Retinoic Acid Produces Rod Photoreceptor Selective Apoptosis in Developing Mammalian Retina

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Purpose. All-trans retinoic acid (ATRA) or 9-*cis* retinoic acid (9CRA), added to dissociated developing neural retinal cells, induces progenitor cells to adopt the rod cell's fate. Retinoic acid (RA) also produces apoptotic cell death in developing tissues. The effects of retinoids on mouse retinal development were examined.

Methods. Retinas were explanted on postnatal day (PN)1 and cultured with or without the retinal pigment epithelium (RPE) attached. Retinas were cultured for 3 weeks in the absence or presence of 100 or 500 nM ATRA or 9CRA. Morphologic development and apoptotic cell death were examined using cell-specific immunocytochemical markers, the TdT-dUTP terminal nick-end labeling (TUNEL) method, and a caspase assay.

Results. Retinal explants, with and without RPE, had similar age-dependent increases in opsin expression. In contrast, explants with RPE had less apoptosis during the first week than retinas without RPE. In explants with RPE, ATRA or 9CRA produced rod-selective apoptotic cell death in which 20% to 25% were lost by PN7 with no further loss by PN21. 9CRA-treated explants without RPE had a decreased number of apoptotic cells and a higher number of (rhod)opsin-positive cells at PN3.

Conclusions. Factors in RPE appear to regulate rod apoptosis in developing retina. Retinoids produce rod-selective apoptotic cell death during normal rod differentiation. In contrast, retinoids accelerate the expression of opsin in retinas without RPE. These differential effects of RA on rod photoreceptors—apoptosis and differentiation—are similar to those observed in other developing tissues and play an important role in both normal and pathologic development. (*Invest Ophthalmol Vis Sci.* 2000;41:937–947)

Retinoids are small, lipophilic, morphogenic signaling molecules that are derived from vitamin A and are essential for the normal development of the central nervous system including the retina.[1–3] Retinoids exert their biologic activity by binding to nuclear receptors: retinoic acid (RA) receptors (RARs) and/or retinoid X receptors (RXRs).[4–6] The RARs bind both all-*trans* retinoic acid (ATRA) and 9-*cis* RA (9CRA), whereas the RXRs bind only 9CRA. RARs and RXRs form heterodimers, and RXRs form homodimers that act as transcriptional regulators and bind to *cis*-acting DNA elements termed retinoic acid response elements (RAREs).[7] The spatial and temporal pattern of retinoic acid expression during development, the interactions with RAR and RXR receptor subtypes, and the interaction of the cognate proteins with the RAREs are each essential for normal cell differentiation, proliferation, development, and apoptosis.[8–12]

The RA system undergoes dramatic spatiotemporal and biochemical changes during the transition from the embryonic to the postnatal period of retinal development.[13,14] The concentration of RA and maximal activity of the RA-synthesizing aldehyde dehydrogenases are highest in the prenatal retina and decrease several fold during postnatal development.[15,16] RA exhibits a ventrodorsal gradient only in the embryonic retina, whereas during postnatal development, several different retinal cell types, including the retinal pigment epithelium (RPE), appear capable of synthesizing RA.[17–19] Moreover, the mammalian RPE contains a cytochrome P-450 monooxygenase that catabolizes RA.[20]

Retinoids help regulate cell proliferation in the nervous system and other tissues by modulating the balance between mitosis and apoptosis.[21–23] The effect of RA on the developing retina appears to be age-, cell type-, receptor subtype-, and preparation-specific. Exposure of whole mouse embryos to low doses of RA during early development results in microphthalmia and anophthalmia.[24] Similarly, RA induces apoptosis of cultured embryonic rat or murine cerebral neurons, hepatocytes, and thymocytes.[25–27] In contrast, RA increases the number of cells expressing rod specific opsin in dissociated embryonic chick and rat neural retinal cells.[28–30] Moreover, rod cell death results from vitamin A deficiency during development[1,2] and in mutant mice without different RA receptors or with an impaired retinoid metabolism in the RPE.[12, 31–34] Taken together, these results clearly suggest that RA affects retinal cell fate by modulating proliferation, differentiation, and survival during development.

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The retinal organ culture system has several advantages, compared with in vitro dissociated retinal cells, for studying the effects of intrinsic and extrinsic chemical factors on mammalian retinal development.[35,36] First, the retina retains its three-dimensional structure and cell–cell contacts throughout growth and development. Second, it can be cultured with the RPE intact, and this preserves the photoreceptor–RPE interactions. Proteins secreted by the RPE are necessary for the survival, proliferation, and development of photoreceptor cells.[37–39] Third, when the explant is cultured with the RPE intact, rod outer segments (ROSs) and cone outer segments form in the photoreceptors.[35,36]

The main goal of the present study was to examine the effects of ATRA and 9CRA on the developing mouse retina, with and without the RPE attached. The specific purposes were to determine whether there were any age-, cell type-, receptor subtype, or preparation-specific effects. To accomplish these four specific goals, retinas from newborn mice were cultured with or without the RPE attached in the absence or presence of ATRA or 9CRA. The retinas were examined at different developmental ages using morphologic, histochemical, and immunocytochemical methods using cell-specific antibodies, the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method and a caspase assay. We report that ATRA and 9CRA produced rod-selective apoptotic cell death in developing retinas with the RPE attached and accelerated opsin expression in retinas cultured without the RPE present. Some of the results of this study have been published in abstract form.[40,41]

METHODS

Animals and Ex Vivo Culture Conditions

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The organ culture has been described in detail.[35,36,42] Briefly, pigmented $C3H^{++/++}$ mice were decapitated, and the eyes were removed 12 to 24 hours after birth. After a rinse in 70% ethanol, the eyes were incubated in culture medium supplemented with 0.025% proteinase K (Sigma, St. Louis, MO) at 37°C for 15 to 25 minutes. The anterior segment, vitreous body, and sclera were removed, and the retina with or without RPE was flat mounted with the photoreceptor side down on a cellulose filter attached to a polyamide grid.

Control retinas were incubated in R16 medium (Gibco, Gaithersburg, MD)[43,44] supplemented with 10% fetal bovine serum (Gibco). The retinoid content of this medium was 0.35 μM retinol and 0.3 μM retinyl acetate. For the RA treatment groups, the medium was supplemented with 100 or 500 nM ATRA or 9CRA (Sigma). Retinal explants without RPE were cultured in control medium or medium containing 500 nM 9CRA. The RA-containing media were prepared from stock solutions made with dimethyl sulfoxide (final concentration 0.01%) in dim light. For each RA treatment group, three to nine explants were examined at each age for each dependent measure.

Histochemistry, Morphometrics, and Immunocytochemistry

The morphology of the retinal explants was evaluated after 2, 4, 6, 13, or 20 days in culture, corresponding to postnatal day (PN)3, PN5, PN7, PN14, or PN21. The retinal explants were fixed in 4% paraformaldehyde, infiltrated with 25% sucrose in Sörensen's phosphate buffer, and cryosectioned (8–10 μm). Sections were collected from the central and peripheral quadrants of the retinal explant. Photoreceptor counting procedures were performed on hematoxylin-eosin–stained sections using light microscope. The number of rows of nuclei in the outer nuclear layer (ONL) and inner nuclear layer (INL) of the central retina were counted once in each explant. Data were collected from three to eight explants in each group.

The identity and number of individual cell types in the retinal explants were evaluated with immunocytochemistry. Three antibodies were used: a polyclonal opsin antibody (AO; 1:10,000),[45] a monoclonal protein kinase C antibody (clone MC-5, 1:100; Amersham, Little Chalfont, UK) that recognizes rod bipolar cells, and a monoclonal glial fibrillary acidic protein (GFAP) antibody (clone 6F2, 1:800; Dako, Glostrup, Denmark) that stains upregulated Müller cell GFAP in injured retinas.[46,47] The bound antibodies were detected by secondary antibodies conjugated with either fluorescein isothiocyanate (FITC; Dako) or biotin that was reacted with avidin-horseradish peroxidase and diaminobenzidine (DAB; Vector, Burlingame, CA). Lectin cytochemistry was performed on cryosections with FITC-conjugates of peanut agglutinin (Dako).

To quantify the amount of opsin in the retinal explants, the explants were categorized into four groups, depending on the number of opsin-positive cells. Explants containing no opsin-positive cells in the photoreceptor layer were given a rank of 0, explants containing single opsin-positive cells were given a rank of 1, retinas with several opsin-positive cells were given a rank of 2, and retinas with a confluent layer of opsin-positive cells were given a rank of 3. Data were collected from four to eight explants in each group.

The TUNEL method (Boehringer–Mannheim, Mannheim, Germany) was performed on cryosections according to published procedures.[48,49] The bound biotin was reacted with streptavidin conjugated with Cy-3 (Jackson ImmunoResearch, West Grove, PA).

All histochemical and immunocytochemical reactions were examined and photographed with a photomicroscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

Caspase Assay

Retinas were removed from cultures at PN3, immediately frozen at −20°C, and analyzed for DEVD(Asp-Glu-Val-Asp)ase (DEVDase) activity within 1 week. The frozen retinas were placed in 400 μl of a room temperature buffer (50 mM Tris-HCl [pH 7.3], containing 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.2% 3[3-cholaminopropyl diethylammonio]-1-propane sulfonate [CHAPS], 3 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, and 10 μg/ml aprotinin), vortexed, incubated in the absence or presence of 50 μM z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk; Bachem, Bubendorf, Switzerland) at 37°C for 45 minutes, and centrifuged in a microfuge for 5 minutes. Samples of supernatants (50 μl) were mixed with 50 μl of 50 μM DEVD-7-amino-4-methylcoumarin (DEVD-AMC) substrate (Bachem) in the same buffer (without CHAPS), and cleavage of DEVD-AMC was measured at room temperature using a luminescence spectrometer (model LS 50B; Perkin–Elmer, Norwalk, CT) with an excitation wavelength of 380 nm (slit 10 nm) and emission wavelength of 460 nm (slit 15 nm). DEVD-AMC cleavage was

linear for 2 hours, and recovery of AMC was more than 95%. Fluorescence readings were obtained at 60 minutes and compared with a standard curve of AMC in the same buffer. Protein was measured by using the BCA protein assay (Pierce, Rockford, IL) using BSA as the protein standard. The values presented represent data from three retinas per treatment condition and are expressed as picomoles AMC formed per minute per milligram protein.

Statistical Analysis

All data are presented as means ± SEM. Morphometric data were analyzed using one-way analysis of variance (ANOVA) and Fisher's protected least-significant difference post hoc comparisons. The ranked values of opsin content were analyzed using Kruskal–Wallis one-way ANOVA by ranks followed by nonparametric multiple comparisons.[50] Statistical analyses were performed according to procedures provided by a statistical package (StatView; Abacus Concepts, Berkeley, CA). All statistical analyses were performed on untransformed data, and the difference between groups was regarded as significant at $P < 0.05$.

Results

Differentiation and Apoptosis in Control Retinal Explants Cultured with and without the RPE Attached

Mouse retinal explants were cultured for 1, 2, or 3 weeks in the presence of RPE or 1 week in the absence of RPE. From the time of explantation on PN1 throughout the culture period, the retinal explants with RPE (Figs. 1A, 1D, 1G) and without RPE (data not shown) developed and maintained morphologic characteristics comparable to the in vivo retina.[35,36,42] All retinal layers were present by PN5, were clearly visible on PN7, and remained well organized throughout the culture period. By PN7 the ONL contained seven to eight rows of photoreceptor nuclei, and this did not change over time (Fig. 2). At PN21,

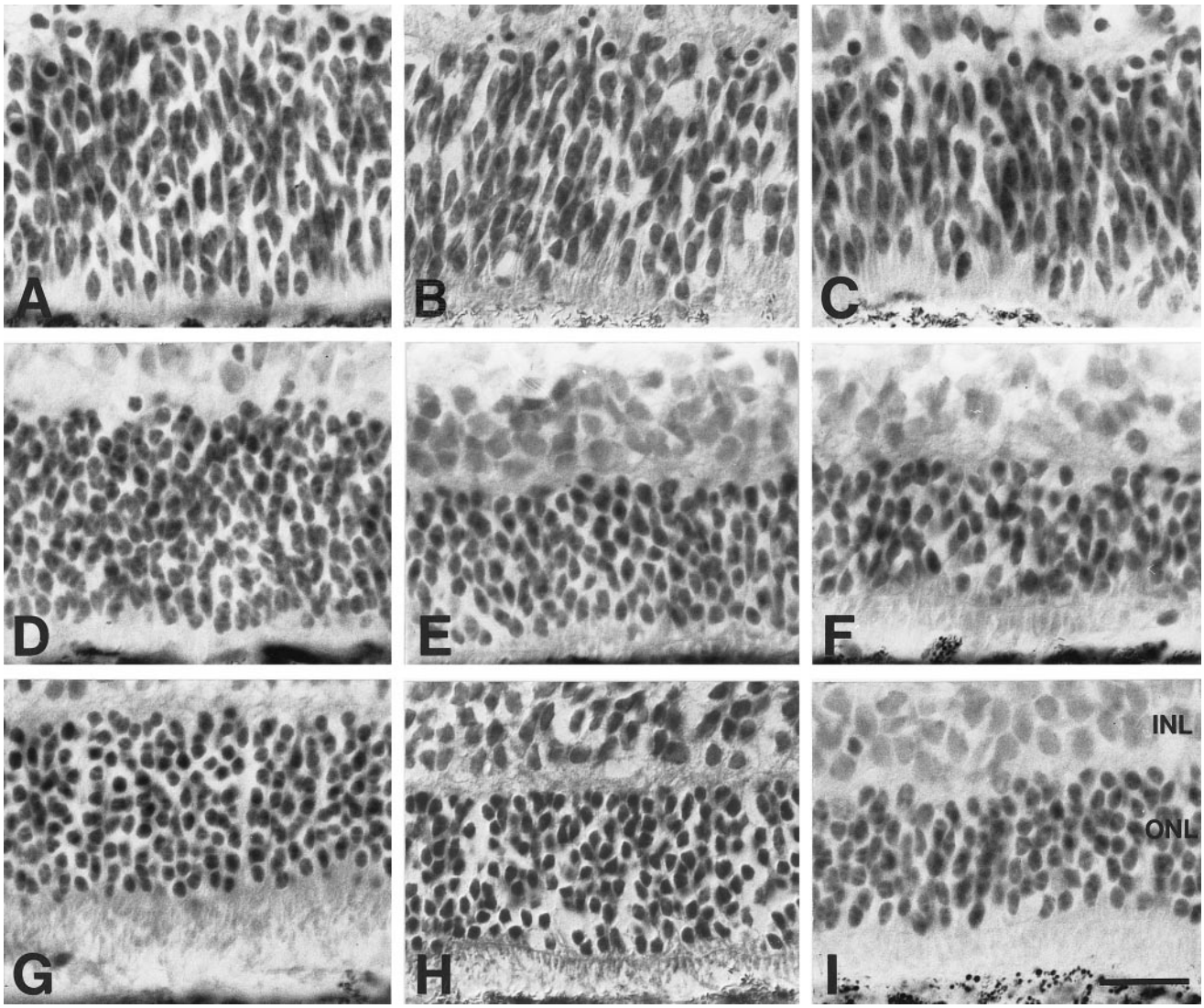

Figure 1. Light micrographs of developing mouse outer retina from retinal explants cultured with RPE attached: (**A**, **B**, and **C**) PN7, (**D**, **E**, and **F**) PN14, and (**G**, **H**, and **I**) PN21. Explants were cultured in R16 medium (**A**, **D**, and **G**), R16 containing 500 nM ATRA (**B**, **E**, and **H**), or 500 nM 9CRA (**C**, **F**, and **I**). Note that explants containing ATRA or 9CRA had fewer rows of photoreceptor nuclei than control retinas. Scale bar, 20 μm.

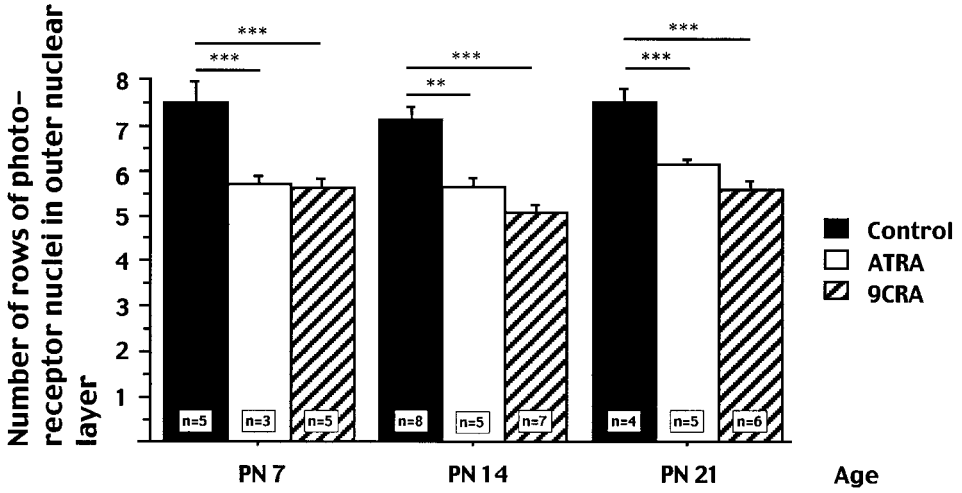

FIGURE 2. Quantitative analysis of the number of photoreceptor nuclei in the developing outer nuclear layer after the addition of 500 nM ATRA or 9CRA to the retinal explants. The data are presented as the mean number of rows of photoreceptor nuclei ± SEM for three to eight explants with RPE attached per age per treatment. There were no differences in the number of cone nuclei in any treatment, and the differences therefore reflect selective decreases in the number of rods. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.5$.

there were approximately four cone nuclei per 100 μM of central and peripheral retina, consistent with the in vivo observations.[51] However, in the INL there was an age-dependent decrease in the mean number of nuclei from five to six on PN7 to four to five on PN14 and PN21 (Fig. 3), reflecting the normal apoptotic cell death of bipolar and Müller cells between PN5 and PN18.[52,53]

In retinal explants, with and without the RPE attached, there were similar age-dependent increases in opsin expression (Fig. 4). Opsin-positive cells were first detected on PN3: consistent with the in vivo observations.[54] By PN5 the amount of rod visual pigment had increased significantly in all retinas, and by PN7 it had increased further (Fig. 4). At PN7 the developing ROS and many of the rod perikarya were opsin-positive (data not shown). During the following 2 weeks as the ROS continued to develop and elongate, the intensity and number of opsin-positive cells further increased, so that by PN14 discrete rods could no longer be detected but instead a confluent fluorescent band of opsin-positive ROS were observed (Figs. 5A, 5B).

In contrast to the finding that the presence of RPE was not important for opsin expression during the first week of development, the RPE was important for the pattern of apoptotic cell death in the developing retina. For example, on PN3 there were approximately four to five TUNEL-positive cells in the ventricular layer per 300 μm of retina cultured with the RPE attached compared with seven to nine TUNEL-positive cells per 300 μm in retinas cultured without the RPE attached (Figs. 6A, 6B). In both groups of retinas, the TUNEL-positive cells were predominantly localized to the GCL, due to axotomy at explantation, and to the ventricular layer. The TUNEL-positive cells in the ventricular layer exhibited typical characteristics of apoptotic cells. That is, the cells were scattered throughout this layer, were shrunken, and had condensed chromatin. In addition, there was zVAD-inhibitable DEVDase (caspase 3–like) activity in both groups of retinas, although there was no statistically significant difference between the two groups (Fig. 7). The absence of difference in DEVDase activity is most likely due to the contribution of caspase activity from the large number of apoptotic ganglion cells present at PN3 (Fig. 6).[52,55]

Rod-Selective Apoptosis in Retinal Explants with RPE Attached Cultured in the Presence of ATRA and 9CRA

Retinal explants with the RPE attached were exposed to 100 or 500 nM ATRA or 9CRA during the 3-week culture period. Only the 500 nM data for ATRA and 9CRA are presented, because there were no concentration-response effects of these retinoids. Exposure to ATRA or 9CRA selectively decreased the number of rod photoreceptors by approximately 20% to 25% in PN7 to PN21 retinal explants cultured with the RPE attached compared with cultured control retinas with the RPE attached

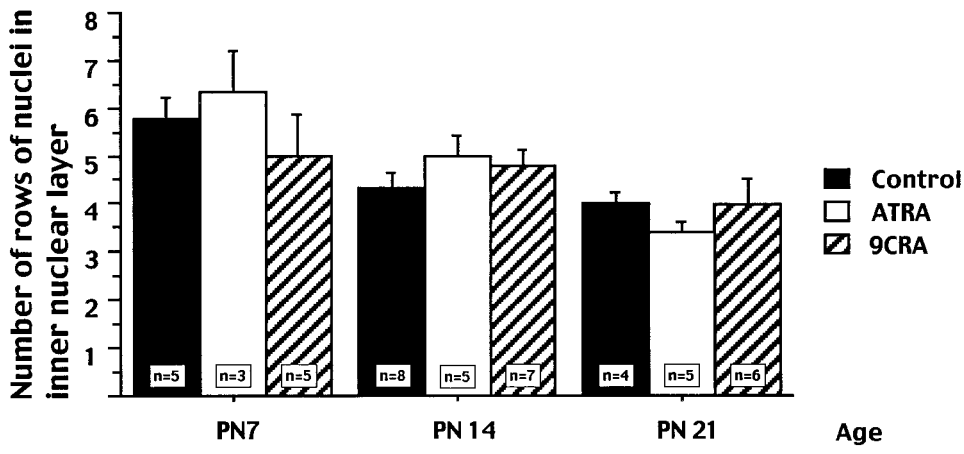

FIGURE 3. Quantitative analysis of the number of nuclei in the developing inner nuclear layer after the addition of 500 nM ATRA or 9CRA to the retinal explants. The data are presented as the mean number of rows of inner retinal nuclei ± SEM for three to eight explants with RPE attached per age per treatment. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.5$.

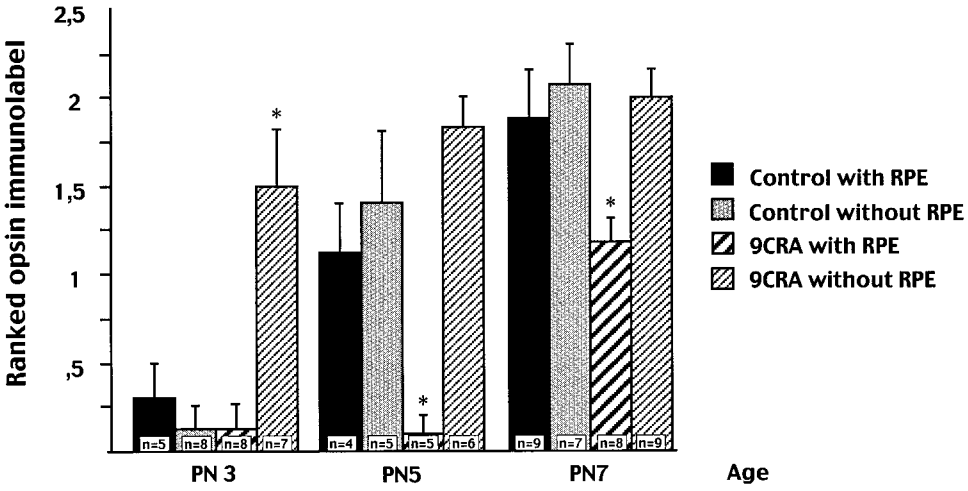

FIGURE 4. Ranked values of opsin-positive cells in mouse retinal explants cultured with or without RPE attached at PN3, PN5, and PN7. The Kruskal–Wallis test followed by nonparametric multiple comparisons between the groups at each age showed that the amount of opsin varied significantly within each of the three age groups. $^*P \leq 0.01$. The data represent values from four to nine explants per treatment group.

(Figs. 1, 2). There were no changes in the number of cones per 100 μm of retina (data not shown) or in the number of INL cells throughout the exposure period (Fig. 3). In addition, there were no differences in the number of peanut agglutinin–stained cone sheaths in the control or 9CRA-treated retinas (data not shown). To examine the onset of this rod-selective loss, the effects of 9CRA on PN3 to PN7 explants were examined. The 9CRA-induced rod loss was observed after only 2 days in culture (PN3) as evidenced by several cells with shrunken cell bodies and condensed chromatin as well as the occurrence of scattered TUNEL-positive cells (13–15 TUNEL-positive cells per 300 μm of retina) in the ventricular layer (Figs. 6A, 6C). The occurrence of TUNEL-positive cells, in conjunction with the recent finding that caspase-3 mediates the formation of internucleosomal fragments through the activation of caspase-activated DNase,[56] suggests that caspase-3 may be involved in retinoid-induced apoptotic rod cell death. Therefore, we conducted DEVDase assays in control- and 9CRA-treated explants. DEVDase activity increased 2.2-fold after the addition of 9CRA, and this increase was completely blocked when 50 μM zVAD-fmk was added to the culture medium at the same time as 9CRA (Fig. 7). In addition, the apoptotic rod cell death was blocked by zVAD-fmk (data not shown).

In addition, there were less opsin-positive rods in the 9CRA-treated retinal explants with RPE on PN5 and PN7 (Fig. 4) as well as in ATRA- and 9CRA-treated retinas on PN14 and PN21 than in age-matched control retinas (Figs. 5C through 5F). This further supports the finding of early and selective rod loss. Similar to the PN7 developing retina, the rod perikarya were stained in the 9CRA-treated retinas on PN21 (Fig. 5F). This is consistent with findings of opsin redistribution to the nuclei in degenerating rods.[57] Furthermore, the rod-selective decrease observed at PN7 did not change with two additional weeks of exposure to ATRA or 9CRA (Figs. 1, 2). Moreover, there were no retinoid-induced changes in the histologic appearance or immunoreactivity of protein kinase C–positive rod bipolar cells or GFAP-positive Müller glial cells at any age, evidenced by the micrographs from PN14 (Fig. 8). The current results are consistent with previous work showing that GFAP is upregulated in control explants.[42] Taken together, these results demonstrate that ATRA and 9CRA produced rod-selective apoptotic cell death in the developing retina and that the results were not due to a general cytotoxic effect of these retinoids.

Acceleration of Opsin Expression in Retinal Explants without RPE Attached Caused by 9CRA

Retinal explants cultured without the RPE attached were exposed to 500 nM 9CRA from PN1 to PN7. As previously noted, control retinas cultured without RPE exhibited an increase in the number of TUNEL-positive cells in the ventricular layer at PN3 (Figs. 6A, 6B). In contrast, at PN3 9CRA-treated retinal explants cultured without RPE exhibited a decreased number of TUNEL-positive cells in the ventricular layer (Figs. 6C, 6D). Moreover, in the 9CRA-exposed retinal explants without RPE there was a significantly higher number of opsin-positive cells present at PN3 compared with control retinas with or without RPE or to 9CRA-treated retinas with RPE (Figs. 4, 5G). By PN5 the number of rhodopsin-positive cells was similar in control explants and 9CRA-treated retinal explants without RPE. However, in 9CRA-treated explants with RPE the amount of opsin was significantly lower than in the other groups on PN5 and PN7 (Fig. 4).

DISCUSSION

The overall goal of the present study was to examine the effects of ATRA and 9CRA on the developing retina in culture, with and without the RPE attached. There were two major findings. First, ATRA or 9CRA produced rod-selective apoptosis in retinal explants cultured with the RPE attached. Second, 9CRA caused an earlier onset of opsin expression in explants without the RPE attached, compared with both control and 9CRA-treated explants with RPE, and control explants without RPE.

In addition, neither the onset of expression nor the amount of opsin expressed was affected by the presence or absence of RPE in control explants during the first postnatal week.

However, in control explants the RPE was found to protect the retina from apoptosis during the first week of postnatal development because in its absence there was an increase in apoptotic cell death in the ventricular layer.

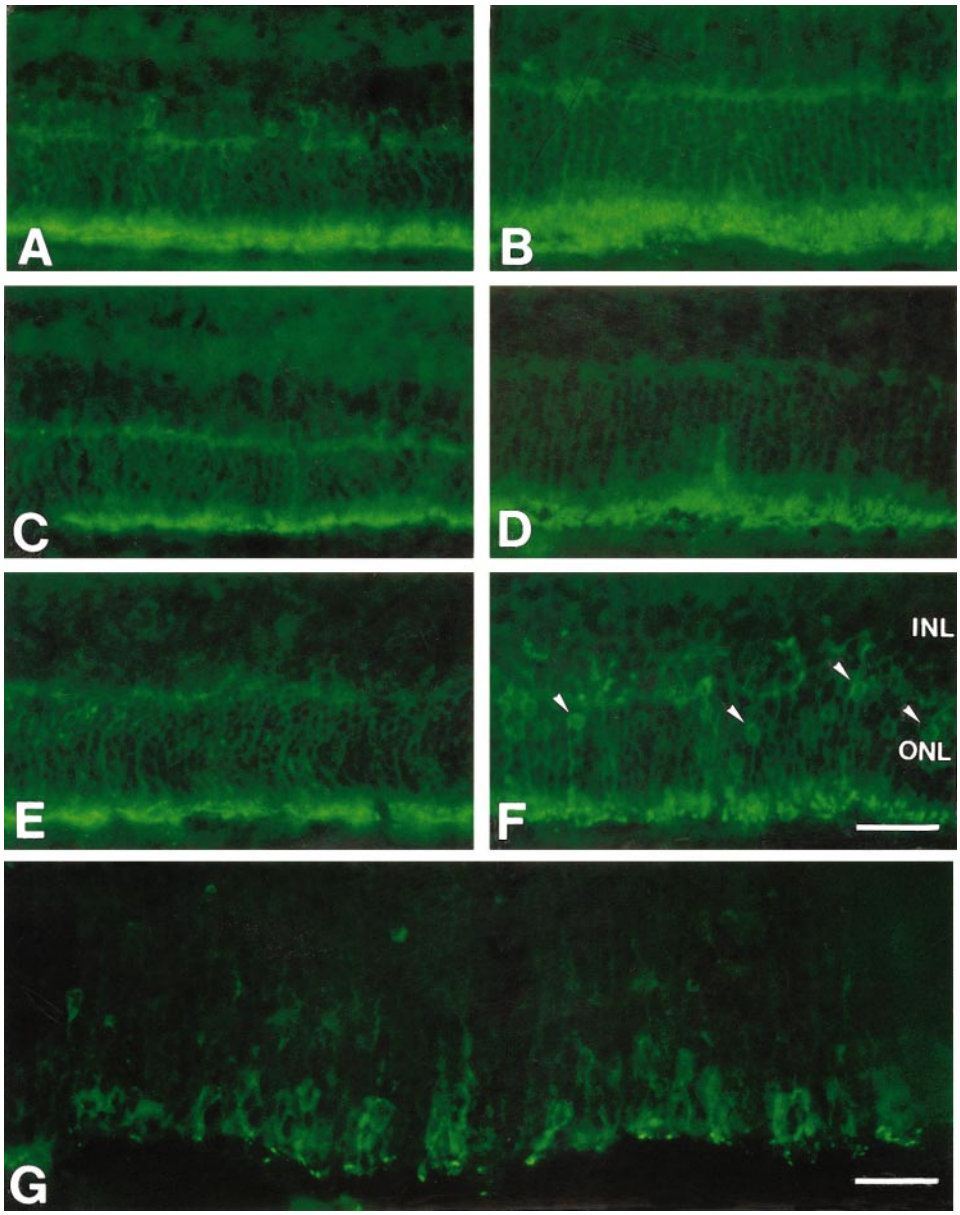

FIGURE 5. Immunohistocytochemical staining of developing mouse retina from retinal explants cultured with RPE attached (**A** through **F**) or without RPE attached (**G**) and stained with antiopsin antibody (AO): (**A, C**, and **E**) PN14, (**B**, **D**, and **F**) PN21, and (**G**) PN3. Explants were cultured in R16 medium (**A** and **B**), R16 medium containing 500 nM ATRA (**C** and **D**), or 500 nM 9CRA (**E**, **F**, and **G**). The reactions were visualized with FITC. *Arrowheads* indicate immunolabeled cell bodies. Scale bars, (**A** through **F**) 35 μm; (**G**) 30 μm.

High-performance liquid chromatography measurements have shown that the embryonal mouse retina has a total RA content of 500 nM and that 310 nM is ATRA, and that in adult retinas, the RA content is four times lower.[58] We thus believe that the RA concentrations used in the present study were in the physiological range. Apart from the RA that was added to the treatment groups, additional sources of retinoids were the putative contribution of retinol from the added 10% fetal calf serum, which can contain approximately 0.5 μM retinol, and the 0.35 μM retinol and 0.3 μM retinylacetate that are constituents of the R16 medium. These sources were equal for both treatment and control groups.

In the retinal explants the RA content is the sum of the amount of RA added to the medium, synthesized mainly by the RPE but also by the neural retina,[18] and catabolized by the RPE cells.[58] During the postnatal period aldehyde dehydrogenases capable of synthesizing RA from retinol are mainly present in the RPE and to a lesser extent in the neural retina.[18] In the explants of the present study, it can thus be expected that retinol can be converted to RA mainly in explants with RPE. However, RA cannot be converted back to retinol because the second oxidation step in the conversion of retinol to RA is an irreversible reaction.[58] RA appears to be catabolized only by a cytochrome P-450 monooxygenase present in high concentrations in bovine RPE cells.[20] In embryonic chick retinal cells without RPE attached, exogenous RA was recovered unmetabolized after 24 hours in culture.[59] In our culture system, degradation of RA can thus probably only be expected in explants with RPE. We speculate that the concentrations of RA in the retinal explants with and without RPE are comparable, because an endogenous synthesis of RA can be balanced by an endogenous degradation in explants with RPE present.

The finding that exposure to ATRA or the prototypical panagonist 9CRA produces a similar degree of rod-selective cell death suggests that the effects of these retinoids were mediated through the RARs, because 9CRA and ATRA have similar affinities to this receptor-type,[4] whereas only 9CRA is a high-affinity ligand at RXRs.[5,6,60] In support of this suggestion are

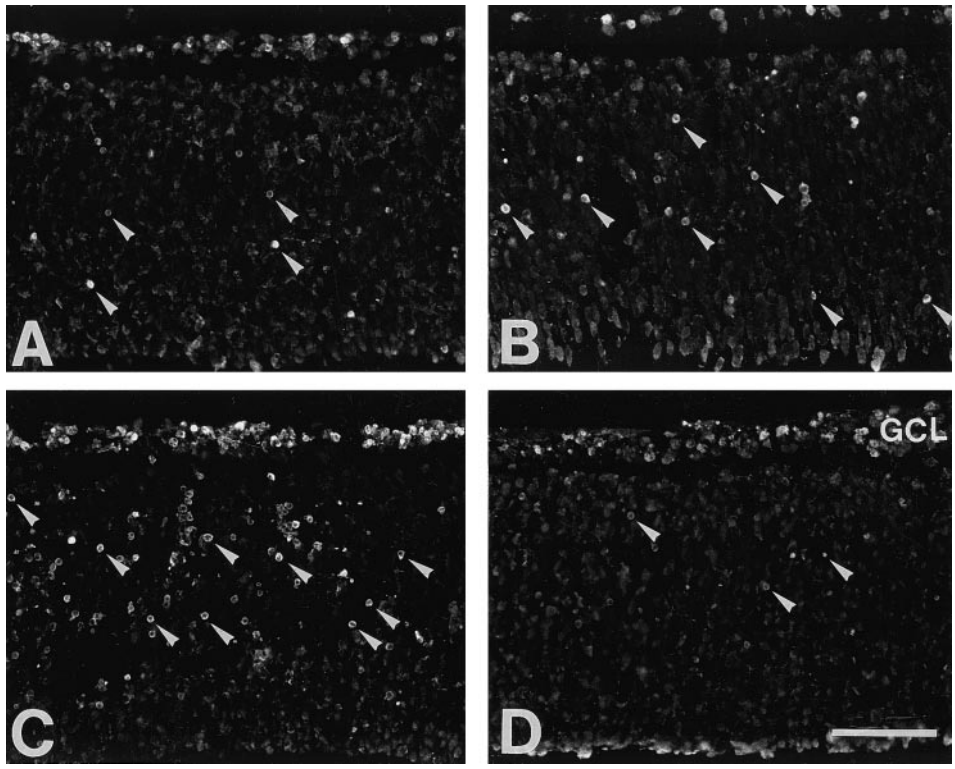

FIGURE 6. TUNEL staining of PN3 mouse retina from retinal explants cultured with RPE attached (**A** and **C**) or without RPE attached (**B** and **D**). The cultured control with RPE (**A**) contained fewer TUNEL-positive cells than the cultured control without RPE attached (**B**). In explants cultured in 500 nM 9CRA the retina with RPE attached (**C**) contained more TUNEL-positive cells than the explant without RPE attached (**D**). *Arrowheads* indicate examples of TUNEL-positive cells. Scale bar, 70 μm. GCL, ganglion cell layer.

findings that RARs are present in the developing postnatal neural retina and RPE of mice.[8,10,32]

However, when immunolabeled opsin in explants treated with 9CRA or ATRA are compared, there is a tendency toward a greater potency of 9CRA in decreasing the amount of opsin (Fig. 5C through 5F). Also the number of labeled rod cell bodies were more numerous at PN21 in 9CRA-treated explants than in ATRA-treated ones, indicating a higher degree of degenerating rods in these explants.[57,65] These results indicate that the effects of ATRA and 9CRA are mediated through RXR receptors. Because ATRA does not bind RXRs, it first requires isomerization into 9CRA, which may explain the lower effect of ATRA in decreasing the opsin amount.

Little is known about the presence of RXRs in the postnatal mouse retina. In the embryonic mouse neural retina RXRs are present until at least embryonic day 16.5.[11] However, in other species RXRs have been found also at later stages, such as RXRγ in photoreceptors in 2-week-old chicks[61] and RXRα and RXRγ in mature human retina.[62]

Taken together, these results are not conclusive enough to decide through which receptor RA exerts its apoptosis-producing effect. It could be mediated by either RARs or RXRs.

In the developing mouse retina, opsin-positive cells are first detected on PN3,[54] whereas ROS first appear by PN6.[63,64] In our ex vivo culture system, opsin was first detected on PN3 in explants with or without RPE. Similarly, opsin expression at PN5 and PN7 did not depend on the presence of RPE. Thus, during the first postnatal week most of the opsin protein in our explants, similar to that found in vivo,[65] was localized to the rod inner segments. In addition, opsin-positive immunoreactivity was found in our PN14 retinas, with (Fig. 5A) or without RPE (data not shown). Opsin in PN14 explants without RPE was still localized to the rod inner segments. Our results are different from those showing that photoreceptor progenitors from PN2 rat retinal neural pieces express little or no opsin on PN9 in the absence of medium conditioned with RPE cells.[39,66] Preparation differences may underlie this discordance.

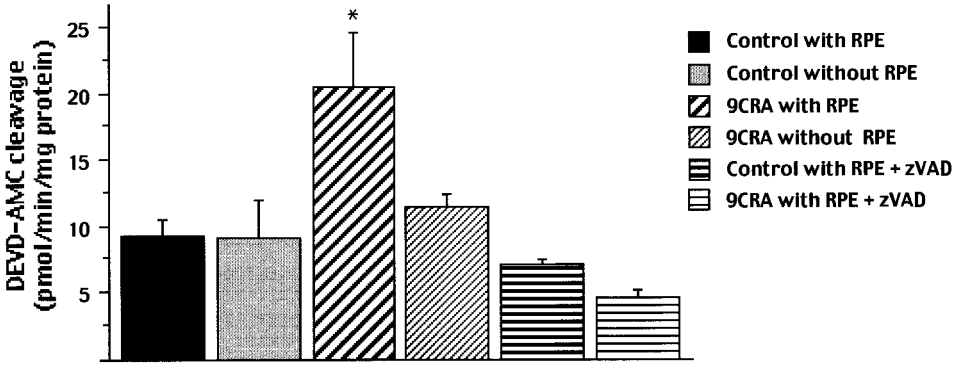

FIGURE 7. DEVDase specific activity of PN3 retinal explants cultured with or without the RPE attached in the presence or absence of 9CRA. On PN1, 50 μM zVAD-fmk and/or 9CRA were added. The data presented are the mean DEVDase enzyme activity $\pm$ SEM for three explants per treatment group. $^*P < 0.05$.

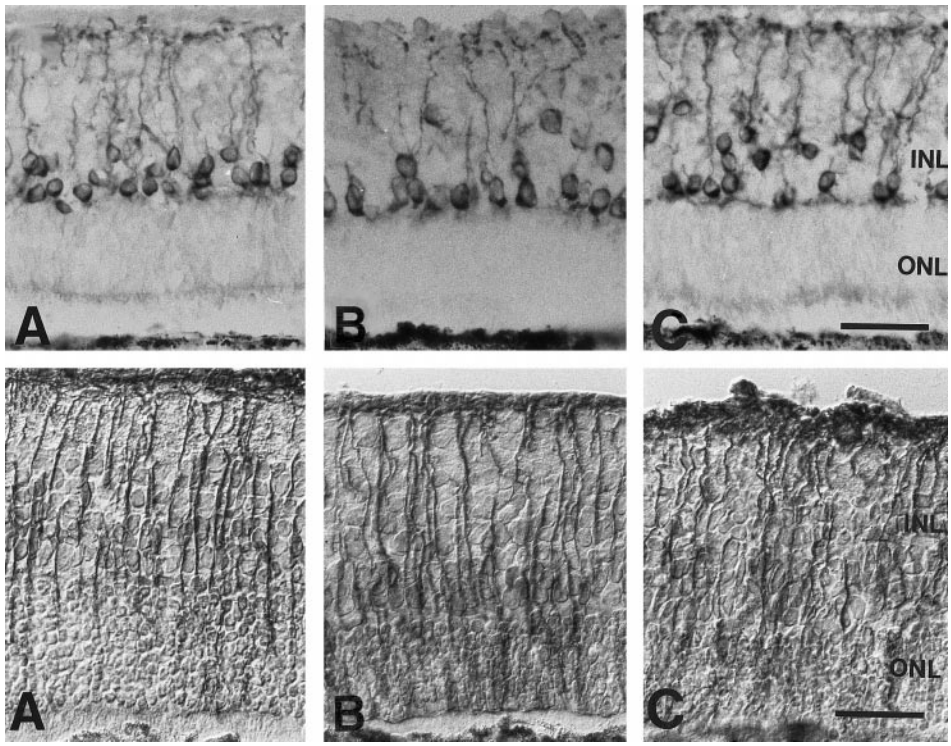

FIGURE 8. *Top:* Immunohistocytochemical staining of rod bipolar cells of PN14 retinal explants cultured with RPE attached with anti-protein kinase C antibody: control (**A**), 500 nM ATRA (**B**), and 500 nM 9CRA (**C**). The reactions were visualized with DAB. *Bottom:* Immunohistocytochemical staining of Müller cells of PN14 retinal explants cultured with RPE attached with anti-GFAP antibody: control (**A**), 500 nM ATRA (**B**), and 500 nM 9CRA (**C**). The reactions were visualized with DAB. Scale bar, 35 μm.

Although the absence of RPE did not affect the expression of opsin during the first postnatal week of development, retinal explants cultured without RPE contained significantly more TUNEL-positive cells in the ventricular layer on PN3 than explants with RPE. During normal retinal development in the mouse, there are approximately two apoptotic cells per 300 μm of retina in the ventricular layer on PN3.[52] In PN3 explants with the RPE attached, we found a similar but slightly higher number of apoptotic cells (i.e., four to five apoptotic cells per 300 μm of retina). In contrast, in retinas cultured without the RPE attached, the number of apoptotic cells in the ventricular layer was doubled. This is in agreement with findings that intravitreal injection on PN7 of an antibody raised against proteins in RPE-conditioned medium has a thinning effect on the ONL in rats.[39] It thus seems that factors synthesized by the RPE protect the developing rods from apoptotic cell death. The results of the present study showing that RA produced a decrease in ONL thickness, an increase in number of apoptotic cells, and an increase in DEVDase activity on PN3, only in explants with RPE, suggest that the expression of such a putative protective factor could be regulated by RA. Our results can be explained if RA switches off the synthesis of the factor, which then leads to photoreceptor cell death.

Likely candidate proteins for this protective factor include basic fibroblast growth factor (bFGF), epidermal growth factor, platelet-derived growth factor, and pigment epithelial-derived factor.[67–71] Recent evidence, using a purified rat photoreceptor culture system, shows that bFGF directly increases photoreceptor survival of developing rods.[71]

ATRA and 9CRA each produced a 20% to 25% selective loss of rods in PN7 retinas cultured with RPE attached. Two additional weeks of exposure to ATRA or 9CRA did not result in any further rod loss. Morphologic, histologic, and biochemical evidence demonstrated that the rod-selective cell death, observed during the first week, occurred by apoptosis. Specifically, the dying cells had shrunken cell bodies, condensed chromatin and nucleosomal DNA fragmentation, evidenced by a threefold increase in TUNEL-positive cells. In addition, there was a significant decrease in the number of opsin-positive cells in the 9CRA-treated retinas with RPE during the first postnatal week that most likely reflects the decreased number of rod cells. Moreover, at PN3 there was a twofold increase in DEVDase activity. Finally, the DEVDase activity and apoptotic rod cell death were inhibited when zVAD-fmk was added to the culture medium. Consistent with findings that caspases are necessary for the final activation stages of apoptotic cell death[72] and that caspase-3 mediates the formation of internucleosomal fragments,[56] we interpret our results to suggest that caspase-3 is involved in the retinoid-induced apoptotic rod cell death pathway. The molecular mechanism by which ATRA and 9CRA initiate the retinoid-induced rod cell apoptosis is unknown. Recent results show that Fas antigen expression in differentiating cerebral cortical cells results in neuron selective cell death[73] and that ATRA upregulates Fas antigen expression.[74] Thus, we are investigating whether rods use the Fas pathway of caspase-3 activation and apoptosis[72] to initiate retinoid-induced rod cell death in developing retina.

The results from the present study share some important similarities with the findings on the vitiligo mouse. This mutant mouse has an impaired retinoid metabolism, in which the levels of all-*trans* retinol and retinyl palmitate in the RPE are increased several times during development.[33,34] After prolonged elevation of retinoids, the rod photoreceptors are impaired and eventually die by apoptosis.[75]

In contrast to the finding that ATRA and 9CRA produced rod-selective apoptotic cell death in retinal explants with

the RPE attached, 9CRA accelerated the expression of opsin in retinas cultured without the RPE attached. This finding is similar to results showing that ATRA accelerates the appearance of opsin by 1 day in embryonal rat retinal explants without RPE and increases the number of cells expressing opsin.[30] The mechanism mediating this effect on opsin expression is unknown. However, the opsin promoter in the *Drosophila Rh1* opsin gene contains a binding site for RA and promoter activity is increased by RA.[76] Thus, it is possible also in mammals that vitamin A and its derivatives not only provide the chromophore to opsin but also control its expression. In teleost fish ATRA has been found to have effects on both apoptosis and rhodopsin expression. In the developing zebra fish retina ATRA accelerates the expression and increases the amount of opsin in rods in vivo, but there the RPE was present. However, it was also found in this species that ATRA affected the cone population by inhibiting cone maturation,[77] and in rainbow trout the developmentally normal loss of UV-cones was accelerated.[78] These findings are in contrast to findings of the present and other studies on human[79] and rat[29,30,80] retinal cells, in which no effects of RA on cones have been reported. Species differences may underlie these discrepancies.

Moreover, there was a significant decrease in the number of apoptotic cells in PN3 retinas cultured without RPE and exposed to 9CRA compared with the corresponding control retinas. These finding are consistent with results obtained with dissociated and cultured developing rat or chick retinal cells without the RPE showing that retinoids increased the number of rod cells in culture.[28,29] In the rat neural retinal cells, RA increased the proportion of cells that became photoreceptors with no change in overall cell number,[29] whereas in the chick neural retinal cells, RA significantly increased the number of differentiated rods and also slightly increased the number of nonphotoreceptor cells.[28]

In summary, results from the present study show that the effects of RA were dependent on whether the developing retinas were cultured with or without the RPE attached. In the presence of RPE, RA produced rod-selective apoptotic cell death, whereas in its absence it appeared to promote the expression of opsin and survival of rods. RA, in several other developing neural and nonneural tissues, has been shown to induce both apoptosis and differentiation.[22,26,81] The apoptotic effects of RA may underlie the craniofacial abnormalities observed in humans and animals after perinatal administration of retinoids.[24,82] On the contrary, RA plays an important role in the differentiation and development of the normal retina.[3,58]

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