In vitro studies on shedding and phagocytosis of rod outer segments in the rat retina: effect of oxygen concentration

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The processes of outer segment shedding and phagocytosis were found to be significantly enhanced in explants of rat retina and pigment epithelium (PE) newly placed in culture. The number of phagosomes reached a maximum at 2 hr of incubation and then rapidly decreased, as is observed in rats entrained in a normal light-dark schedule when the lights are turned on in the morning. This pattern was not qualitatively affected by the time of day the tissues were excised or by the presence of a high concentration of melatonin in the culture medium. The shedding peak in vitro was observed only when the retina-PE unit was incubated in an atmosphere composed of 45% oxygen, 5% CO₂, and 50% air. When the atmosphere contained lower (95% air and 5% CO₂) or higher (95% O₂ and 5% CO₂) oxygen, PE cells contained only a few scattered phagosomes. These functional differences may be caused by different arrangement of PE cell microvilli at a "moderate" (45%) O₂ concentration vs. "low" or "high" O₂ concentrations. A balanced O₂ level thus appears to be critical for phagocytosis in explant culture. (INVEST OPHTHALMOL VIS SCI 22:439-448, 1982.)

Key words: retina, pigment epithelium, photoreceptor disc shedding, phagocytosis, oxygen concentration, organ culture

Shedding of retinal outer segments and phagocytosis of the shed discs by the pigment epithelium (PE) are important steps in the process by which the retinal photoreceptor cell undergoes constant renewal. Although it is known that outer segment membranes are shed and phagocytized in a circadian manner, little is known about the actual control of these processes or in fact whether there are separate signals for disc shedding and for phagocytosis.

Shedding and phagocytosis have recently been reported in vitro in tissue culture, eyecup preparations, and organ culture of retinal and PE tissue. Since the signals for disc shedding and phagocytosis may be intrinsic to the eye and not directly controlled from an extraocular source, it is reasonable to assume that significant information about these processes can be gained in such in vitro experiments. In the present study, we use our previously described culture technique to give evidence that microenvironmetal conditions such as O₂ concentration may play an important role in controlling the functional expression of phagocytosis.

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Materials and methods

Animals. Sprague-Dawley albino rats were obtained from the NIH animal colony. They were maintained under a cyclic lighting condition (i.e., lights were turned on between 6:00 A.M. and 6:00 P.M.). The intensity of the fluorescent light was 40 to 50 foot candles at the top of the cages. Animals were sacrificed at three different times during the cyclic light-dark schedule: (1) 6:15 A.M., (2) 11:00 A.M., and (3) 9:00 P.M. In the experiments at 9:00 P.M., animals were killed under dim red light, with all the subsequent procedures (e.g., dissection) performed under an infrared image converter (Noctovision). Animals were sacrificed with sodium pentobarbital (Diamond Laboratory, Des Moines, Iowa) or with ether, and the eyes were rapidly enucleated and rinsed several times in Hanks' Minimal Essential Medium (MEM; GIBCO, Grand Island, N.Y.).

Tissue culture. The anterior segments of the globes were removed at the equator. The posterior retina, along with adherent PE, choroid, and sclera, was dissected into quarters in Hanks' MEM. Cultures were kept in an incubator (Heinicke Instruments Co., Hollywood, Fla.) at 37° C with a continuous flow of atmosphere composed of 5% CO₂, 45% O₂, and 50% air unless otherwise noted. The medium consisted of a 3:3:1 mixture of Hanks' MEM, fetal calf serum (GIBCO), and chick embryo extract (GIBCO).

In experiments designed to study the effects of differing concentrations of oxygen, animals were sacrificed at 11:00 A.M., and tissues were dissected and immediately incubated in one of three different atmospheres: "A," 5% CO₂, 45% O₂, and 50% air; "B," 5% CO₂ and 95% air; "C," 5% CO₂ and 95% O₂ for periods of time given in the figure legends. When appropriate, melatonin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol and added to the medium at a final concentration of 0.2 mM. Control cultures in this series of experiments received the same amount of ethanol and were incubated in atmosphere "A."

Microscopy. Tissues were sampled just before incubation was started ("0" hr control) or after 1 to 4 hr of incubation and were fixed with 1% glutaraldehyde and 1% paraformaldehyde in 0.075M phosphate buffer, pH 7.2 overnight. They were then postfixed with 1% osmium tetroxide in 0.15M phosphate buffer for 1 hr and dehydrated in a graded ethanol series, which was exchanged with propylene oxide, and were finally embedded in epoxy resin. The number of phagosomes was counted in a 200 μm field of PE cells with thick (1 μm) sections stained with 1% toluidine blue. Criteria for phagosome counting was as previously defined by LaVail; that is, all inclusion bodies in the PE soma as well as intensely staining structures among PE processes that were greater in any dimension than 0.75 μm were defined as phagosomes and were counted. For each experimental condition, tissue specimens were taken for three animals and 10 sections from each were examined for numbers of phagosomes. Each experiment was repeated two times and the mean values and standard deviations were calculated. For transmission electron microscopy, thin sections were stained with uranyl acetate and lead citrate and observed with a JEOL 100C electron microscope.

Results

Tissues dissected and fixed before in vitro incubation ("0" hour control) are shown in Fig. 1, a. Only occasional phagosomes are seen. Fig. 1, b to d, are tissues incubated for 1, 2, and 3 hr, respectively, in atmosphere "A." The number of phagosomes increased remarkably after transferring in vitro. The peak value was obtained at 2 hr of incubation; subsequently, the number of phagosomes sharply decreased (Fig. 1, d).

In vivo, a burst of shedding occurs in the rat soon after lights are turned on in the morning. The in vitro shedding sequence demonstrated in Fig. 1, b to d, however, was the same as that in tissues excised at different times during the day (Fig. 2, a). The peak number of phagosomes (40 to 50 phagosomes per 200 μm of PE) was observed at 2 hr of incubation in tissues excised at 6:15 A.M. (15 min after lights on), 11:00 A.M., and 9:00 P.M. (dark period). The burst of shedding in vitro was also not affected by the presence of 0.2 mM melatonin in the culture medium (Fig. 2, b). Shedding was neither enhanced nor suppressed at this high concentration of the
Fig. 1. Light micrographs of retina-PE explants dissected approximately 5 hr after lights were turned on. a, Rat tissue dissected and fixed just before starting incubation ("0" hr control). b, c, and d, Retina-PE explant incubated for 1, 2, and 3 hr, respectively. Incubations were in atmosphere "A." (Bar = 100 μm.)

neurohormone. Melatonin also had no noticeable effect on retinal structure in the cultured explants (not shown).

Fig. 3 shows the effect of different compositions of atmosphere on disc shedding in culture. Control atmosphere "A" shows the typical pattern of shedding at 2 hr of incubation (Fig. 3, a). When the explant was cultured in atmosphere "B," large numbers of inclusion bodies were present in the PE that stained greenish yellow with toluidine blue (Fig. 3, b). Photoreceptor nuclei showed some de-
generative changes. In atmosphere "C" the neural retina and PE cells were well preserved but very few phagosomes were evident (Fig. 3, c). The phagosome number under the three atmospheric conditions over a 4 hr period in culture is shown in Fig. 4. Only in atmosphere "A" was there a burst of shedding comparable to the situation observed in vivo.

Ultrastructurally, retinas incubated in atmosphere "A" were found to be well preserved with numerous phagosomes (2 hr of incubation) in the apical portion of PE cell soma (Fig. 5, a). Phagosomes were usually surrounded by optically empty space. Phagocytized disc membranes were in various degrees of degradation. Some phagosomes still contained several dozen well-defined discs; other phagosomes contained clumped, electron-dense masses. PE cell microvilli were generally found grouped together and elongated perpendicularly to cell soma, surrounding adjacent outer segment fragments. Microvilli expanded only around rod outer segments (ROS) located near the PE cell. Primary and secondary lysosomes were abundant in the apical half of the PE cell and in the microvilli; most mitochondria were located just above the basal infoldings. The basal infoldings were tall and were often surrounded by large optically empty spaces. Outer segments were well preserved (Fig. 5, b) but exhibited a segmented appearance every few decades of discs (see also Fig. 1, b to d), although this could very well have been caused by the natural cleavage characteristics of fixed rat ROS. Several ROS appeared to have recently shed their terminal discs; shed packets of discs were not of uniform size.

Retina and PE cultured in atmosphere "B" showed a generally similar ultrastructure to that discussed above, e.g., well-preserved disc membranes and tall basal infoldings (Fig. 6). In general, preservation of organelle structure actually seems to be somewhat better than that seen in atmosphere "A." At least three characteristic differences were observed, however. First, there was an obvious paucity of phagocytic activity. Second, numerous sheets of microvilli elongated parallel to PE soma and had somewhat less tendency to envelop adjacent ROS fragments. Third, large numbers of homogeneous inclusion bodies were contained in the PE. They had no limiting membrane and relatively high electron density.

The tissues incubated in atmosphere "C" showed distinctly different morphologic characteristics (Fig. 7, a). Microvilli were more plentiful than in atmospheres "A" or "B";
Fig. 3. Effect of different compositions of atmosphere on disc shedding in culture. a, Tissue incubated in atmosphere “A”; b, tissue incubated in atmosphere “B”; c, tissue incubated in atmosphere “C.” Tissues were excised at approximately 11:00 A.M.; incubations were for 2 hr. (Bar = 10 μm.)

they were elongated, multilayered, and parallel to the apical surface of the PE soma. Few phagosomes were found. Multilayered cell processes containing cytoplasm and cell organelles were observed; these often “sandwiched” microvilli between them and the PE cell soma. Basal infoldings were elongated but only small optically empty spaces existed between them. Some microvilli were elongated and surrounded ROS fragments. These fragments were usually very large and had irregularly arranged, vacuolized discs.

Discussion

The retina-PE unit shows an enhanced sequence of disc shedding and phagocytosis when placed in organ culture under proper conditions. These phenomena occur independent of lighting conditions and of the time of day the samples are taken, since the numbers of phagosomes in the control (0 hr) retinas are quite different from each other but uniformly increase several times within 2 hr in culture. It thus appears that the precipitation of the shedding sequence is not acutely
dependent on lighting conditions or on circadian rhythm.

The present results are in accord with data reported by Goldman and O'Brien in an eyecup preparation, although they used a different strain of rats, different dissection technique, and different medium as well as a different gassing atmosphere. Their incubation atmosphere was 5% CO₂ and 95% O₂, an atmosphere similar to our atmosphere "C," where we observed no shedding. We assume, however, that the O₂ tension at the retinal surface within the eyecup would be somewhat more comparable to our atmosphere "A" (i.e., 45% O₂ where shedding is observed), since the entire eyecup was immersed in buffer under the Goldman-O'Brien conditions, whereas the explant sits at the atmosphere/liquid interface in the present experiments. No attempt was made to actually measure O₂ tension in the buffer under our conditions, since only a thin film of liquid separates the tissue explant from the gas phase. Flannery and Fisher have also reported that disc shedding occurs in cultured whole-eye explants of Xenopus laevis within 1 hr of light stimulus.

The ultrastructural changes and lack of phagocytosis observed with atmosphere "B" could be ascribed to pH differences or to simple anoxia. These changes include the arrangement of PE microvilli parallel to the cell body instead of perpendicular and their inability to fully engulf ROS fragments. These possible explanations are simplistic, however, since the buffer capacity of the enriched medium used is high and the effects are observed within 2 hr of incubation. Similarly, both low (atmosphere "B") and high (atmosphere "C") oxygen concentrations result in changes in microvillus orientation and inhibition of phagocytosis. It thus appears that a relatively delicately balanced O₂ concentration is necessary for ROS shedding and phagocytosis at least in vitro.

A somewhat parallel situation is observed in RCS rats that demonstrate an inherited form of retinal degeneration. In this animal, early development and elongation of ROS appear normal, but PE cell microvilli

Fig. 4. Number of phagosomes in tissues incubated under different atmospheric conditions. o, Tissue incubated in atmosphere "A"; a, tissue incubated in atmosphere "B"; •, tissue incubated in atmosphere "C." Tissues were excised at approximately 11:00 a.m. Data points are mean values ± S.D. from two experiments.
Fig. 5. Electron micrographs of retina-PE cultured for 2 hr in atmosphere "A." a, PE cell soma containing many phagosomes surrounded by optically empty space. Some phagosomes are clumped into electron-dense masses. (Bar = 1 μm.) b, Outer segment area of retina. (Bar = 1 μm.)
are deranged and phagocytosis is minimal. It is hoped that the presently described in vitro system may ultimately yield information relevant to this disease.

The basic mechanism by which a cascade of disc shedding and phagocytosis is triggered in vitro is presently unknown, as are the signals that initiate the process in vivo. In vitro, it appears that the simple act of enucleation is not sufficient to precipitate the burst of disc shedding and phagocytosis, since these events can be inhibited by manipulation of the $O_2$ concentration in a subsequent incubation period. Similarly, Besharse et al.\textsuperscript{15} have recently shown that the bicarbonate ion concentration of the culture medium is of critical importance in supporting disc shedding in incubated eyecup preparations of \textit{X. laevis}. The present and previous work\textsuperscript{16, 17} appears to rule out the influence of circulating neurohormones such as melatonin. Teirstein et al.\textsuperscript{11} have recently presented in vivo evidence for the presence of independent oscillators regulating outer segment phagocytosis within each eye. It is clear from the present work that the supply of $O_2$ available to the PE cells and ROS from the choroid are critical in maintaining a normal shedding cycle. Moreover, it may be that a simple change in $O_2$ concentration may itself act as one of the
Fig. 7. Electron micrographs of retina-PE explants cultured for 2 hr in atmosphere “C.” a, PE cell soma and parallel arrays of cell processes. (Bar = 1 μm.) b, Outer segment area of retina and ROS fragments. (Bar = 1 μm.)
signals in the processes of shedding and phagocytosis.

REFERENCES