows indicate finger-like projections of the macrophage.
EF: Epithelial cell fragment, 7400X

subgroups was difficult due to the presence of an intermediate type, the majority of the macrophages within either spherical or convexo-concaval cavities were S1- and S2-type, and S3-type macrophages increased in number within narrowing convexo-concave cavities. Both S1- and S2-type macrophages appeared to express active phagocytosis to epithelial cell fragments.

DISCUSSION

Our present SEM study on early lens formation showed that, inside the lens vesicle, numerous macrophages and small cell fragments from the lens epithelium were observed on the inner surface of the lens epithelium. Spherical cell fragments appeared early in the lens placode invagiation stage, and macrophages showed active phagocytosis to remove them from the cavity at the primary lens fiber elongation stage.

Macrophages as professional phagocytes have a scavenging function of removing apoptotic bodies formed during the degenerative process in apoptosis[8]. In the developing eyes in mammals, apoptotic cell death has been recognized in the neuroepithelium of the optic cup and lens epithelium[9~12], and, during lens morphogenesis, apoptosis occurs not only in the epithelium at the border of the lens placode but also in the lens stalk epithelium. The apoptotic processes in the developing lens are known to involve a caspase-3 dependent pathway[12], and apoptotic cell fragments have been observed in the lens pit and lens cavity. Present SEM observation revealed small spherical bodies of the cell fragments not only on the surface of the lens pits but on the inner surface of the anterior wall of the lens vesicle, and quite numerous free macrophages, which had a scavenging function to eliminate them, were seen inside the lens vesicle. By removing cell fragments from the lens cavity, macrophages play an essential role in early lens formation.

Regarding the surface morphology of the macrophages, the majority were of S-type, and these were subdivided into S1, S2 and S3 subgroups. The macrophages in the cavity predominantly consisted of both S1-and S2-types at the primary lens fiber elongation stage. Thereafter the proportion of S3-type macrophages showed an increase. As is well known, macrophages showing active phagocytosis have numerous lamelopodia and filopodia, forming small pits on the cell surface for phagocytosis[13~15]. Our present observations showed that, in lens formation, S1- and S2-type macrophages having the characteristic morphology of active phagocytosis changed to S3-type cells as the cavity became narrower. Due to the surface morphology, F-type macrophages might be moving to the inner surface of the anterior wall of the vesicle to become S-type cells. Macrophages changed their surface morphology inside the lens cavity with development of the lens epithelium and primary lens fiber probably due to their phagocytotic activities. Between the lens vesicle formation stage and the lens cavity closure stage, 11-13 days of gestation, most of circulating cells in the peripheral circulation of mouse embryo are primitive erythroblasts from yolk sac[16], and various embryonic tissues including fetal liver contain numerous scavenger macrophages to remove degenerating primitive erythroblasts and their debris[17,18]. Since these scavengers are categorized as fetal macrophages, macrophages in the lens cavity also appear to the same fetal macrophage group.

Since the lens vesicle was formed by invagination of surface ectoderm, the cavity originally was connected with the amniotic cavity. In human fetuses, amniotic fluid physiologically contains macrophages[19,20], so that it may be possible for macrophages inside the lens cavity to move directly from the amniotic cavity at the lens placode invagination stage. However, as shown in our previous report[7], macrophages were distributed not only in the mesenchyme around the lens primordium but also within the

lens epithelial layer, and their mitotic figures could be identified in the cavity. Therefore, it appears to be reasonable that macrophages migrate into the lens cavity from outside mesoderm around the lens primordium to proliferate inside the vesicle. In apoptosis, dying cells themselves secrete chemotactic factors, i.e."eat-me" signals, on the cell surface[21], and neighboring cells that engulf apoptotic cells have also revealed several chemokines for attraction of macrophages for apoptotic cell clearance[22,23]. In addition to the variety of macrophage surface morphology, their migration and emigration in the lens, and, their localization and proliferation within the cavity, all might be considered to be under the influence of chemokines, although cells responsible for chemokine production in developing eyes should be the subject of further investigation.

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