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Sensitivity of photoreceptors to elevated levels of cGMP in the human retina.

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When isolated human retinas were cultured in the presence of a phosphodiesterase inhibitor or dibutyryl cyclic guanosine monophosphate (dbcGMP), degenerative changes occurred which were proportional to the concentration of drug used and the period of exposure. Low concentrations of either drug did not alter retinal morphology compared to controls. Higher concentrations provoked vesiculation of rod inner segments and rounding up of cones. Numerous pyknotic nuclei were noted in the outer nuclear layer of those preparations. Combining IBMX and dbcGMP in the same medium destroyed virtually every rod in the specimen within 8 hr of incubation. Under those conditions, cones remained structurally intact although somewhat rounded. In all treatments, cells of the inner retinal layers maintained normal morphology.

Our results suggest that elevated levels of cGMP in the human retina can alter certain metabolic processes in photoreceptors, which leads to degenerative changes and cell death uniquely in rod photoreceptors.

Several inherited retinal diseases in animals are found to be associated with elevated retinal levels of cyclic guanosine monophosphate (cGMP). It is thought that the basis for these disorders is a phosphodiesterase (PDE) in the outer retina which is unable to effectively hydrolyze cGMP. For example, in rd (retinal degeneration) mice, rod photoreceptors begin to degenerate around 10 days postnatally. Elevated levels of cGMP occur 2 to 3 days before this time, peaking at day 10 when degenerative changes are most evident. Furthermore, the PDE in the outer retina of the rd mouse shows decreased activity for cGMP hydrolysis as compared to PDE activity in normal mice. A strain of Irish setter dogs is afflicted by a disease which has a similar etiology and, like the disease in the rd mouse, has abnormally low PDE activity and correspondingly high levels of cGMP.

Lolley et al. and Hollyfield et al. utilized an in vitro system and the eye rudiment of the tad, Xenopus laevis, to directly assess the effects of high levels of cGMP on developing photoreceptor viability. When retinas were cultured in the presence of a phosphodiesterase inhibitor or dibutyryl cyclic guanosine monophosphate (dbcGMP), degenerative changes occurred which were proportional to the concentration of drug used and the period of exposure.
Figs. 1 and 2. Control retinas. (Bar = 40 μm for both.)

Fig. 1. After 4 hr incubation. All layers are present and appear normal as compared to an unincubated control. Photoreceptors are intact, and cones (arrows) are easily distinguished from rods.

Fig. 2. After 8 hr incubation. No pathological changes have occurred. Minor swelling is visible in the tips of some rod outer segments.

ence of PDE inhibitors, cGMP levels increased 3.5- to 8.5-fold, which was accompanied by the degeneration and death of the developing photoreceptor cells. Similarly, when retinas were cultured in the presence of dibutryl or 8'-bromo-analogues of cGMP, similar photoreceptor degenerative changes followed. Thus, in a normal retina, the accumulation of cGMP caused the specific destruction of developing photoreceptor cells in a manner analogous to that which occurs in inherited retinal diseases of mice and dogs.

Most degenerative photoreceptor diseases of the human retina differ from those of the animal models in that they occur relatively late in life, long after photoreceptor cells have differentiated and begun functioning. Documentations of the etiology and pathogenesis of these disorders have not been made in man due to the unavailability of specimens during early stages of affliction. In this paper we report that altered cyclic nucleotide composition in adult human retina can cause profound changes in the viability of photoreceptors.

Materials and methods. The two eyes utilized in the study were obtained from the University of Texas, M. D. Anderson Tumor Institute, following exenteration procedures. One was from a 73-year-old woman and the other from a 57-year-old man. Both patients had orbital squamous cell carcinoma. Neither individual had undergone chemotherapy or radiation treatment prior to surgery. The eyes were wrapped in aluminum foil and placed on ice for transfer to the laboratory. Within 23 or 45 min following enucleation, dissection of the retina was initiated. The entire neural retina and adhering pigment epithelium-choroid complex were gently dissected from the globe and vitreous body in the culture medium described below. After removal of the optic disc and foveolar regions, each retina was cut into thin, pie-shaped strips radiating from the center to periphery, and each strip in turn was cut into three fragments comprising central, equatorial, and peripheral retina which could be distinguished at later stages of processing and study. The culture medium utilized was based on the Ringer's-bicarbonate-glucose solution described by Basinger and Hall with the exceptions that the NaCl concentration was 140 mM, and that 2% sucrose and 0.1% case amino acids were present. Approximately 10 pieces per flask were cultured in this medium alone or in a medium to which were added the following: 4, 2, or 1 × 10^{-3} M isobutylmethylxanthine (IBMX); 3.2, 1.6, or 0.8 × 10^{-2}M dibutryl cGMP (dbcGMP); or combinations of IBMX and dbcGMP at these concentrations. All media were gassed with 95%:5% O_2:CO_2 for 20 min prior to culturing. Cultures were maintained for 4 or 8 hr at 37°C in a Dubnoff metabolic shaking incubator.
Figs. 3 to 6. Retinas after 4 and 8 hr incubation. (Bar = 40 μm for all.)

Fig. 3. Incubated in 4 mM IBMX for 4 hr. Although rod cell morphology appears relatively unaltered when compared to controls, cones (arrows) have distinctly spherical inner segment profiles. Other cells in the preparation appear normal.

Fig. 4. Incubated in 4 mM IBMX for 8 hr. Cones remain in a rounded shape, whereas rod inner segments have become vesiculated and swollen. Several pyknotic cells are present in the outer nuclear layer. The inner retina appears normal.

gassed with the above mixture at a rate of 1 ft³/min. For the 8 hr incubations, the tissues were placed in fresh media at 4 hr.

Following incubation the tissues were fixed in 3% formaldehyde in 0.1M Sorenson’s buffer for 20 min followed by 1% formaldehyde and 2% glutaraldehyde in 0.1M Sorenson’s buffer for at least 3 hr. Samples were then postfixed in 1% osmium tetroxide for 1 hr, dehydrated, and embedded in Epon 812. Sections were cut at 1 μm and stained in 1% toluidine blue.

Results. Incubating human retinas in the modified Ringer’s medium had no adverse effects on cell morphology after 4 or 8 hr of incubation. Indeed, after 4 hr in culture (Fig. 1) retinal morphology appeared virtually identical to that of unincubated retinas. After 8 hr minor swelling was visible in tips of rod outer segments (Fig. 2). No changes in inner retinal morphology were detected after 8 hr incubation in the control medium. When IBMX and/or dbcGMP were added to the control medium, retinal changes were evident which were proportional to both the concentration of drug utilized and the period of exposure. Low concentrations of either drug (2 mM IBMX or 8 mM dbcGMP) produced no significant modification in retinal morphology even after 8 hr incubation. A 4 hr incubation period in 4 mM IBMX produced rounding of the normally elongate cone inner segments, although no significant changes in rod morphology were appreciated (Fig. 3). After 8 hr in this medium (Fig. 4), however, most rod inner segments appeared swollen and vesiculated. Outer segments were not significantly different from 8 hr controls. Many pyknotic nuclei, generally held to be an indication of cell death, were also seen in the outer nuclear layer under these conditions.

Combining IBMX and dbcGMP in the same medium produced profound effects on photoreceptor morphology at all times and concentrations tested. Within 4 hr incubation (Fig. 5), most rods were severely affected; that is, inner segments were filled with many small vesicles, and many outer segments appeared distorted and discontinuous with the inner segments. Numerous pyknotic nuclei and empty spaces normally occupied by rod nuclei were seen in the outer nuclear layer. In contrast to rods, cones remained relatively in-
Fig. 5. Incubated in 2 mM IBMX and 16 mM dbcGMP for 4 hr. Cones remain morphologically healthy although somewhat distorted when compared to controls. Many rods have vesiculated or missing inner segments, and their outer segments have lost their orientation to a great extent. Pyknoses and swollen spaces are apparent in the outer nuclear layer.

Fig. 6. Incubated in 2 mM and 16 mM dbcGMP for 8 hr. Virtually every rod in the preparation is destroyed. Inner and outer segment debris lies outside a layer of structurally intact but rounded cone inner segments. The outer nuclear layer is filled with degenerating rod cell bodies and nuclei and many swollen spaces. Cone nuclei in that layer and all inner layers of the retina appear normal.

tact. After 8 hr in the combined treatment (Fig. 6), virtually every rod in the retina was destroyed or undergoing degeneration. Some rod inner and outer segment debris lay outside a well-defined layer of intact cones. The outer nuclear layer contained many pyknotic nuclei and spaces previously occupied by rod nuclei. Cone nuclei were unaffected and formed a well-defined stratum adjacent to the external limiting membrane. Incubation in the presence of dbcGMP also produced specific lesions in the photoreceptor layer, although the degenerative changes were patchy and not uniformly distributed as was observed when IBMX was utilized. For example, incubating with 16 mM dbcGMP for 4 hr resulted in no morphological changes in about nine tenths of the photoreceptors (Fig. 7), although isolated groups of rods and cones appeared to be minimally affected (Fig. 8). Culturing in 32 mM dbcGMP for 8 hr resulted in the death of most of the rods with some structural changes in cones (Fig. 10), whereas small isolated groups of photoreceptors appeared only minimally damaged (Fig. 9). It therefore appears that certain photoreceptors may be more resistant than others to dbcGMP at the concentrations tested. In all treatments neurons of the inner retinal layers were morphologically unaffected.

Discussion. The pathological changes in human photoreceptors noted in the present study resemble those induced by elevation of cyclic nucleotide levels in differentiating retina of Xenopus1,7 and those reported in the retinal dystrophies of the mouse4 and Irish setter.5 In all these studies degenerative changes occur in the photoreceptor layer in the absence of changes in the cells of the inner retina. It has also been observed in the rd mouse that rods are more severely affected than cones. Evidence has previously been presented which indicates that cGMP is produced and localized primarily in rod photoreceptor.6 The evi-
Figs. 7 to 10. Retinas incubated in dbcGMP. (Bar = 40 µm for all.)

Figs. 7 and 8. Incubated in 16 mM dbcGMP for 4 hr. Although 90% of this sample appears normal (Fig. 7), groups of photoreceptors show morphological changes in isolated patches (Fig. 8). Note misshapen cone and damaged rods.

Figs. 9 and 10. Incubated in 32 mM dbcGMP for 8 hr. Most of the preparation contains damaged rods and misshapened cones (Fig. 10). In other regions of this same section only minimal damage to photoreceptors was observed, i.e., rounded cones and vesicles in several rod inner segments (Fig. 9).
dence from studies on the rd mouse and the findings presented in this study with the human retina indicate that rod photoreceptors are also highly sensitive to elevated levels of cGMP.

That cones tend to round up in the presence of cyclic nucleotides indicates that the latter may influence the shape of these cells. In fact, investigators have recently shown that treatments which raise cyclic nucleotide levels also enhance myoid microtubule assembly in photoreceptors,10 a finding which suggests that cyclic nucleotides may mediate photomechanical events in photoreceptors of species, the rods or cones of which elongate or contract as a function of lighting conditions.

The changes produced in human retina after 8 hr in culture with IBMX-dbcGMP medium are also morphologically similar to several documented cases of photoreceptor dystrophies in man. In advanced cases of retinitis pigmentosa, for example, Szamier and Berson11 report that cones persist long after rods degenerate and completely disappear. Those cones which remain appear rounded and have truncated or absent outer segments but intact inner segments, cell bodies, and synaptic pedicles, not unlike those reported in the present study. In a study on an eye from an individual with a younger stage of this disease, Szamier et al.12 observed that photoreceptors in the foveal and parafoveal regions had inner segments with twice normal diameter which contained swollen mitochondria and large autophagic vacuoles. The outer nuclear layer in that retina was reduced to a single row of nuclei bordering on the external limiting membrane. The latter condition is similar to that which we observed in 8 hr cultures exposed to 2 mM IBMX and 16 mM dbcGMP.

Unfortunately, we do not know whether altered cyclic nucleotide metabolism underlies photoreceptor degeneration in any of the forms of retinitis pigmentosa. Measurements of cGMP levels in retinas with an advanced stage of the disease will probably prove to be inadequate in providing this information, since photoreceptor degeneration has already taken place and the conditions which caused these changes are probably no longer evident. This study is the first to provide direct information regarding the sensitivity of rod photoreceptors in the human retina to elevated levels of cGMP. This model system should prove to be a valuable tool for evaluating the mechanism of cGMP toxicity to rod photoreceptors.

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