Characterization of the Macrophages Associated with the Tunica Vasculosa Lentis of the Rat Eye

Paul G. McMenamin, 1 Jenny Djano, 1 Rosamund Wealthall, 1 and Brendan J. Griffin 2

PURPOSE. The tunica vasculosa lentis (TVL) is a transient vascular network surrounding the developing lens that regresses prenatally in humans and postnatally in rodents. Macrophage-like cells, sometimes referred to as hyalocytes, have been postulated to play a role in the regression of this vascular system; however, the precise identity of these cells is still unclear. The aim of the present investigation was to provide phenotypic data on these cells in combination with their threedimensional distribution on the lens surface during the period when regression of the TVL is taking place. To this end the authors have used a novel combination of silver-enhanced immunogold labeling and environmental scanning electron microscopy (ESEM).

METHODS. The eyes of Wistar rats at various pre- and postnatal ages (E20, postnatal days [D]0, 2, 5, 7, and 10) were studied by either conventional scanning electron microscopy (SEM) or ESEM. The latter was used to study specimens that had been incubated with various antileukocyte monoclonal antibodies (ED1, ED2, Ox6, Ox42), followed by immunolabeling with gold-conjugated secondary antibody visualized by silver enhancement.

RESULTS. Conventional SEM of the developing lens revealed a pattern of radial and interconnecting vessels in the TVL similar to previous studies. In addition, large numbers of cells with the morphologic characteristics of macrophages were noted on the lens surface closely associated with the vessels. The gradual attenuation and regression of vessels was noted over the course of the time period investigated. Immunolabeled specimens examined by ESEM revealed that most of the macrophage-like cells were indeed ED1+ and ED2+ (both pan-macrophage markers) and MHC class II+ (Ox6) and CD11b/18+ (Ox42): a phenotype characteristic of macrophages. This phenotype altered little between E20 and D10.

CONCLUSIONS. The cells surrounding the developing lens that are postulated to play a role in regression of the TVL have the morphologic and immunophenotypic characteristics of resident tissue macrophages similar to those previously identified in the adult rodent uveal tract and the vitreous (hyalocytes). This phenotype differs from that of dendritic cells and microglia; however, it is postulated that lens-associated macrophages are ideally located to act as a source of retinal microglia after completion of their role in TVL regression. (Invest Ophthalmol Vis Sci. 2002;43:2076–2082)

From the 1School of Anatomy and Human Biology and 2Centre for Microscopy and Microanalysis, The University of Western Australia, Crawley (Perth), Australia.

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Corresponding author: Paul McMenamin, School of Anatomy and Human Biology, The University of Western Australia, Crawley (Perth), 6009, Australia; mcmenamin@anhb.uwa.edu.au.

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During mammalian normal ocular development a transitory network of vessels, the hyaloid vascular system, is important for providing nourishment for developing ocular structures especially the lens and the retina. The posterior component of the capillary network around the developing lens, the tunica vasculosa lentis (TVL), which is derived from the hyaloid artery, communicates via a series of equatorial placed capsulopupillary vessels with the vessels of the pupillary membrane on the anterior surface of the lens. In humans these vascular arcades around the lens regress in the last trimester, whereas in some laboratory animals they regress postnatally. 2–5 This vascular regression is characterized by the presence of wandering phagocytic cells around the lens that have been variably termed “vitreal cells,” “hyalocytes,” or simply macrophages. 2–5 Conventional histologic and ultrastructural studies suggest these cells are active in the phagocytosis of cellular debris resulting from the regression of the TVL. 2–5 Recently Lang and colleagues 10–13 provided experimental evidence in a transgenic mouse model that these macrophages played an active role in the initiation of programmed cell death of the endothelial cells of the TVL. The conclusions of this study have been disputed by Mitchell and coworkers, 9 who suggest a more passive role for the macrophages and postulate the importance of withdrawal of VEGF produced by the developing lens, which they believe may act as a “survival factor” for the vascular endothelial cells.

Environmental scanning electron microscopy (ESEM) is a relatively new technique that makes it possible to examine practically any material, including biological tissues, wet or dry, insulating or conducting, because it allows the introduction of a gaseous environment in the specimen chamber. A gaseous detection device enables images to be obtained in secondary (SE) mode. This signal is mixed with the backscattered (BSE) electron image to provide strong material and morphologic contrast. 14,15 Because there is no need for conductive coating of biological material immunophenotypic characterization of cells can be achieved by staining with colloidal-gold conjugated immunoreagents followed by silver enhancement. 16 Immunopositive cells can then be readily detected by ESEM (because of the silver staining) in the context of the surrounding surface tissue microarchitecture. In a brief report we demonstrated the value of ESEM in the immunophenotypic characterization of macrophages associated with the TVL in the developing rat eye. 17 The present study is a more complete immunophenotypic analysis of the macrophages associated with the TVL using a panel of antileukocyte antibodies in combination with a more extensive conventional scanning electron microscopy (SEM) and ESEM analysis.

MATERIALS AND METHODS

A total of 88 Wistar rats (E20, postnatal days [D]0, 2, 5, 7, and 10) were used in the study. Animals were obtained from the Biological Sciences Animal Unit at The University of Western Australia. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ages of rat fetuses were determined by counting from day 1, the morning after mating in which...
the vaginal plug is evident. Pregnant rats were killed by an overdose of nembutal (pentobarbitone sodium, 60 mg/ml; Merial Australia, Parramatta, NSW, Australia), and the fetuses were removed immediately from the uterus. The fetuses were decapitated, and the eyes were enucleated and fixed in either a mixture of glutaraldehyde (2.5%) and paraformaldehyde (2%) in 0.1 M phosphate buffer (for conventional SEM) or paraformaldehyde alone (4% in 0.1 M phosphate buffer; for ESEM). Rat pups (D0 to D10) were also killed by decapitation before enucleation.

In larger (older) eyes the posterior segment of the eye was dissected from the anterior segment containing the lens and enveloping TVL. The lens was then gently removed from the anterior segment by careful dissection, from a posterior approach, of any adhesions, developing zonules or vascular connections around the equator or pupil/optic cup margin. This freed the lens, thus allowing its removal intact with the TVL. In small eyes dissection was found to cause unacceptable damage thus the entire lens was left in situ in the anterior segment and was preserved intact for processing. Tissues destined for conventional SEM (48 animals: E20, D0, 2, 5, 7, and 10; D5, n = 10; D10, n = 8) were processed in the usual fashion (dehydration, critical point drying, and sputter coated with gold). These specimens were examined in a Philips 505 SEM (Philips, Eindhoven, The Netherlands) at 15 to 30 kV in secondary emission mode. Lenses destined for ESEM examination (40 animals; n = 8 per age group) were washed (three times in phosphate-buffered saline (PBS)), blocked with 50 mM of glycine, and again washed (3 times in PBS/0.5% bovine serum albumin (BSA)) before incubation in one of a range of anti-rat leukocyte primary monoclonal antibodies (mAbs; Table 1). After further washes (PBS/0.5%BSA), tissues were incubated in goat anti-mouse IgG-1nm gold conjugate (British Biocell International, Cardiff, United Kingdom), and washed in PBS followed by distilled water before being incubated in 2% glutaraldehyde followed by silver enhancement (British Biocell International).55 Tissues were then washed in distilled water and dehydrated before being critical-point dried. Lenses were mounted on carbon tape with the posterior surface upward. Entire anterior segments were mounted corneal surface downward. No coating of specimens is required for ESEM. Specimens were examined with either an ElectroScan E-5 or Philips FEI XL30 ESEM using BSE (backscattered electron) imaging at 30 kV. In light of the fact that the tissue is not coated and imaging is performed by the backscatter detector, it should be pointed out that sharpness of images is slightly different form conventional SEM, where surface topography is highlighted because of the presence of a thin sputter coating of gold.

Lenses from each time point were incubated with the range of mAbs in quantitative analysis. Negative controls in which PBS was substituted for the primary mAb were processed in parallel and examined by ESEM. Iris or choroid whole mounts were processed in parallel and acted as positive controls.

### Table 1. Monoclonal Antibodies Used in the Study

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Reference(s)</th>
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<tr>
<td>ED1</td>
<td>CD68, lysosomal antigen present in monocytes, tissue macrophages and some dendritic cells</td>
<td>18–20</td>
</tr>
<tr>
<td>ED2</td>
<td>Antiresident tissue macrophages, target antigen/molecule unknown</td>
<td>18</td>
</tr>
<tr>
<td>OX6</td>
<td>Anti-major histocompatibility complex Class II (α) molecule present on mature Dendritic cells and activated macrophage subpopulations</td>
<td>21</td>
</tr>
<tr>
<td>OX42</td>
<td>Anti-CD11b/18, present on neutrophils, macrophages, especially microglia</td>
<td>22</td>
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The conventional SEM component of the present study revealed changes in the developing rat TVL that are essentially similar to those described in previous studies2,3,23; however, a brief description of changes in the vessels of the TVL and the distribution of macrophages/hyalocytes during the period of regression will be presented as it is considered essential to the interpretation of the data from the ESEM study.

The mean diameter of the lens increases from approximately 750 μm at E20 to 1400 μm at D2 to 2050 μm at D10. Early experiments demonstrated that removal of the lens from the anterior segment caused damage to the delicate vessels of the TVL and the lens itself. Therefore, in subsequent experiments (conventional SEM and ESEM) the lens was left in situ once the posterior segment was removed. This allowed clearer visualization of the lens and surrounding TVL with minimal damage. Conventional SEM revealed the characteristic alterations in vessel morphology during development of the lens and TVL. The anteroposterior directed vessels that radiate from the hyaloid artery on the posterior pole of the lens (and therefore referred to as radial vessels) are interconnected by obliquely orientated interconnecting vessels (Figs. 1A, B). In the younger eyes (E20–D2) these vessels form a dense mesh around the lens (Figs. 1A–D). With growth of the lens the distance between radial vessels increases and the length of the interconnecting vessels increases. At later time points (D5–D10) the interconnecting vessels become attenuated (Figs. 1E–H) and less numerous, which has the effect of making the radial vessels appear straighter with fewer lateral anastomotic connections. At later time points (D10) some of the main radial vessels become attenuated and show evidence of degeneration (Figs. 1G and H).

Numerous rounded and ovoid cells with ruffled cell membranes and cell protrusions or processes of various thicknesses were identified throughout the surface of the lens from E20 to D10. They were particularly numerous in the equatorial region of the lens and were associated with the vessels or lens surface (capsule) between the vessels or were free within the vitreous (Figs. 1D–H). They often appeared in clusters and were common close to the equator where interconnecting vessels were more frequent and around the terminal portion of the hyaloid artery at the posterior pole of the lens. Although quantitation of macrophage density per frame indicated a decrease between prenatal/newborn eyes and postnatal eyes (Fig. 2) when the size and growth of the lens are considered the total number increases from 1025 to 1205 on the entire posterior surface of prenatal lenses to 2222 to 3216 on D5 and D10 lenses. These values may be an underestimate as some vessels suffered damage or were lost during tissue dissection and preparation/processing, although when possible we avoided the inclusion of such lenses in quantitative analysis.

### Immunophenotypic Analysis of Intraocular Macrophages using ESEM

Lenses at all developmental ages (E20, D0, 2, 5, 7, and 10) were investigated using a panel of antileukocyte mAbs, including ED1, ED2, OX6, and OX42, with the aim of characterizing the macrophages associated with the developing TVL and revealing their three-dimensional disposition. Whole lenses in situ (Fig. 3A) were incubated intact in the various reagents. Immunolabeled cells are visualized as bright features against a dark background.

The mAb ED1 recognizes a heavily glycosylated protein of 97,000 MW expressed predominantly on cytoplasmic granules such as phagolysosomes, and the degree of staining can be...
correlated to the phagocytic/endocytotic activity of the cell and it has been postulated to be similar to CD68 in humans. \(^{20}\) Immunopositive intracytoplasmic lysosomes occur in monocytes, macrophages, and to a lesser extent dendritic cells (DC) that usually contain a single perinuclear focal immunopositive region. \(^{20}\) The degree of staining of macrophages and the characteristic pattern of granular intracellular staining in light microscopic immunohistochemistry with less obvious resolution of the cell margins is the typical appearance of ED1\(^+\) cells. The general pattern of immunogold/silver-enhanced stained material in the present study was similar (Figs. 3B–F) in that focal points of silver were the usual evidence of positive staining. This was evidence that the silver enhancement reagents were able to penetrate cell membranes and stain intracellular compartments and cytoplasmic contents. Macrophages associated with the TVL at all ages were found to be ED1\(^+\) (Figs. 3B–F). Most ED1\(^+\) cells were of a rounded or ovoid shape and were both closely associated with vessels and with the lens capsule surface between vessels (Figs. 3A–D). They were more restricted in distribution to vessels from D5 onward.

Staining with the mAb ED2, which is a pan-specific tissue macrophage marker\(^{18}\) recognizing a membrane associated antigen, revealed an identical staining pattern to ED1 with numerous immunopositive cells around the TVL (Figs. 4A–F). A more even membrane-associated staining pattern similar to that observed in previous light microscopic studies of ocular ED2\(^+\) macrophages in adult\(^{24}\) and developing rat eyes\(^{25}\) was observed. The distribution of macrophages, namely large clusters in the early age groups (Figs. 4A, 4C) or more evenly distributed cells associated with vessels (Figs. 4E, 4F), was similar to the ED1-stained preparations. It is worth noting that on the basis of the ESEM examination that almost all cells that would have been classified as macrophages on the basis of their relationship to the vessels of the TVL and their morphologic

**Figure 1.** Rat lenses at various pre- and postnatal ages as seen by conventional scanning electron microscopy (SEM). The ages of the animals are indicated on the micrographs. Note the density of vessels in the TVL of the E20 lenses (A and B) and how the area of exposed lens surface increases in postnatal specimens (C–H). Note that the two major types of vessels, radial (R) and interconnecting (I), are of equal diameter in E20 and D0 lenses; however, by D5–D10 the interconnecting vessels have started to regress (arrows in E, G, and H). Also note the large numbers of hyalocytes associated with the vessels and lens surface. Magnification bars, 100 \(\mu\)m.
appearance and shape in conventional SEM studies were generally ED1/H11001 or ED2/H11001.

Staining of lenses of all age groups with the mAb Ox6, which is specific for the rat MHC class II (Ia) molecule present on dendritic cells and activated macrophages, revealed that most macrophages around the lens were Ox6⁺ (Figs. 5A, 5B). In D10 animals a few rare Ox6⁺ were noted in one animal (Fig. 5C).

Staining with the mAb Ox42, which recognizes rat C3b receptors (CD11b/18) present on neutrophils and some macrophage subpopulations such as retinal microglia,21 failed to demonstrate any reactivity with the macrophages around the developing lens in all age groups studied.

Controls

No silver-labeled cells were observed in control specimens. Some background silver deposits evenly distributed on the lens surface were a feature of some experiments especially if the incubations with the silver enhancement reagents were slightly longer than optimal. This is a well-recognized complication of this method.16

DISCUSSION

The temporal changes in the growth and regression of the tunica vasculosa lentis (TVL) during pre- and postnatal development of the rat lens observed in the present study using conventional SEM confirm several previously published electron microscopic investigations.2,3,23,26,27 Our data confirmed that with increasing growth in diameter of the fetal lens, the rich plexus of vessels forming the TVL gradually regresses by an attenuation of first the interconnecting vessels and later the radial vessels. In addition we also confirmed the presence of large numbers of macrophage-like cells (hyalocytes) on the lens surface close to these regressing vessels. The collagenous

FIGURE 2. Macrophage densities present on lens surfaces at magnification ×526 (an area of 39700 μm²) at age groups E20–D10. Bars without a common letter are significantly different (P < 0.05; one-way ANOVA and post hoc testing). Bars, SEM.

FIGURE 3. (A) D5 rat anterior segment containing the lens viewed from the posterior aspect and examined by conventional SEM. This illustrates the nature of the specimens processed for immunohistochemistry and the orientation of specimen mounting for ESEM examination. (B–F) Rat lenses at various ages stained with the monoclonal antibody ED1 and visualized with silver-enhanced immunogold labeling and examined by ESEM. Note the highly contrasting ED1 immunopositive macrophages associated with the vessels of the TVL. Some minimal background silver deposits are visible in some specimens. Note the view of erythrocytes within the lumen of the vessels of the TVL afforded by ESEM, a perspective not seen by conventional SEM (cf. Fig. 1). Magnification bars are present on each micrograph.
and filamentous remnants of regressing vessels surrounded by macrophage-like cells observed in the present study were similar to those described by previous investigators.\(^3\) The rat TVL has been described as having completely regressed by D11 to D12,\(^2,23\); however, in our study the oldest animals were D10 and therefore still possessed remnants of vessels. From the quantitative and qualitative data, it is apparent that large numbers of macrophage-like cells are still present at later time points when regression of the TVL seems almost complete. This raises the possibility that not all the cells seen by conventional SEM are indeed phagocytic macrophages, whose function is to remove debris. Cells around the TVL may represent a more heterogeneous population. Furthermore, in light of the evidence that macrophages are themselves a phenotypically and functionally heterogeneous group of cells, it is clear that a better understanding of the immunophenotypic nature of the macrophage-like cells associated with the TVL was required. This was the stimulus for the second part of the study which took advantage of the novel perspective offered by the combination of immunolabeling and ESEM.

There have been only a restricted number of previous investigations of the phenotype of macrophages associated with the developing lens and TVL. The majority of these have been performed in the mouse, and all have demonstrated that the hyalocytes are F4/80\(^+\).\(^5,11,12\) However, because this mAb is not truly macrophage specific (i.e., it labels small immature DC subpopulations as well as macrophages),\(^28,29\) and other monoclonal antibodies have not been investigated, the presence of other cell types could not be conclusively ruled out from these previous rodent studies. Recent morphologic and immunohistochromic data suggest that hyalocytes, in humans at least, may represent a heterogeneous group of cells.\(^30\) These authors have suggested that hyalocytes fall into two main morphologically distinct categories: type I, “dendritic hyalocytes” with two to five long primary cell processes; and type II, hyalocytes with a more rounded/pleomorphic cell body. They believed the latter group resembled natural killer lymphocytes capable of mediating cytotoxicity. It was postulated that the type I cells may be related to “dendritic antigen-presenting cells and microglia.” Furthermore, these authors stated that some of the hyalocytes might constitute populations of mast cells, although no evidence of the latter was presented. This is, to the author’s knowledge, the first time it has been speculated that antigen-presenting cells, NK cells, and mast cells are associated with the developing lens and vitreous. The present authors find it difficult to envisage a functional role for such cells in the early development of the eye. This is particularly true of mast cells, which although have been widely reported in the choroid of the eye,\(^30\) have never, to our knowledge, been described within the retina, vitreous or lens, i.e., within the internal aspect of the blood-ocular barrier (for review see McMenamin\(^32\)).

In light of the speculation cited above that there may be dendritic cells (professional antigen-presenting cells) among the macrophage populations associated with the TVL, we chose to investigate the distribution of MHC class II\(^+\) cells. However, other than a very rare immunopositive cell in one D10 rat eye, there was little evidence of MHC class II\(^+\) cells in the many rat lens specimens studied. This supports recent data in which no MHC class II\(^+\) dendritic cells were identified in the

![Figure 4](https://example.com/fig4.png)
developing rat eye other than the well-described populations in the uveal tract\textsuperscript{25} (iris, ciliary body, and choroid).\textsuperscript{24,53,54} The observation that cells associated with the TVL in the developing human eye\textsuperscript{30} are CD45\textsuperscript{−} MHC class I\textsuperscript{+} and class II\textsuperscript{−} does not exclude the possibility that these cells are mononuclear phagocytes/macrophages, because it well established that activated macrophages may express MHC class II. The consistent finding in the present study of large numbers of pleomorphic ED1\textsuperscript{−} and ED2\textsuperscript{−} hyalocytes around the TVL is consistent with the conclusions of previous morphologic studies that these cells are indeed macrophages. Both these mAbs, particularly ED2, are pan-macrophage-specific markers, present on most rat macrophage subpopulations except those resident in non- connective tissue microenvironments such as the microgial of the retina and brain parenchyma.\textsuperscript{33–36} Therefore, immunoreactivity of hyalocytes with these mAbs is strong evidence of their macrophage phenotype. The strong immunoreactivity with ED1, an antigen associated with lysosomes, indicates a high degree of active phagocytic activity during TVL regression. This conclusion is consistent with the role of macrophages in debris removal in other areas of the developing central nervous system,\textsuperscript{37–39} including the retina.\textsuperscript{29}

Wolters\textsuperscript{40} in a descriptive morphologic study first postulated that a relationship may exist between hyalocytes and microglia, namely the former being precursors of the latter. It is now well established in experimental animals that microglia are long-lived bone-marrow-derived specialized CNS macrophages that invade the brain parenchyma and neural retina during early development.\textsuperscript{33,41,42} Microglia in rats are ED2\textsuperscript{−} ED1\textsuperscript{−} Ox42\textsuperscript{−} (CR3 [complement receptor 3] or CD11b), yet we observed no Ox42 immunoreactivity among the ED1\textsuperscript{−} ED2\textsuperscript{−} hyalocytes in the present study. We postulate, based on the data from the present study and our previous data, of the distribution of macrophages in the developing eyes of eutherian mammals and marsupials,\textsuperscript{25} that on completion of their role as debris removers, some hyalocytes likely migrate into the peripheral neural retina and differentiate into microglia. This would require a change in phenotype such as the downregulation of phagocytic activity and loss of the surface determinant recognized by ED2, but a similar change is thought to occur when the bone-marrow precursors initially invade the central retina via the developing vasculature and spread in a centrifugal manner toward the periphery.\textsuperscript{41}

Trafficking of mononuclear phagocytes from the ciliary body vasculature into the peripheral retina and vitreous close to the ora serrata was noted by Balazs et al.\textsuperscript{6} The presence of large pleomorphic and dendriform macrophages in the periperal subretinal space of the human fetal eye\textsuperscript{33,44} was thought to possibly represent such cells during this infiltration. This second site of microglial origin was later confirmed by Diaz-Araya et al.,\textsuperscript{35} who noted in early human retinal development that microglia appear to infiltrate both the central retina and retinal margin. This proposal that mononuclear cells derived from the ciliary body or indeed the hyalocyte population around the lens may contribute to the final retinal microglial population is further supported by our recent demonstration of subretinal macrophages in the peripheral retina in several species of eutherian mammals and marsupials.\textsuperscript{25}

In summary, the data provided by ESEM in combination with immunolabeling has shown that the hyalocytes associated with the regressing TVL in the pre- and postnatal rat eye have the immunophenotypic and morphologic characteristics of macrophages. No evidence was obtained that any other leukocyte or immune cell population was present during lens development.

References


