Reports

Application of Miniosmotic Pumps For Liposomal Drug Delivery to the Ocular Lens

Judith M. Megow and Sidney Lerman

The feasibility of using miniosmotic pumps in conjunction with specifically targeted liposomes to deliver agents directly to the ocular lenses of rabbits in vivo was examined in a pilot study. Concanavalin A was used to provide specific targeting of the vesicles to the lenses of the treated eyes. Seven days after pump installation, the animals were sacrificed and tissue samples from both control and treated eyes were examined for the presence of the administered agent. Four animals were treated with liposomally encapsulated Sorbinil and specific delivery to the lenses of the treated eyes was demonstrated by fluorescence spectroscopy. Two other animals were treated with liposomally encapsulated 3H-8-MOP, and specific delivery of the drug to the treated lenses was demonstrated by phosphorescence spectroscopy and by liquid scintillation counting. The drugs delivered to the treated eyes could not be detected in any of the tissues or fluids of the contralateral control eyes. Except for the ocular lens, these agents could not be demonstrated to any substantial degree in the other tissues of the treated eyes. Invest Ophthalmol Vis Sci 28:1429-1433, 1987

We have demonstrated that Concanavalin A (Con A) in conjunction with negatively charged large unilamellar liposomes results in highly specific delivery of the vesicles and their contents to the ocular lens both in vitro and in vivo. In previous in vivo studies, direct intracameral injections into the anterior chamber were employed. The present pilot project examines the feasibility of using miniosmotic pumps to provide continuous delivery of liposomally encapsulated agents to the ocular lens for in vivo experimentation, without having to resort to multiple direct injections. Eliason and Maurice have reported on the feasibility of employing osmotically driven pumps to provide continuous delivery of agents to other ocular tissues. Six animals were employed for this study. However, as will become apparent, the data obtained are of an "all or none" nature, essentially obviating the need for large numbers of animals or subsequent statistical evaluation.

Materials and Methods. Liposomes: Con A-containing large unilamellar vesicles were prepared as previously reported, using a 1:4:4 molar ratio of phosphatidyl serine: dipalmitylophosphatidyl choline: cholesterol. For the Sorbinil studies, the concentration of Sorbinil in the swelling medium was 4.0 mM. In the 3H-8-methoxypsoralen (3H-8MOP) studies, the concentration of 3H-8MOP was 1.0 mM. Sorbinil was provided by Dr. Shambu Varma (Department of Ophthalmology, University of Maryland). 3H-8MOP (specific activity 2.2 Ci/mMol) was purchased from HRI Associates, Inc. (Emeryville, CA).

To assess the stability of the liposomes used in this study, vesicles containing entrapped carboxyfluorescein (CF) were incubated in Earle's Balanced Salts Solution (EBSS), in normal rabbit aqueous humor, and in protein-containing secondary aqueous collected from rabbit eyes 15 min after a small-gauge needle had been inserted into the anterior chamber. Leakage of CF was monitored by fluorescence spectroscopy, as previously detailed. Additional assessment of vesicle leakage was made by liquid scintillation counting to determine the amount of 3H-8-MOP remaining in unused liposomes retrieved from the osmotic pumps that had been implanted in rabbits for 7 days.

Placement of miniosmotic pumps: New Zealand white rabbits weighing 4.5-4.7 kg each were used in this study—four treated with liposomally encapsulated Sorbinil, and two treated with 3H-8MOP. One eye of each animal received the osmotically delivered, liposomally encapsulated agent. The contralateral, untreated eye served as the control. Use and treatment of the animals complied with the ARVO Resolution on Use of Animals in Research. Alzet 2ML1 miniosmotic pumps (ALZA Corp., Palo Alto, CA) capable of pumping 10 µl/hr for up to 7 days were implanted in the animals' shoulder region and a dermal pocket was made between the scapulae. A Menghini needle was run forward from the incision site under the skin, across the neck and cheek, exiting at the internal surface of the lower eyelid near the temporal corner. Polyethylene tubing (PE 60 with the intraocular end tapered to the diameter of a 30 g needle) was threaded through the needle and the nee-
### Table 1. Results of liposome leakage assays*

<table>
<thead>
<tr>
<th>Duration of incubation (hr)</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>96</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EBSS</strong></td>
<td>94-96</td>
<td>93-94</td>
<td>92-93</td>
<td>91-93</td>
<td></td>
</tr>
<tr>
<td>Normal rabbit aqueous</td>
<td>95-96</td>
<td>90-93</td>
<td>88-92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit aqueous from sham-injected eyes</td>
<td>92-94</td>
<td>90-93</td>
<td>88-92</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3H-8MOP-containing liposomes†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pump experiment 1</td>
<td>92-93</td>
<td>91-93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pump experiment 2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Expressed as % of the originally entrapped agent retained in the vesicles after incubation. Ranges reflect the variation among three separate liposome preparations evaluated at each time interval. All incubations were carried out at a total liposomal lipid concentration of 2 mg/ml.

† Since only one liposome preparation was used in each pump experiment, these determinations are the average of three assays performed on each of the two preparations.

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Figure 1. (a) Fluorescence spectra of the intact treated lens from a rabbit receiving liposomally encapsulated sorbinil, showing the red shifted sorbinil fluorescence emission at 400-405 nm with both 264 nm (- - - -) and 290 nm excitation ( — — ). The intrinsic tryptophan emission at 335 nm is also seen with both the 264 nm and 290 nm excitation and the previously reported intrinsic visible fluorescence (360 nm excitation) is also present ( - - - -). (b) Fluorescence spectra of the lens in (a) after the capsule was removed. The sorbinil with both 264 (- - - -) and 290 nm ( — — ) excitation is still present (400-405 nm emission) as is the intrinsic tryptophan (335 nm emission), and the 360 nm excited intrinsic visible fluorescence is also present ( - - - -).
examined, vesicle retention of CF was 83% or better. Thus, the vesicles showed marked stability under the conditions anticipated in vivo.

No Sorbinil fluorescence could be detected by fluorescence spectroscopy in the corneas, aqueous humor or vitreous from either the treated or the untreated eyes, nor could any be detected in lenses form the untreated eyes. However, in all four lenses from the treated eyes, two emission peaks were demonstrated with both 290 nm and 264 nm excitation, which is characteristic of the presence of Sorbinil in this tissue (Fig. 1a). When the capsules were removed and these lenses were re-examined, the Sorbinil fluorescence was still clearly demonstrable (Fig. 1b).

In the animals receiving liposomally encapsulated 3H-8MOP, there was no detectable 8MOP by phosphorescence spectroscopy in the corneas, aqueous or vitreous samples from either the control or treated eyes, nor was any detected in the control lenses. In the treated lenses, however, distinct phosphorescence peaks characteristic of the drug in free form were present (Fig. 2a), which could still be detected after the capsules had been removed (Fig. 2b). A photoproduct was also detected in the treated lenses which was similar to that previously reported in lenses following in vivo 8-MOP administration. Liquid scintillation counting of tissues from the 3H-8MOP treated animals corroborated the phosphorescence findings (Table 2). No 3H-8MOP could be detected in the corneas or aqueous from either control or treated eyes, or in the control lenses or retinas. Small amounts of 3H-8MOP, however, were demonstrable in the retinas from the treated eyes (approximately two times the background levels found for comparable samples of retina from completely untreated animals). Counting of the lens capsule and homogenates of the remaining lens matter (cortex and nucleus) from the treated eyes revealed substantial activity (CPM) in both. The activities detected in the cortical and nuclear fractions from both of the treated lenses corresponded to the presence in that part of the lens of 0.039 μg of 8-MOP, while the capsular material for each lens contained activity equivalent to that in 0.025 to 0.027 μg of the drug.

**Discussion.** We demonstrated previously that Sorbinil encapsulated in Con A-containing liposomes could be delivered to rabbit lenses in vivo by a single direct injection of the vesicles into the anterior chamber. Sorbinil fluorescence in the lens always persisted for considerably longer time periods when the drug was administered in liposomes. The levels of Sorbinil lens fluorescence in the present pump experiments were comparable to those seen 1 hr after a single intracameral administration of the vesicles.

Thus, use of the miniosmotic pumps in conjunction with targeted liposomal delivery of Sorbinil to the lens provided and sustained higher levels of the drug that could be achieved employing comparable levels of the drug administered in either free form or in targeted liposomes as a single bolus dose.

With the 3H-8MOP treated animals the lenticular levels of 8-MOP (as detected by phosphorescence spectroscopy) were comparable to the levels detected in earlier studies within 2 hr after a single parenteral administration of the agent in free form. Moreover, a photoproduct was found in the treated lenses similar to that seen in animals receiving single parenteral injections of 8-MOP. Thus the levels of 8-MOP
delivered in liposomes via the miniosmotic pumps are more than adequate for studying this drug with respect to its phototoxic properties in the lens.

Interpretation of data derived from experiments employing osmotic pump delivery into the eye clearly depends upon the extent to which conditions in the eye have returned to normal after surgery. In these pump experiments, comparison of the 280 nm absorbances of aqueous from completely untreated rabbits, with the aqueous from both the control and treated eyes of the experimental animals 7 days after surgery yielded almost identical results (Table 3), indicating that the treated eyes had returned to normal or near normal condition.

The efficacy of liposomal delivery to the ocular lens strongly depends upon whether the agents in the vesicles actually get into the lens. In the present work, penetration through the capsule of both the liposomally delivered Sorbinil and 3H-8MOP was clearly shown, (Figs. 1b and 2b). Assays for liposome leakage demonstrated that vesicles of the type employed with the osmotic pumps retained 88% or better of their contents, regardless of the incubation conditions. Since previous studies have shown that the liposomes themselves do not penetrate into the vitreous, detection of the encapsulated agents themselves in either the vitreous or retina would be a means of assessing vesicle leakage in the eye in vivo. With the Sorbinil-treated animals, fluorescence spectroscopy failed to detect this drug in the vitreous of the treated eyes. Liquid scintillation counting of the retinas from the treated eyes of the animals receiving liposomally encapsulated 3H-8MOP, however, did indicate the presence of small amounts of 3H-8MOP. Thus, as expected, some leakage of vesicle contents does occur in the eye in vivo. Nevertheless, the very low amounts of 3H-8MOP activity in the treated retinas, (two times background) indicate that leakage occurring is minor, compared to the amounts of drug delivered to treated lenses.

These data demonstrate the feasibility of employing miniosmotic pumps in conjunction with specifically targeted liposomal delivery of agents to the ocular lens for experimental applications.

Key words: miniosmotic pumps, liposomes, ocular lens, 8-methoxypsoralen, sorbinil, fluorescence, phosphorescence

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References

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Table 2. Average CPM in ocular specimens from 3H-8-methoxypsoralen-treated rabbits*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount assayed</th>
<th>CE†</th>
<th>EE‡</th>
<th>CE†</th>
<th>EE‡</th>
<th>Untreated§</th>
<th>Untreated§</th>
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</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>100 µl</td>
<td>27.5</td>
<td>26.8</td>
<td>26.8</td>
<td>28.2</td>
<td>27.0</td>
<td>29.2</td>
</tr>
<tr>
<td>Vitreous</td>
<td>100 µl</td>
<td>30.9</td>
<td>31.5</td>
<td>28.8</td>
<td>27.8</td>
<td>34.2</td>
<td>31.0</td>
</tr>
<tr>
<td>Cornea</td>
<td>20 mg (±2 mg)</td>
<td>27.2</td>
<td>33.2</td>
<td>24.8</td>
<td>30.2</td>
<td>22.4</td>
<td>29.7</td>
</tr>
<tr>
<td>Retina</td>
<td>5 mg (±0.1 mg)</td>
<td>22.2</td>
<td>54.8</td>
<td>33.7</td>
<td>74.7</td>
<td>20.8</td>
<td>34.6</td>
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<tr>
<td>Lens capsule</td>
<td>5.5 mg (±0.1 mg)</td>
<td>40.8</td>
<td>28.102</td>
<td>62.0</td>
<td>28.011</td>
<td>42.0</td>
<td>61.0</td>
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<tr>
<td>Lens cortex and nuclear matter</td>
<td>25 mg (±1.0 mg)</td>
<td>22.5</td>
<td>64,433</td>
<td>49.1</td>
<td>63,878</td>
<td>22.3</td>
<td>45.0</td>
</tr>
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</table>

* Samples in amounts indicated were subjected to liquid scintillation counting in 9.0 ml RIAFLOUR scintillation cocktail (New England Nuclear, Boston, MA). Tissues were minced and/or homogenized prior to counting. Results are expressed as the averages of the CPM found for four separate 5 min counting cycles. Average background levels for 100 µl samples of water were 27.3 and 25.2 for the two series of experiments.
† CE = Control eye.
‡ EE = Experimental eye.
§ Specimens from completely untreated rabbits were employed to establish negative controls.

Table 3. 280 nm absorbance of rabbit aqueous humor*

<table>
<thead>
<tr>
<th>Aqueous samples†</th>
<th>No. of animals</th>
<th>Control eyes</th>
<th>Treated eyes</th>
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<tr>
<td>Sorbinil-treated rabbits</td>
<td>4</td>
<td>0.306-0.322</td>
<td>0.311-0.322</td>
</tr>
<tr>
<td>3H-8MOP-treated rabbits</td>
<td>2</td>
<td>0.329-0.330</td>
<td>0.298§</td>
</tr>
<tr>
<td>Untreated rabbits</td>
<td>3</td>
<td>0.292-0.331</td>
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</tr>
</tbody>
</table>

* Expressed as the range found for the control and treated eyes within each group.
† For all samples, 50 µl aqueous was diluted to a final volume of 2.5 ml in Earle's balanced salts solution.
‡ Only aqueous from one treated eye was examined, since all of the aqueous from the other treated eyes was required for phosphorescence spectroscopy and liquid scintillation counting.

The Influence of Calcium on Glucose Metabolism in the Rabbit Lens

Kenneth R. Hightower and Sharon E. Harrison

Results were obtained which demonstrate that calcium accumulation in the rabbit lens may suppress glycolysis, not only by its inhibitory effect on cation transport but by its direct effect on glycolytic enzymes. In lenses cultured in calcium-enriched medium, lactate production declined in proportion to the increase in free and bound levels of calcium. In lens homogenates to which varying amounts of calcium were added, lactate production also decreased. To insure that excess calcium was not simply chelating ATP, homogenates were exposed to calcium and then dialyzed against a calcium-free buffer prior to addition of ATP. Under these conditions, lactate production diminished maximally by approximately 50% as bound calcium increased five-fold. Invest Ophthalmol Vis Sci 28:1433–1436, 1987

Altered glycolytic activity has been reported in the mammalian lens during growth and maturation 1–3 and during cataract development. 4–6 In brain cortex, it is well documented that the absence of calcium stimulates glycolysis while an increase in calcium levels inhibits a variety of glycolytic enzymes. 7 In muscle, hexose phosphate metabolism is regulated by physiologic concentrations of calcium. 8 The present investigation is concerned with the potential cytotoxic effects of elevated levels of calcium on glycolysis in the rabbit lens.

Materials and Methods. Freshly excised lenses from New Zealand rabbits, aged 4–6 weeks were employed in all experiments. To assess glycolysis, lactate concentrations were measured in 5 ml of TC199 culture medium in which normal or calcium-loaded lenses were incubated at 37°C. Calcium-loaded lenses were obtained by preculturing lenses in a HEPES-buffered ion medium containing 20 mM calcium chloride, as previously employed. 9 During incubation, medium samples of 0.05 ml were taken at 0, 30, 60, and 180 min to establish linearity of lactate production. Lactate assay was performed using Sigma kit #826-UV (Sigma Chemical Co., St. Louis, MO) with a Gilford Spectrophotometer (Model 250; Oberlin, OH). Lactate levels are expressed as micromoles per hour per lens.

Total calcium levels in cultured lenses were measured using atomic absorption spectroscopy, 9 and were used to provide a measure of bound calcium (total minus free calcium). Free calcium measurements were performed using calcium microelectrodes made according to specifications published previously. 9 The measurements involved simultaneous determination of membrane voltage and calcium-sensitive voltage in the intact lens while monitoring the voltage difference with a two-channel differential amplifier (FD 223 WPI, New Haven, CT). Glass microelectrodes were silanyzed in N,N-dimethyltrimethylsilylamine vapors for 1 hr at 205°C to insure adequate binding of the calcium-sensitive cocktail (Fluka, Hauppauge, NY). Calcium and voltage electrodes were calibrated in pairs in a series of calcium buffers 9 with a resulting calibration curve shown in Figure 1.

Homogenate experiments were accomplished by homogenizing a lens in 2 ml of Tris-ion buffer containing 230 mM Tris-HCl (pH 7.45), 200 mM KCl, 30 mM NaCl and 4 mM MgCl2. The homogenate was divided into four aliquots of 0.5 ml each and 0.5 ml substrate (4 mM ATP, 2 mM AMP, 1 mM NAD and 6 mM glucose in deionized water) was added. Calcium in 0.01 ml volumes was added to experimental aliquots prior to substrate addition to obtain a final concentration of 1 or 5 mM. One aliquot was