The Potential Usefulness to Research of Retina Obtained by Biopsy

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Retinal biopsy has been performed on normal rabbits and dogs. It was shown that retinal samples could be obtained by internal and external routes in rabbits, but in dogs inability to achieve adequate vitrectomy precluded useful retinal biopsy by the internal route. A single external biopsy specimen of 3 mm diameter was more than adequate to undertake standard histopathological examination, immunocytochemical experiments and determination of cyclic nucleotide levels. The quality of the micrographs, immunocytochemical labelling of rhodopsin and phosphodiesterase, and cyclic nucleotide analyses were similar to those obtained with retinas from freshly enucleated eyes. The surgical exercise was well tolerated by most eyes and does not preclude serial biopsies being undertaken. It is concluded that retinal biopsy provides material of sufficient quantity and quality to satisfy many laboratory needs in retinal research. Invest Ophthalmol Vis Sci 29:2-11, 1988

The search for the pathogeneses of retinal diseases has been hampered by the shortage of tissue available for laboratory studies. This has been particularly damaging in the study of degenerative and genetically determined disorders. Despite major efforts, only a limited amount of material has been obtained from patients after death; moreover, in many instances this material has given only limited information because either the time interval between death and enucleation resulted in considerable autolysis, or the advanced state of disease allowed the conclusion that cell death had occurred but prevented analysis of the cause of metabolic failure. Correlation between the functional characteristics of the diseased retina and the laboratory findings has been hampered further by the prolonged time interval between the last clinical examination and death of the patient, since few if any of the common inherited retinal disorders are associated with a shortened life expectancy.

Retinal biopsy has been considered as a possible solution to this problem. Such an exercise might be justified with fully informed consent if it could be shown that search for therapy for retinal dystrophies was unlikely to be successful without biopsy and that various objectives could be met. First it must be shown that the eye would not be put at great risk by the operation of the biopsy. Surgical techniques have been devised in rabbits and primates which appear to minimize this risk; recently this work has been extended to humans. It has been shown that hypotensive anesthesia and hyperventilation prevent choroidal bleeding. Vitrectomy has been undertaken by some workers to reduce the risk of postoperative retinal detachment due to proliferative vitreo-retinal disease although others have found this unnecessary. Furthermore, good wound closure prevents postoperative ocular hypotony and incarceration of ocular tissues. In some instances the tissue has been obtained by an internal approach, while in others an external approach has been used. Development of these surgical techniques has allowed successful removal of large intraocular tumors.

Second, biopsy would be unhelpful if laboratory expertise was inadequate for useful information to be obtained from the specimen. Laboratory techniques have now advanced sufficiently such that the activity of various enzymes and the concentration of many proteins and lipids can be assessed on small quantities of tissue. Immunocytochemistry and in situ hybridization studies can be performed with great precision. In addition, a limited number of metabolic attributes can be tested in organ support systems, and
retinal pigment epithelium has now been successfully cultured in many centers. However, most laboratory work on animal retinas has been undertaken on relatively large quantities of material removed under controlled conditions from freshly enucleated eyes, which contrasts with the limited volume of tissue which could be removed by biopsy.

Finally, it is clear that damage occurring at the time of surgery should not render the specimen unusable in the laboratory. The prolonged period of high illumination required by the procedure could cause pathological changes due to heating, drying or photocotoxicity. Good preservation of retinal morphology in biopsy specimens has been demonstrated by several authors, but no other attributes of retinal function have been tested.

In order to address this last potential problem, a series of experiments have been undertaken to assess the suitability of biopsy material obtained for laboratory analysis. Tissue was collected from normal rabbits and dogs and analyzed using biochemical and histological techniques.

**Materials and Methods**

**Surgical Technique**

Internal biopsy: Vitrectomy was performed initially using an Ocutome Model 800A (Berkeley Bioengineering, San Leandro, CA), with the cutting probe, infusion port and light pipe inserted through paralimbal sclerotomy sites. Internal biopsy was performed with a 1 mm trephine which was hollow throughout its length so that axial suction could be applied to the tissue being cut. The trephine was inserted through an enlarged paralimbal sclerotomy and the retinal sample was obtained under direct vision through the operating microscope by rotating the trephine with slight pressure against the inner surface of the wall of the eyeball. Light suction was applied to the tissue throughout the cutting. The tissue was recovered by washing the retinal disc from the trephine using balanced salt solution.

External biopsy: After opening the conjunctiva, sutures were passed under the rectus muscles to allow the globe to be manipulated. A full thickness 5 mm scleral flap with a stepped edge was reflected, exposing the outer surface of the choroid. If possible, the material was obtained using a 3 mm trephine with axial suction; if that failed, the sample was excised with scissors. After each procedure any prolapsed vitreous was abscised and the sclera was repaired with 8-0 interrupted sutures. The tissue obtained was placed in a petri dish in balanced salt solution, the retina separated from the choroid, and processed immediately.

Large samples obtained by the external route were divided into two or four pieces for the various analyses while the small internal biopsies were used intact. The retinal samples obtained by the external route were restricted to the equatorial fundus by the limitations imposed by exposure. In order to avoid damage to the lens, more posterior tissue from within the central 20 degrees was obtained by the internal route. All procedures were in keeping with the ARVO Resolution on the Use of Animals in Research.

**Preparation of Rabbit Anti-bovine Opsin Antibodies**

Rod outer segments were isolated from 40 fresh bovine eyes according to the method of Papermaster and Dreyer. Opsin was separated from other proteins on a 4 mm sodium dodecyl sulfate polyacrylamine gel (SDS-PAGE) according to the method of Laemmli and the position of the opsin band determined by scanning the gel at 280 nm. The region was excised from the gel and protein was extracted and further purified by hydroxyapatite chromatography according to previously published methods. Subsequent SDS-PAGE analysis and silver staining of the gel showed no contaminants other than opsin multimers. One hundred micrograms of purified opsin in complete Freund's adjuvant was injected intradermally into each of two rabbits on a weekly basis for 6 weeks. Animals were bled from the marginal ear vein on the fifth, sixth, and seventh weeks. The presence and specificity of opsin antibodies were determined by labelled antibody staining of nitrocellulose replicas (Western blots) obtained by electrophoretic transfer of solubilized outer segment proteins from SDS gels.

Staining of outer segment proteins was limited to opsin and its multimers. Specific antiopsin IgG was affinity-purified from immune serum by coupling purified bovine opsin to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) following instructions provided by the manufacturer, except that NaCl was omitted from the buffer that was used to bind the antigen. After the serum sample was loaded onto the affinity column and washed to remove unbound proteins, specific IgG was eluted with 4 M MgCl2 in phosphate buffered saline (PBS; 10 mM sodium phosphate in 0.9% NaCl). One milliliter fractions were eluted into 5 ml volumes of PBS in order to achieve rapid dilution of the MgCl2. IgG was dialyzed against PBS and concentrated prior to use.

**Preparation of Rabbit Anti-Bovine Cyclic Guanosine Monophosphate Phosphodiesterase (cGMP-PDE) Antibodies**

Rod outer segments were isolated from 500 frozen-thawed bovine retinas (Hormel, Austin, MN)
and homogenized in 10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM MgCl₂, 0.1 mM PMSF and 1 mM DTT, pH 7.5 (buffer A). The suspension was centrifuged at 40,000 g for 30 min and the supernatant was discarded. The pellet was resuspended in 5 mM Tris, 0.5 mM MgCl₂, 0.1 mM PMSF, 1 mM DTT, pH 7.5 and bleached on ice for 10 min. After centrifugation at 40,000 g for 30 min, the resulting pellet was re-extracted three more times as before, and the four supernatants were combined and centrifuged at 40,000 g for 3 hr. The clear supernatant was applied to a DEAE 50-A Sephadex column and eluted with a 0-1 M NaCl gradient in 20 mM MOPS, 1.5 mM MgCl₂, 0.1 mM PMSF, and 1 mM DTT, pH 7.5. Fractions (0.5 ml) were collected and aliquots were assayed for cGMP-PDE activity as described previously. 14 Fractions containing enzyme activity were pooled, dialyzed against buffer A, concentrated to 1 ml and subjected to HPLC on a TSK-3000 column. cGMP-PDE was eluted with buffer A and the fractions containing activity were pooled and concentrated. Subsequent SDS-PAGE and Coomassie blue staining of the gel showed mainly the typical 84-88 kDa doublet corresponding to cGMP-PDE together with three very minor contaminants (120, 95, and 67 kDa proteins). From this enriched preparation 750 µg of cGMP-PDE in complete Freund’s adjuvant were injected intradermally into two rabbits. The animals received a 500 µg boost after 2 weeks and 200 µg boosts every month thereafter. They were bled from the marginal ear vein on the fifth week and thereafter every 2 weeks.

Polyclonal antibodies were affinity-purified from the immune serum following the procedure described by Talian et al.15 with minor modifications. One percent pigskin gelatin and 2% BSA in PBS was used as the blocking buffer for the nitrocellulose sheets and incubation in the buffer was carried out for 4 hr at 37°C. Excised nitrocellulose strips containing the electrophoretically transferred cGMP-PDE were incubated overnight at 37°C in immune serum diluted 1:15. The bound antibodies were eluted and concentrated to 0.3 OD (1 cm light path) for immunocytochemical staining. Specificity of the affinity-purified antibody for cGMP-PDE was determined by Western blot analysis. 13 Bound antibody was visualized by horseradish peroxidase bound to goat anti-rabbit IgG. Development of the colored reaction product was carried out with 0.05% 4-chloronaphthol and 0.01% H₂O₂ in PBS.

Analysis of Tissue

Histology and routine electron microscopy: Following biopsy, the specimens were fixed at 21°C by immersion for 1 hr in a mixture of 2.5% glutaraldehyde and 2% formaldehyde buffered at pH 7.4 with 0.1 M sodium phosphate. They were fixed additionally for 1 hr in 1% osmium tetroxide, dehydrated in a graded ethanol series (50%–100%), further treated with propylene oxide and embedded in Araldite 502 (Ciba Products Co., Summit, NJ). Silver-gold sections were cut with a diamond knife and stained with lead and uranium salts.

Immunocytochemistry: Specimens for immunocytochemistry were fixed in mixed aldehydes as described above and then stored for 5 days at 4°C in phosphate-buffered 4% formaldehyde. Thereafter, they were dehydrated at room temperature in a graded series of dimethyl formamide (50%–100%) and embedded in Lowicryl K4M (Polysciences, War- rington, PA). The Lowicryl was polymerized with ultraviolet light for 24 hr at 4°C and additionally for 48 hr at room temperature. After washing in 0.1 M phosphate buffer (pH 7.4) and blocking with 4% bovine serum albumin (BSA) in phosphate buffer, sections supported by Formvar and carbon on copper grids were reacted for 1 hr with affinity-purified rabbit polyclonal IgG antibodies directed against bovine opsin or bovine light-activated cGMP-PDE. The concentration of affinity-purified primary antibody was 0.1 OD (A380, 1 cm light path) for opsin antibodies and 0.3 OD for cGMP-PDE antibodies. In each case, the antibodies were diluted in 0.1 M phosphate buffer containing 1% BSA. After washing in 0.1 M phosphate buffer, the sections were washed in saline (0.9%) containing 20 mM Tris and then incubated in goat anti-rabbit IgG antibodies adsorbed to 10 nm colloidal gold (Janssen Life Sciences Products, Piscataway, NJ). The colloidal gold-labelled antibodies were diluted 1:49 with Tris saline and incubation was for 1 hr. The sections were washed in phosphate buffer, fixed for 20 min in phosphate-buffered 2% glutaraldehyde, washed with distilled water and dried before viewing in the electron microscope.

Cyclic nucleotides: Each biopsy sample was homogenized in 200 µl of 0.1 N HCl. Two 10 µl aliquots were separated for the determination of protein by the method of Lowry et al.16 The rest of the homogenate was boiled for 1 min, centrifuged at 4000 g for 10 min, and the supernatant fraction was removed. Cyclic GMP and cAMP were measured by radioimmunoassay as described by Farber and Lolley.17 For cGMP determinations, two 30 µl aliquots of each supernatant were diluted to 100 µl with 50 mM sodium acetate, pH 6.2 and were acetylated with 5 µl of a mixture of acetic anhydride: triethylamine (1:2) prior to assay. For cAMP measurements, two 20 µl aliquots were used. The dilution buffer in this case was 50 mM sodium acetate, pH 6.2 containing 21 mM CaCl₂; acetylation was done in the same way.
The succinyltyrosine [I^{125}] methyl ester derivatives of cyclic GMP and cyclic AMP and the antibodies against these cyclic nucleotides were purchased from New England Nuclear (Boston, MA). After completion of the radioimmunoassay and counting of the samples, nonspecific binding was subtracted, total cpm/bound in the standards were plotted against increasing femtomoles of standard added and the cyclic nucleotide concentration in the tissue samples was read from the linear plot. The results were expressed as picomoles of cyclic nucleotide per milligram protein.

Results

Surgical Results

In each of two rabbits external biopsy was undertaken in one eye and internal biopsy on the other as a non-survival experiment. No particular surgical difficulties were encountered and good samples of retina and pigment epithelium were obtained by each method. Six additional rabbits were allowed to survive following unilateral internal biopsy of three and unilateral external biopsy of the remaining three. There were few external signs of inflammation 1 week after surgery and the ocular media remained clear. After 3 weeks the wounds appeared clean by ophthalmoscopy, and the retina elsewhere remained flat in all but one, in which there was limited detachment around the internal biopsy site. Two animals with flat retinas were sacrificed at this stage and four were observed for a further 6 months. At this time the appearance of the wounds was unchanged (Fig. 1) except for one in which complete retinal detachment developed in the animal that had previously had limited detachment. In addition, one animal had limited posterior lens opacities.

In two dogs internal biopsy was attempted in one eye using identical techniques; it was found that the nature of the vitreous precluded adequate vitrectomy and the specimens obtained were unsatisfactory. In both instances the dog was allowed to survive the operation. Uveitis developed and complete retinal detachment followed within 7 days. The dogs were sacrificed at that time. In two additional dogs, external biopsy was performed. Apart from bleeding from the sclera no surgical difficulties were encountered during the exposure. It proved impossible to obtain adequate samples with a trephine and tissue was removed using scissors. The retina remained flat and the ocular media clear during the 4 weeks following the procedure. External biopsy was undertaken in the fellow eye of each dog prior to sacrifice. The tissue obtained was relatively easy to handle, although in dogs the vitreous adherent to the inner retina made it difficult to free the samples from surgical instruments.

Histopathology

The morphology of the biopsy material both from dogs and rabbits was well-preserved apart from minor changes in the intra- and interdiscal spaces (Fig. 2). After surgery, examination confirmed that the retina was flat on either side of the biopsy site (Fig. 3a, d) with the exception of the animal with detachment. The inner surface at one internal biopsy site of a rabbit in which the choroid had not been disturbed was covered with a monolayer of retinal pigment epithelial cells (Fig. 3c), and with fibrous tissue at the other sites. On either side of the lesion there was a 1 mm "die back" zone (Fig. 3a, d) with loss of receptors on its central edge and absence of outer segments more peripherally. Beyond the margins of this zone the receptors appeared quite normal (Fig. 3a, b, d).

Immunocytochemistry

Ultrathin sections of Lowicryl K4M-embedded biopsies were readily stained by the two-stage colloidal gold method. Localization of two antigens, namely opsin, an intrinsic membrane protein and cGMP-PDE, a soluble enzyme, were detected in tissue sections of both species under study (Figs. 4, 5). Rod outer segments were heavily labelled with opsin antibody and significant binding was evident in inner segments (Fig. 4a), the sites of opsin synthesis and
Fig. 2. Light and electron microscopy of rabbit (a, b) and dog (c, d) biopsies. Apart from some distortion of the inter- and intradiscal spaces, the retinal morphology shows good preservation of structure. Rabbit retina removed by internal biopsy retained some pigment epithelium (short arrows). Dog retina removed by the external route lacked pigment epithelium and contained adherent red blood cells (long arrows). Magnification Figures 2a and 2c ×770; Figure 2b ×44,000; Figure 2d ×13,750.

transport.18-20 Cyclic GMP-PDE, an important enzyme in phototransduction whose activity is depressed in Irish Setter dogs and rd mice with inherited retinal degeneration,21,22 was also readily detectable in rod outer segments (Figs. 4b, 5a). The number of copies per cell for cGMP-PDE is smaller than opsin by about ten-fold,23 hence the labelling density for this protein was considerably less than that for opsin.
Cyclic Nucleotides

Although the protein content of the rabbit or dog biopsies varied from one another, the levels of cGMP or cAMP expressed per mg of protein were quite reproducible in the different specimens, particularly for the type of surgical procedure used (Table 1). Rabbit specimens 1, 2 and 3 were external biopsies whereas samples 4, 5 and 6 were obtained internally. The levels of cGMP were in the same range for all of the rabbit samples, although cAMP levels were considerably lower in the external biopsies. Only external biopsies were obtained from dogs and the levels of cGMP and cAMP measured were in agreement with those reported in the literature for normal dog retinas of different species.22,24
Fig. 4. Electron microscope immunocytochemistry of dog rods showing the junctinal zone between the inner and outer segment joined by the connecting cilium (C). Goat anti-rabbit antibodies adsorbed to colloidal gold particles were used to demonstrate binding sites for rabbit antibodies to opsin (a) and light-activated cGMP-PDE (b). Labelling of the rod outer segments (OS) is in accordance with the relative density of antigen as measured by biochemical means. Labelling of newly synthesized opsin in transit from inner segment (IS) sites of origin is also evident in Figure 4a. Magnification X27,500.
Discussion

Our experience shows that it is possible to obtain samples of retina from the rabbit and dog without destroying the integrity of the sample. The only technique which failed was the internal approach in the dog eye due to the difficulty in achieving an adequate vitrectomy. The surgical difficulties were somewhat greater in the dog than in the rabbit because of bleeding during exposure of the choroid and adherent qualities of the vitreous. Hypotensive anesthesia and controlled hyperventilation, neither of which were available to us, would have minimized the bleeding tendency.

Tissue loss in the eye due to the surgical procedure comprised the sample of retina removed and a 1 mm zone around the biopsy site. Such loss of peripheral retina would not be expected to cause appreciable visual loss and there were no detectable behavioral changes in the animals. Apart from a small area around the biopsy site, the retina appeared normal after biopsy such that the exercise would not prejudice the usefulness of subsequent biopsies. In all but one eye in this series it would have been possible to

Table 1. Protein and cyclic nucleotide content of rabbit and dog biopsies

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<tr>
<th>Animal</th>
<th>Specimen</th>
<th>Protein (mg)</th>
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* Picomoles per mg protein. Values represent the mean of two aliquots from each sample; each measurement was done in duplicate.
undertake a second or more biopsies from one eye or alternately from the other eye, since the fellow eye had recovered sufficiently from surgery. Clearly the procedure is not totally innocuous, as shown by the detachment in one rabbit after internal biopsy. However, there is sufficient evidence from this study as well as from previous work to suggest that biopsies can be taken without high risk of destroying the eye. There is little doubt that the risk would vary from one species to another. The observation that the procedure is relatively safe in one species does not allow the conclusion that it would be safe in others.

This experience shows that the specimens taken were more than adequate in size for laboratory examination. Three of the four quarters of a 3 mm sample of retina would allow histological examination, multiple immunocytochemical experiments and analysis of cyclic nucleotides. A remaining sample would be available for additional analysis such as in situ hybridization or could be kept in reserve if one sample proved inadequate. Alternatively, it is possible that a slightly smaller specimen might be obtained.

It is also shown that the removal of a retinal biopsy under normal surgical conditions does not prejudice the usefulness of the sample for research. The appearance of the tissue by electron microscopy showed little artifact, and the distribution and concentration of antigen label was in accord with that found in retina obtained by other means. Two analyses of each specimen were undertaken to determine the levels of each cyclic nucleotide. Interestingly, the results from the rabbit biopsies indicated some variation in cGMP levels among the different samples independent of the type of surgery performed, whereas cAMP levels seemed to be considerably lower in the external biopsies than in the internal ones. This may be related to the relative distribution of each cyclic nucleotide in the rabbit retina. cGMP is known to be concentrated mainly in the outer segments of rod photoreceptors,25 which are present in abundance throughout the rabbit and dog retina, whereas cAMP is enriched in the cone visual cells most of which are located in the central retina and in the cells of the inner retina.26,27 Furthermore, most vertebrate retinas, including that of the rabbit, are thicker in the posterior or central region than in the periphery. Thus, samples obtained by the internal procedure had more cellular components enriched in cAMP than did the external biopsies.

The potential usefulness of this biopsy technique is not limited to the study or diagnosis of human retinal disease. When animals are in short supply, as is the case with some breeds of dog with inherited retinal degeneration, biopsy could be used to good advantage in these cases as well as in humans. Although biopsy would not be useful in the documentation of regional distribution of change at a given point in the evolution of the disease, it would allow serial documentation of degeneration in the equatorial retina without having to sacrifice the animal when retinal material is needed.

With the current level of expertise it has been possible to identify fundamental metabolic abnormalities in animals with early onset, genetically determined retinal dystrophies. While progressively more information is becoming available on human retinal degeneration, the irregular and unpredictable supply of material which is frequently not ideally suited for laboratory analysis will not allow advances in the understanding of human disease at the same rate as has occurred in animals. That laboratory investigative techniques may reveal important new information concerning the pathogenesis of retinal dystrophies in man was illustrated recently by the demonstration of abnormal incorporation of sugars by receptors of normal morphology derived from a patient with retinitis pigmentosa.28 It is only by biopsy that retina could ever be obtained prospectively from patients with inherited retinal receptor disease. Such an exercise might not be justified before clinical and laboratory sciences have provided sufficient information to formulate hypotheses concerning the pathogenesis of human retinal dystrophies. When such a time comes it is important that the laboratory techniques will have been adapted to make maximum use of the small quantities of tissue likely to be available from biopsy.

**Key words:** retinal biopsy, photoreceptors, pigment epithelium, retinal glia, retinitis pigmentosa, immunocytochemistry, cyclic nucleotides

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### References


