Effects of Silicone Oil and Hyaluronic Acid on Cultured Human Retinal Pigment Epithelium

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An experimental model was developed to evaluate the effects of several vitreous substitutes on human retinal pigment epithelial (RPE) cells. Cultured RPE cells were grown into confluent monolayers on semipermeable membranes and nourished from the basal side only, while the apical surface was directly and continuously exposed to preparations of hyaluronic acid and silicone oil for up to 4 weeks. In the silicone oil-treated group, multilayer sheets formed within 2 weeks of exposure and incorporation of \(^{3}\text{H}\)-thymidine was twice that of controls. Exposure of RPE cells to silicone oil and certain preparations of hyaluronic acid resulted in a three- to fourfold decrease in cellular polarity, as measured by assaying the cell-surface enzyme, gamma-glutamyl transpeptidase. These results suggest that a model using cultured RPE-cell monolayers is useful in identifying and understanding alterations in ocular tissues triggered by exposure to vitreous replacement materials. Invest Ophthalmol Vis Sci 31:1761-1766, 1990

The physical properties of vitreous replacements, particularly silicone oil, have been valuable in reattaching the retina in severe cases of proliferative vitreoretinopathy (PVR). However, while silicone oil has been used for almost three decades as a retinal tamponade, our understanding of possible related complications is still evolving. The development of epiretinal membranes at the silicone-oil interface, secondary to cellular proliferation, is a cause of surgical failure. Eyes with large areas of bare retinal pigment epithelium (RPE), such as seen after retinotomies, or eyes with large tears may be especially susceptible to the untoward effects of vitreous substitutes on the RPE. The mechanism of pathologic changes associated with silicone oil remains largely unknown. As a trigger for cellular proliferation, it might act directly as a mitogen, concentrate growth factors which are already present, or stimulate the production of such factors. It is difficult to determine whether the alterations associated with silicone oil occur directly or are mediated through the inflammatory process associated with a breakdown of the blood–retinal barrier. Alternatively, the silicone-oil interface may somehow mechanically affect the RPE. Hyaluronic acid has been used as a vitreous substitute, although there is no surface tension at an aqueous–hyaluronate interface.

Other models using cell culture to study the toxicity of viscoelastic materials have been of short duration. This study was undertaken to determine what effects various vitreous substitutes had on the gross morphology and polarity of human RPE cells cultured for up to 4 weeks. The effects of three hyaluronic-acid preparations (Amvisc (Medchem, Woburn, MA), Viscoat (Cooper, Bellvue, WA), Healon (Pharmacia, Pasadena, CA) and silicone oil (Wacker, Germany; 1000 cs) were studied.

Materials and Methods

Human eyes were obtained from the Medical Eye Bank of Western Pennsylvania, and RPE cells were isolated from the eye cups as described elsewhere. First-passage cells were plated on 12-mm diameter, opaque, 0.45-µm semipermeable nitrocellulose membranes (Millicell, Millipore Products Division, Bedford, MA) at densities of 10-20,000 per insert. Dulbecco's modified Eagle's medium and Ham's F-12 were used in a 1:1 ratio, supplemented with insulin (5 mg/l), transferrin (5 mg/l), and selenium (5 µg/l) (ITS; Collaborative, Bedford, MA) and 5% fetal calf serum. This medium was used to bathe the basal surface of the RPE monolayers. Hank's balanced salt solution (HBSS) was used to cover the apical surface. Fresh medium was added to the basal side every 72 hr. At confluence, as assessed by Giemsa staining of the membranes, the HBSS was replaced with 0.5 ml of either nonemulsified silicone oil or commercial preparations of hyaluronic acid (Amvisc or Healon).

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or a combination of hyaluronic acid and chondroitin sulfate (Viscoat). Care was taken to maintain the column of vitreous substitute inside the inserts above the level of the culture medium (Fig. 1) to achieve constant contact between the assayed product and the RPE surface. Controls were incubated in HBSS. All experiments were done in triplicate.

Cellular integrity was determined by measuring the release of the cytoplasmic marker, lactate dehydrogenase (LDH), into the basal media over 48 hr. Samples of the basal medium were obtained on days 1, 3, 5, 7, 9, 14, 21, and 28 and assayed as previously described.12 On days 1, 7, 14, 21, and 28 the membranes were rinsed, stained, and mounted for light and transmission electron microscopy to determine morphologic status.

Incorporation of 3H-thymidine in an acid-insoluble fraction was used to assess cell proliferation. The cells were incubated with 2 µCi of 3H-thymidine per ml of medium for 48 hr. The vitreous substitute was then carefully removed. The inserts were rinsed three times in phosphate-buffered saline (PBS), incubated in 5% trichloroacetic acid for 10 min at 4°C, and rinsed three times in 90% ethanol for 5 min at 4°C. Those membranes containing the acid-insoluble material were carefully removed from their plastic supports and dissolved by overnight incubation in 0.5 ml of 1 M NaOH. Levels of incorporation were determined by liquid scintillation counting (Beckman LS 3801, Irvine, CA).

Data were analyzed statistically using the paired t-test to compare values between control and the different vitreous substitutes.

The ability of the cell layers to retain their polarity with respect to gamma-glutamyl transpeptidase (γ-GTP) was measured after 2 and 7 days of incubation. The γ-GTP is preferentially located on the apical surface of a number of cell types,13 including cultured RPE cells. Each insert, containing cells which had been incubated with the vitreous substitutes, was rinsed three times in PBS. The apical and basal surface activity was measured by incubating the apical and basal surfaces in an assay mixture containing glycyl-glycine and glutamyl-P-nitroanilide for 15 min at 37°C. The reaction was followed by measuring the change in absorbance at 410 nm and done under conditions where the reaction rate was linear with respect to time and enzyme concentration.

Results

Cellular Integrity

Incubation of the RPE cells in the presence of Amvisc, Healon, Viscoat, or silicone oil for periods up to 4 weeks did not increase extracellular LDH activity. Extracellular LDH activity was always less than 1% of the total LDH activity (data not shown).

Cellular Morphology

At 1 week, flattening of the monolayer was seen in the presence of hyaluronic-acid preparations compared with controls. This became more apparent at 3 weeks (Figs. 2A-B) along with a greater loss of microvilli compared with controls (Figs. 3A-B). The RPE layer under silicone oil maintained a similar thickness compared with controls at 1 week; however, by 3 weeks the cells had formed sheets of three or four cell layers (Figs. 4A-B). This was not seen in controls. We observed no increase in the number of cytoplasmic vacuoles in cells grown under silicone oil.

Cellular Proliferation

Incubation of the RPE monolayers under silicone oil resulted in a higher rate of 3H-thymidine incorporation (Fig. 5). The cell layers exhibited a twofold increase after 1 week of incubation, and this level remained significantly above control levels for at least 4 weeks. In contrast, the rate of incorporation of 3H-thymidine in hyaluronic acid-treated cultures remained at or below that of controls. Cultures treated with Amvisc and Healon had proliferation rates similar to controls (HBSS). Cultures treated with Viscoat showed a transient decrease in 3H-thymidine incorporation for the first 2 weeks of incubation but returned to control levels by 3 weeks of incubation. These levels remained at control values through week 4.

Cellular Polarity

The incubation of vitreous substitutes decreased cellular polarity as measured by γ-GTP activity, except for Healon-treated cells which remained unaltered (Fig. 6). Control cultures had apical to basal ratios of approximately 10:1. After only 2 days of
Fig. 2. (A) HA at 3 weeks (LM, ×60). (B) HA at 3 weeks (TEM, ×7160).
Fig. 3. (A) Ctr at 3 weeks (LM, original magnification ×60). (B) Ctr at 3 weeks (TEM, original magnification ×6280).

Fig. 4. (A) Sil at 3 weeks (LM, original magnification ×60). (B) Sil at 3 weeks (TEM, original magnification ×6920), multilayer appearance with microvilli in the intercellular space (arrow).
incubation the apical:basal ratio decreased significantly ($P < 0.05$) in cultures incubated with silicone oil, Amvisc, and Viscoat. A four to fivefold reduction in the ratio was observed at 7 days of incubation. The individual vitreous replacement materials had no direct inhibitory effect on the enzyme activity (data not shown).

**Discussion**

Previous reports using animal models, histopathologic observations of eyes exposed to silicone oil, and clinical observations suggest that the presence of silicone oil in the vitreous cavity may lead or contribute to complications and pathology.\textsuperscript{14-18}

We found that the presence of silicone oil leads to increased cellular proliferation as shown by $^3$H-thymidine incorporation and histologic analysis. However, the mechanism of this response is still unclear. Our results support the premise that the observed increase in proliferation is a direct result of the interaction of the cells with silicone oil rather than an event which is secondary to an inflammatory response which may have been produced in an in vivo model. Lambrou and colleagues\textsuperscript{6} demonstrated previously that silicone oil promotes proliferation of membranes in vitrectomized rabbit eyes and that vitreous aspirates from these eyes exhibit increased mitogenic activity.

There are several ways by which the silicone oil-induced proliferation could be triggered. The silicone oil may induce the synthesis and secretion of growth factors which may act in an autocrine fashion on the RPE cell layer. The RPE cell is known to synthesize growth factors.\textsuperscript{19} Since these growth factors are not lipid soluble, the presence of silicone oil at the apical surface could increase their concentration and thereby enhance proliferation.\textsuperscript{7} Lastly, the silicone oil or its impurities may act directly as mitogen. Experiments are underway to distinguish among these possibilities.

Although our experiments were done in a manner to avoid emulsification, one could argue that proteins could produce emulsified droplets of silicone oil at the cell surface and allow the RPE cells to phagocytize silicone particles. However, we have no histologic evidence suggesting that silicone oil actually entered the cells.

We also observed a loss of the polar distribution of the enzyme $\gamma$-GTP under vitreous substitutes. Incubation in the presence of Amvisc, Viscoat, and silicone oil for as little as 2 days reduced the polar distribution of this enzyme. Healon had no significant effect on polarity, but Amvisc and Viscoat reduced polarity. The RPE-cell polarity plays a key role in the cell’s ability to function normally physiologically. The cell must maintain the ability to pump fluid unidirectionally from its apical to basal surface while transporting nutrients from the choroidal circulation to the apical surface. If the loss of the cell’s ability to maintain the polar distribution of $\gamma$-GTP is characteristic of a general loss of the cell’s biochemical polarity, then exposure to vitreous substitutes in vivo may severely inhibit RPE cell function. More extensive studies will be needed to determine what common characteristic, if any, triggers the loss of cellular polarity.

In summary we found that several vitreous substitutes exert direct effects on the RPE cell. These alterations in cellular polarity and proliferation may adversely affect the ability of the RPE cell to function normally in their presence.
Key words: RPE, silicone oil, hyaluronic acid, proliferation, in vitro

References