otinamide N-oxide reductase activity in bovine eyes, suggesting that the ocular tissues described above are rich in enzyme activities responsible for not only drug metabolism but also intermediary metabolism.

As regards aldehyde oxidase, only the liver enzyme has been extensively characterized. Its molecular weight has been estimated to be about 300,000. This liver enzyme, which contains molybdenum, iron and FAD in its molecule, can catalyze the oxidation of both aldehydic and N-heterocyclic compounds, and can utilize electron acceptors such as molecular oxygen and potassium ferricyanide. In the field of drug metabolism, this liver enzyme has been recognized as the major reductase responsible for the reduction of a variety of xenobiotics, such as sulfoxides, nitrosamines, azo dyes, N-oxides, aromatic nitro compounds, and hydroxamic acids.

Key words: bovine, rabbit, eye, metabolic reduction, nicotinamide N-oxide, aldehyde oxidase

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Methotrexate-Anticollagen Conjugate Inhibits In Vitro Lens Cell Outgrowth

Thomsen J. Hansen,† Roxane Tyndoll,† and David D. Soll†

After-cataract, or posterior lens capsule opacification, is an undesirable but common sequel to extracapsular cataract surgery. We are investigating biochemical means to prevent after-cataract formation, which can be applied at the time of the original surgery. Based on similar research efforts in cancer chemotherapy, we have prepared a conjugate of the antimetabolic agent methotrexate with an antibody specific for basement membrane collagen, the major protein in the lens capsule. The conjugate was evaluated using biochemical measurements, and retained both antimetabolic and antibody activities. When the conjugate was applied to bovine posterior capsules in vitro, or in vivo in rabbits, it was an effective inhibitor of lens epithelial cell outgrowth in cell culture. Invest Ophthalmol Vis Sci 28:1206-1209, 1987

Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidences of both aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber-type intraocular lenses, which are now considered to be the lenses of choice in most cases. A disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within 3 yr after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells which remain after extracapsular lens extraction. This can be treated by additional surgery, but prevention of after-cataract would be preferable to treatment, and could be achieved by physically or chemically destroying the lens cells at the time of the original cataract extraction.

In animal experiments, cytotoxic drugs administered during surgery or intraperitoneally have been investigated as a means of inhibiting lens epithelial cells. Of these, the most successful has been methotrexate (MTX). MTX kills dividing cells preferentially, though not exclusively, and is used in cancer chemotherapy. Because MTX is not specific as to the type of cell that it kills, serious side effects can occur. Targeting MTX to cancer cells has been attempted by covalently linking the drug to an antibody specific for that cell type. Targeting simultaneously allows a greater concentration of MTX in the

References

vicinity of the cancer cells, increasing effectiveness, and a smaller concentration systemically, lessening side effects. Using similar reasoning, it would be desirable, for reasons of both effectiveness and safety, to target MTX to the remaining lens epithelial cells for prevention of after-cataract. Since the cells must divide for MTX to exert its cytotoxic effect, the drug should remain in the eye at least through the generation time of lens epithelial cells. While these cells normally divide very slowly and only at the equator, division occurs within 48 hr after injury, such as would occur during surgery. Untargeted drugs instilled in the aqueous fluid would be continually diluted by inflow of aqueous fluid, which is renewed with a half time of about 3 hr. For our targeting studies, we covalently conjugated MTX with an antibody to basement membrane collagen, which is the major protein of the posterior capsule.

Materials and Methods. Materials: Antibody to human basement membrane collagen was obtained from Australian Monoclonal Development (Australian Monoclonal Development Pty. Ltd., Artarmon, New South Wales). Goat antimouse serum albumin (antialbumin) was from Atlantic Antibodies (Scarborough, ME). 3'-5'-7-3HMTX with a specific activity of 33.7 mCi/mg (15.3 Ci/mol) was from Amersham (Arlington Heights, IL). Other biochemicals, including MTX, 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide (ECDI), basement membrane collagen (as control for determining anticollagen activity), and tissue culture media, were from Sigma (St. Louis, MO).

Bovine posterior capsules, including the equatorial portions, were dissected from calf eyes obtained from a local slaughterhouse. Eyes were removed within 1 hr of slaughter and kept on ice until lens capsule removal, less than 2 hr thereafter. New Zealand white rabbits were obtained from a commercial lab animal supplier. They were about 6 months old and weighed 4.5 to 6.5 kg. Procedures involving live animals were approved by the Drexel University Animal Care Committee, and conformed with the ARVO Resolution on the Use of Animals in Research.

Conjugation: Antibodies (anticollagen and antialbumin) were covalently linked with MTX using the water-soluble carbodiimide ECDI as condensing agent. Both unlabelled and 3HMTX-labelled conjugates were prepared. Ten mg MTX and 2 mg ECDI were mixed in 1.5 mL of buffer (0.01 M sodium phosphate buffer pH 7.5, all references to buffer refer to this). Premixing the MTX and ECDI provided much better protein recovery than when all three reagents were mixed together. After 10 min, the mixture was centrifuged and 150 μL of the supernatant, containing 1 mg MTX, were removed and mixed with 0.5 mg of antibody in a volume of 0.5 mL. This was shaken in the dark for 4 hr, and the conjugate was separated from unbound MTX by gel filtration on a 1-cm × 25-cm column of BioGel P6 (Bio-Rad, Richmond, CA), using buffer for elution. Column fractions were analyzed by absorbance at 280 nm and 370 nm, by inhibition of the enzyme dihydrofolate reductase and, when 3HMTX was used, by scintillation counting.

Anticollagen determination: Antibody activity was measured by enzyme-linked immunosorbent assay (ELISA). For evaluation of the antibody activity of the conjugate, 5 μg collagen in 100 μL 0.1 M NaHCO₃ was placed in a microtiter well, followed sequentially by 1% bovine serum albumin, 0.25 μg conjugate or anticollagen, enzyme-linked anti-IgG, and enzyme substrate (p-nitrophenyl phosphate). Qualitative evaluation of color provided evidence for presence (yellow) or absence (clear) of enzyme and therefore of conjugate. Limit of detection was not investigated. For antibody binding to bovine posterior lens capsule, capsule was placed in the well and treated with 50 μL of solution containing 12.5 μg conjugate (or an equivalent amount of anticollagen or antialbumin or buffer). Rabbit posterior capsules were treated with conjugate in vivo (see below). Following albumin treatment, tissues were dried under vacuum (50 μm Hg) at room temperature for 3 hr and fixed in ethanol for 15 min. Fixing in this manner eliminated false positives from untreated capsules, while retaining antibody activity of conjugate treated capsules. Assay then continued as above.

3H attachment: Attachment of 3HMTX conjugate to bovine posterior capsules was studied in microtiter wells, similar to the first steps of ELISA. 3H conjugate (1500 dpm, or 830 dpm antialbumin conjugate, or 3900 dpm 3HMTX, or buffer) was incubated with the capsule for 60 min at room temperature. The capsule was rinsed, then homogenized in 5 mL Omnifluor (New England Nuclear, Boston, MA) and counted. Rinsings were also collected and counted. Rabbit posterior capsules were homogenized and counted similarly after in vivo treatment.

Cell outgrowth: Bovine posterior lens capsules, including the equatorial portions containing epithelial cells, were placed on glass slides and treated with 50 μL conjugate containing 0.15 μg bound MTX (or 2.5 μg MTX or buffer). These were incubated for 2 hr at 37°C, in a humidified chamber. Each was then transferred, without rinsing, to a 35-mm dish containing 4 mL Minimal Essential Medium Eagle (Sigma), 0.4 mL calf serum, 14 μg sodium bicarbonate, 400 units penicillin G, and 400 μg streptomycin sulfate. Plain polystyrene dishes were used, rather than tissue culture dishes, to prevent cell attachment to the dish.
Table 1. In vivo treatment of rabbit eyes

<table>
<thead>
<tr>
<th>Rabbit #</th>
<th>Eye</th>
<th>Treatment</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R</td>
<td>Anticollagen conjugate (12.5 μg)</td>
<td>ELISA</td>
</tr>
<tr>
<td>1</td>
<td>L</td>
<td>Anticollagen (12.5 μg)</td>
<td>ELISA</td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>Buffer</td>
<td>ELISA</td>
</tr>
<tr>
<td>2</td>
<td>L</td>
<td>Antialbumin (12.5 μg)</td>
<td>ELISA</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>3HMTX anticollagen conjugate (1500 dpm)</td>
<td>3H</td>
</tr>
<tr>
<td>3</td>
<td>L</td>
<td>3HMTX antialbumin conjugate (830 dpm)</td>
<td>3H</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>MTX (2.5 μg)</td>
<td>Cell outgrowth</td>
</tr>
<tr>
<td>4</td>
<td>L</td>
<td>Anticollagen conjugate (12.5 μg. 0.15 μg MTX)</td>
<td>Cell outgrowth</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>Anticollagen (12.5 μg)</td>
<td>Cell outgrowth</td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>Buffer</td>
<td>Cell outgrowth</td>
</tr>
</tbody>
</table>

The only surface available for cell attachment and outgrowth was the glass slide. Rabbit posterior capsules, after in vivo treatment, were placed directly into medium under glass slides. Capsules were examined immediately to confirm presence of cells, incubated for 7 days at 37°C then reexamined for outgrowth.

**In vivo treatment**: Five rabbits underwent bilateral extracapsular lens removal under general anesthesia. Lens material was aspirated/irrigated from the eye with BSS (Alcon Labs, Fort Worth, TX), but no particular effort was made to remove epithelial cells at the lens equator. Each eye received a posterior chamber intraocular lens. Just before closure, 50 μL of solution containing 12.5 μg conjugate and 0.15 μg MTX (or appropriate control) was injected into the anterior chamber, as summarized in Table 1. Eyes were treated externally with a broad spectrum antibiotic ointment after surgery and twice a day thereafter. After 48 hr, animals were sacrificed by injection of an overdose of sodium pentobarbital. Posterior capsules, including equatorial portions, were removed and evaluated by ELISA, scintillation counting, or cell outgrowth, as with in vitro experiments.

**Results.** Conjugation: Tritium incorporation indicated that 2.5 moles of MTX were bound to each mole of antibody. This corresponds to 3.9 μg MTX.

Fig. 1(a) (top). Lack of cell outgrowth from capsule treated in vivo with conjugate (X160). (b) (bottom). Good cell outgrowth from capsule treated in vivo with buffer (X160).

Fig. 2(a) (top). Moderate cell outgrowth from capsule treated in vivo with MTX (X160). (b) (bottom). Moderate cell outgrowth from capsule treated in vivo with anticollagen (X160).
bound to 0.5 mg antibody. Pooled fractions with MTX activity had DHFR inhibition equivalent to 0.34 μg free MTX.

In vitro: Positive ELISA results were obtained after treatment of bovine posterior capsules with conjugate and with free anticollagen. Expected negative responses were obtained from antialbumin and buffer. 3H conjugate treatment of bovine posterior capsule showed 59% attachment of label. 3HMTX and 3H antialbumin conjugate attached less than 1%. In all cases, remaining activity was recovered in the rinsings. Lens epithelial cells from bovine posterior capsules treated with conjugate did not grow. Cells treated with free MTX were only slightly inhibited compared to buffer treated cells which grew well.

In vivo: Using ELISA, both the conjugate and unconjugated anticollagen were found to be attached to the posterior capsule after 48 hr, while antialbumin and buffer controls gave a negative response. Thirty percent of the radioactivity from the 3HMTX-anticollagen conjugate was recovered in the posterior capsule. This not only gives a quantitative measure of conjugate attachment, but also shows that the MTX remains conjugated to the antibody in vivo. An additional 8% was found in the cornea. No radioactivity above background was found in the eye treated with 3HMTX-antialbumin conjugate.

The anticollagen conjugate completely inhibited cell outgrowth when the posterior capsule was placed in tissue culture medium (Fig. 1a). Under the same conditions, good outgrowth was seen from the buffer treated capsule (Fig. 1b). Free MTX (20X the amount in the conjugate) and free anticollagen each depressed, but did not prevent, cell outgrowth (Fig. 2).

Discussion. The MTX-anticollagen conjugate that we have prepared retains both antimetabolic and antibody activities, as shown through biochemical measurements. It is an effective inhibitor of the outgrowth of lens epithelial cells in vitro, and initial animal experiments support the possibility that the conjugate would be effective in vivo. The effects seen were due specifically to the conjugate, as free MTX, free anticollagen, a nonspecific conjugate, or buffer failed to produce the desired results. We believe that the conjugate acts by attaching to the capsule, exposing lens cells to a toxic level of MTX. As with antibody targeting of MTX to cancer cells in vitro, free MTX and free anticollagen somewhat inhibited cell outgrowth, but did not completely suppress it as did the conjugate. Even more effective conjugates may be possible by increasing the molar incorporation of MTX or by using an antibody specific to lens cells, rather than to lens capsule. More complete and longer-term animal testing is needed to determine whether such conjugates are effective in preventing after-cataract, and to evaluate possible side effects.

Key words: lens, cataract, methotrexate, antibody, collagen, rabbit, cell culture

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