donor tissues used in this study and also Dr. W. Richard Green and Dr. Susie Humphreys for their help and advice.


Key words: diabetes mellitus, corneal epithelium, basement membrane, transmission electron microscopy, multilaminated basement membrane

REFERENCES

Effect of arachidonic acid on normal and dystrophic retinal pigment epithelium in tissue culture. BRENTA J. TRIPATHI AND RAMESH C. TRIPATHI.

With carmine particles used as markers, no significant difference was observed between the phagocytic activity of 1- to 3-week-old confluent monolayer cultures of normal and dystrophic retinal pigment epithelial (RPE) cells harvested from 1-day-old Royal College of Surgeons rats. The phagocytic activity of both normal and dystrophic RPE was markedly suppressed in a medium containing 1 mg/ml arachidonic acid (AA), and the cells rapidly assumed a rounded profile. With 100 μg/ml AA, the phagocytic activity of dystrophic RPE was differentially reduced compared with that of the normal sample (p < 0.01); this effect was subsequently accompanied by a gradual change in the shape of the cells. Lower concentrations of AA (3 μg/ml and below) did not produce a significant effect in either group.

Arachidonic acid (AA) is the major polyunsaturated fatty acid (PUFA) constituent of retinal pigment epithelium (RPE), accounting for some 16.6% by weight of total phospholipids. AA is also present in phospholipids of the rod outer segments (5.6% by weight) together with the other PUFAs, docosahexaenoic acid (23% by weight), and trace amounts of linoleic and linolenic acids. 1-2 Elevated levels of plasma AA have been reported in some patients with primary pigmentary degeneration of the retina. 3-4 Fibroblasts cultured from one patient who had pigmentary degeneration of the retina and progressive spinocerebellar degeneration incorporated greater amounts of 14C-arachidonate into triglyceride and esterified cholesterol than did normal human fibroblasts. 3-4 These studies led to the hypothesis that a defect in arachidonate metabolism may adversely affect the normal functioning of the RPE, at least in patients with some types of retinitis pigmentosa. 4

This report is concerned with the effect of varying concentrations of AA on the morphology and phagocytic activity of the RPE cultured from normal and dystrophic rats of the Royal College of Surgeons (RCS) strain.

Materials and methods. Cultures of RPE were initiated from 1-day-old dystrophic rats of the RCS pink-eye strain and from their congenic controls, RCS+dy* rats. 3 The animals were descendants of breeder pairs kindly supplied by Dr. M. M. LaVail. The animals were anesthetized with sodium pentobarbital and were washed in three changes of sterile saline. In a sterile environment and with the aid of a dissecting microscope, the lids were opened and the eyes were enucleated. The globes were opened at the ora serrata, and the retina was dissected out from the posterior segment. The RPE cells were gently recovered from the posterior segment by microdissection and transferred to a cover glass of a Sterilin disposable tissue culture chamber containing TC 199 culture medium (Gibco) with 10% fetal calf serum and 10% penicillin/streptomycin antibiotic at pH 7.4.
Fig. 1. Effects of 100 μg/ml AA on the phagocytic activity of RPE in culture. a, 10-day-old culture of dystrophic cells exposed to AA for 3 hr. Most cells in this field have not phagocytosed carmine particles. b, 10-day-old culture of congeneric control RPE cells exposed to AA for 6 hr. Most cells in this field have engulfed carmine particles (arrows). (Light micrographs ×660.)
Fig. 2. Ten-day-old culture of dystrophic RPE exposed to 100 μg/ml AA for 6 hr. Most cells in this field failed to engulf carmine particles and have assumed a rounded profile. (Light micrograph ×625.)

The chamber was sealed and incubated at 37° C. After 24 hr of incubation the medium was replaced (to remove any nonviable cells and other debris), and thereafter it was changed every 3 to 4 days.

AA (Sigma Co.) was diluted in the above tissue culture medium to provide serial dilutions ranging from 1 mg/ml to 0.5 μg/ml. At these dilutions the pH remained at 7.4. When cultures of RPE attained a confluent monolayer, usually after 7 to 10 days of incubation and up to a maximum of 3 weeks, the medium was changed to one containing AA. The results from normal and dystrophic RPE cell cultures were matched for length of time in vitro. The response of the cells to varying concentrations of AA was observed at hourly intervals by phase-contrast microscopy.

The phagocytic activity of the RPE was evaluated by the ability of the cells to engulf carmine particles, which were added to the culture medium 30 min before the conclusion of the experiment. Prior to fixation in 10% buffered formalin at predetermined time intervals (1, 3, 6, 15, and 24 hr), the cultures were washed in basic salt solution for removal of free carmine particles. The fixed cultures were stained with hematoxylin and eosin. We quantitated the phagocytic activity of both normal and dystrophic RPE exposed to AA by noting the presence or absence of engulfed carmine particles in 100 cells counted at random under the 25× objective of the light microscope. The results of six independent experiments were averaged, and the significance was determined by the Student's t test.

Results. Although no difference was observed between the response of normal and dystrophic cells cultured for 1, 2, or 3 weeks (the latter corresponding to the approximate in vivo age when the phagocytic defect becomes most obvious in dystrophic rats), most cultures incubated for 7 to 10 days provided an optimum number of cells for quantification of phagocytic activity.

Changes in phagocytic activity of RPE. Control
Table I. Quantitative determination of phagocytic activity of cultured RPE with carmine particles used as markers (mean ± S.D.)

<table>
<thead>
<tr>
<th></th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>15 hr</th>
<th>24 hr</th>
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<tbody>
<tr>
<td><strong>100 µg/ml AA:</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Normal RPE</td>
<td>91.5 ± 1.05</td>
<td>93 ± 1.41</td>
<td>90.07 ± 1.63</td>
<td>67 ± 1.78</td>
<td>36.5 ± 2.17</td>
</tr>
<tr>
<td>Dystrophic RPE</td>
<td>88.5 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.16 ± 1.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.6 ± 2.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 1.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 1.51&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Control:</strong></td>
<td></td>
<td></td>
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<tr>
<td>Normal RPE</td>
<td>90.66 ± 1.21</td>
<td>92.2 ± 0.83</td>
<td>93.66 ± 1.97</td>
<td>95.5 ± 1.04</td>
<td>94.16 ± 1.16</td>
</tr>
<tr>
<td>Dystrophic RPE</td>
<td>90.16 ± 1.17</td>
<td>91.66 ± 1.21</td>
<td>94.33 ± 1.37</td>
<td>94.83 ± 1.37</td>
<td>93.5 ± 1.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>p = 0.005.  
<sup>b</sup>p > 0.001.  
<sup>c</sup>No significant difference (p = N.S.) between normal and dystrophic samples.

cultures of RPE from normal rats actively phagocytosed carmine particles. Over a 24 hr period an average of 93.13 ± 1.9 cells per hundred contained engulfed particles. A comparable uptake of carmine particles (average, 92.9 ± 2.09 cells per hundred; p = N.S. compared with normal controls) was noted in RPE cultured from dystrophic RCS rats. On exposure to 1 mg/ml AA for 1 hr, carmine particles were not engulfed by either normal or dystrophic RPE samples. With 100 µg/ml AA, a differential suppression in the phagocytic activity of dystrophic and normal RPE samples was observed (Fig. 1). This effect was subsequently accompanied by a change in cell morphology (Fig. 2). By 3 hr it was apparent that the phagocytic activity of the dystrophic RPE was markedly reduced (p ≥ 0.001 compared with normal). The difference between normal and dystrophic samples remained significant during the 24 hr of incubation in AA medium (Table I).

Incubation up to 24 hr in 5 µg/ml AA and less showed no significant difference from respective control cultures in the phagocytic activity of either normal or dystrophic RPE.

**Morphologic changes in RPE.** Cultures of RPE from normal and dystrophic animals showed no appreciable difference in morphology after 7 to 10 days of incubation. Foci or groups of RPE cells were present as monolayers on the cover glass of the culture chamber. Most cells had a flattened, somewhat epithelioid profile and, at the extreme edge of the colony, had long, slender cytoplasmic processes through which they made contact with adjacent cells.

On incubation in medium containing 1 mg/ml AA, both normal and dystrophic RPE reacted similarly. Within 1 hr the cells lost their flattened appearance and assumed a rounded profile. Further exposure to AA at this concentration resulted in loss of cellular attachment to the coverglass, and by 6 hr all cells were suspended in the culture medium.

On incubation in 100 µg/ml AA, many cells in the dystrophic sample assumed a rounded shape after 3 to 5 hr (Fig. 2), whereas cultures of normal RPE began to show a similar effect after 15 to 24 hours. Even with 24 hr of incubation, neither the normal nor the dystrophic cells detached from the cover glass.

A 24 hr exposure to lower concentrations of AA (5 µg/ml and below) had no appreciable effect on the cellular morphology of the normal or dystrophic RPE samples.

**Discussion.** Our findings show that, with carmine particles used as markers, there was no significant difference in the phagocytic activity of normal vs. dystrophic RPE cultures of 1 to 3 weeks harvested from 1-day-old RCS rats. With a near-threshold dose of AA for normal RPE, however, the phagocytic activity of dystrophic RPE is significantly reduced, indicating its greater susceptibility to AA. It may be argued that any agent that causes rounding of cells will also suppress phagocytic activity as a secondary event. However, in our experiments, suppression of phagocytosis in dystrophic cells was apparent before cell rounding (i.e., 1 to 3 hr compared with 3 to 5 hr exposure to AA, respectively). Similarly, in normal samples, phagocytic activity was reduced significantly by 15 hr, whereas cell rounding became apparent between 15 to 24 hr of exposure. These events may reflect subcellular and biochemical changes not detectable by light microscopy. Because cell membrane formation and its stability are essential prerequisites for both phagocytosis and maintenance of normal cell shape, this site could be the primary target for AA to exert its effect. The precise mechanism of action of AA on the RPE, however, requires elucidation.

PUFAs are known to damage cells by two main mechanisms: detergent-like action and auto-oxidation. In the former process, membrane proteins are denatured in a manner similar to the action of detergents. It has been suggested that, in induc-
ing brain edema in vitro, AA acts predominantly by this mechanism.7 Auto-oxidation, or peroxidation, involves formation of free-radical intermediates from lipids that cause molecular damage to the membranes, especially the cross-linkage of proteins.8 Antioxidants are effective in preventing this process. It has recently been reported that docosahexaenoic acid–induced inhibition of nucleic acid synthesis in cultured lymphocytes could be counteracted by α-tocopherol and superoxide dismutase.9 The RPE is known to have considerable superoxide dismutase activity10 as well as peroxidase enzyme associated with plasma membranes.2 These enzymes within the RPE could provide a physiologic mechanism to neutralize the potential damaging effects of the PUFA-rich outer segment membranes that are shed for phagocytosis. If AA inhibits the phagocytic activity of RPE by release of auto-oxidative products, then the greater susceptibility of dystrophic RPE observed in our present study implies a defect in the protective enzyme system of the cell.

The RCS rat is a model for retinitis pigmentosa in man. The degenerative changes in the retina of dystrophic animals are considered to be caused by a defect in the ability of the RPE to phagocytose outer segment material. Studies in vitro have shown a reduction rather than a total lack of phagocytosis for outer segment material.11-14 However, normally the phagocytic activity of the dystrophic RPE is not reduced appreciably for inert markers such as latex spheres, polystyrene beads, yeast, and oil droplets.15-18 Further work is in progress to determine whether the incorporation of rod outer segments material in the culture media can sufficiently raise the level of AA (as well as other PUFAs) to approximate our experimental situation reported here. In this regard it is noteworthy that a raised level of AA is far more effective in disrupting cellular function than any other PUFA.7

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Key words: arachidonic acid, polyunsaturated fatty acids, retinal pigment epithelium, morphology, phagocytosis, tissue culture, normal rat, dystrophic rat, carmine, retinitis pigmentosa

REFERENCES


The distribution of refractive errors was followed in chicks from hatching to 8 weeks of age. A dramatic progressive decrease in the variability of refractions was observed over this period. In addition, there appeared to be a parallel decline in hyperopia, even when the artificial...