Retinoic Acid
A Key Molecule for Eye and Photoreceptor Development

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Vitamin A has long been known to play a critical role in vision. The aldehyde form of the vitamin (retinal or retinaldehyde) serves as chromophore for all known visual pigments. Specifically, the 11-cis isomer of retinal binds to proteins, termed opsin, to form both the rod and the cone visual pigments. Light isomerizes the bound 11-cis retinal to the all-trans form, initiating excitation of the photoreceptor cell. Deficiency of vitamin A causes night blindness (loss of visual sensitivity) as one of its first symptoms, and reports of nutritional night blindness are found as far back as the ancient Egyptian medical papyri.

The realization that vitamin A also is involved in retinal and eye development stems from studies beginning in the 1930s and extending into the 1960s that showed an excess or a deficiency of vitamin A during development cause eye abnormalities. Recent experiments suggest that the acid form of vitamin A (retinoic acid) is critical for early eye and photoreceptor differentiation and is the subject of this review (see Fig. 1).}

RETINOIC ACID SIGNALING PATHWAY

Retinoic acid (RA) exerts a wide variety of effects during vertebrate development and cellular differentiation. It plays a major role in anteroposterior patterning of the body; in the formation, patterning, and growth of the limb bud; in spermatogenesis; and in the formation and maintenance of the skin. Retinoic acid exerts its effects on cells by binding to cytosolic receptors that serve as transcription factors. The retinoic acid receptors belong to the superfamily of nuclear receptors, which include the steroid hormone, thyroid hormone, and vitamin D3 receptors. The RA-sensitive receptors bind as heterodimers to RA-responsive DNA elements (RAREs) located in the regulatory regions of target genes and, thus, can control gene transcription directly. It has been hypothesized that the capacity of retinoic acid to generate its diverse biologic effects is controlled at various levels of the RA signaling pathway.

Several physiologically active forms of RA, such as all-trans RA, 9-cis RA, all-trans 3,4-didehydro RA, and 4-oxo RA, have been identified, and the synthesis of these forms appears to be cell specific. The two families of nuclear receptors, the RARs and RXRs (consisting of the isotypes α, β, γ and their isoforms) serve as a second source of control for the varied biologic effects of retinoic acid. The RAR family of receptors is activated by all-trans, 9-cis, 3,4 dihydro, and 4-oxo RA, whereas the RXR receptor family is activated exclusively by 9-cis RA. Activated RAR/RXR heterodimers bind to a variety of polymorphic response elements (RAREs), and recent findings suggest that these receptors also interact with multiple coactivators and repressors. Therefore, the complexity of RA signaling is heightened further by an array of combinatorial effects of the ligand bound RAR/RXR heterodimer with various cis-regulatory elements and auxiliary binding proteins.

The first data to identify the spatial and temporal patterns of activated RA receptors in developing eye tissues came from two laboratories that generated transgenic mice carrying a reporter construct containing multiple copies of a RARE from the human RARβ-2 gene. The RARE from the RARβ-2 promoter is a highly responsive indicator element for the presence of activated RARs. By coupling this DNA element to a minimal promoter–reporter construct, distinct and reproducible patterns of reporter gene expression were observed in specific embryonic regions, including the developing optic cup, as early as embryonic day 9.5.

RETINOIC ACID SYNTHESIS IN THE VERTEBRATE RETINA

Retinoic acid is generated in the developing and postnatal mouse eye by three enzymes that convert retinaldehyde to RA. These enzymes are expressed in an ordered spatial arrangement and are temporally regulated during retinal morphogenesis and differentiation.
FIGURE 1. The conversion of retinol to active metabolites. Retinol can be converted to a storage form (a retinyl ester) or to a retinaldehyde. The aldehyde form of the vitamin, required for the visual process, is converted to all-trans retinoic acid through an irreversible reaction.

The V2 enzyme is the first observable enzyme in the embryonic eye and appears in the mouse at approximately E8 within the general region of the early optic primordia. Preliminary results indicate this enzyme is localized at the site ventral to the optic pit. Later it is expressed in the RPE throughout the life of the animal. The ventral retina enzyme, V1, becomes detectable in the early optic primordia at E8.5, but it disappears completely by postnatal day 14 (P14). The dorsal enzyme is expressed at E9 within the dorsal region of the early eye vesicle; it persists throughout life but at levels much lower than those detected during embryonic development. Thus, during embryonic development, retinoic acid levels are highest in the ventral retina. At these early stages, the retina contains a ventrodorsal RA gradient. Because of the disappearance of V1, the postnatal partitioning of RA reverses, and a dorsoventral gradient is present. The developmental expression of the V2 enzyme in the RPE is complementary to that of the retinal enzymes at these early postnatal stages. With continued development, the activity of the dorsal enzyme is reduced greatly. Furthermore, the highest levels of RA are in the photoreceptor cell layer, and the lowest are in the ganglion cell layer.

As in the embryonic mouse eye, the developing zebrafish retina is rich in endogenously synthesized RA, with ventral levels significantly higher than dorsal levels. As in the mouse retina, expression of the ventral enzyme, localized to the ventral two thirds of the retina, precedes that of the dorsal enzyme by several hours. Therefore, only the ventral dehydrogenase is active during the first 24 hours of zebrafish eye development. Retinoic acid synthesis is mediated in a dorsal compartment, covering approximately one third the retinal area, by an enzyme resembling the mouse ADH2 class-1 aldehyde dehydrogenase. In the adult zebrafish, both enzymes persist, and they retain their asymmetrical distribution throughout life. This is probably because in fish, the retina grows throughout life and continues to expand at the periphery. Therefore, any mechanism important for establishing dorsoventral differences in the embryonic retina is likely to persist in the retinal periphery of adult fish.

DEVELOPMENTAL EFFECTS OF ALTERED RETINOIC ACID LEVELS WITHIN THE EMBRYONIC RETINA

To determine the role of retinoic acid in early eye development, perturbations in RA levels during the formation of the optic primordia were examined in zebrafish. As in other animals, the stage in the zebrafish eye most susceptible to retinoid perturbations coincides with the development of the optic primordia. RA excess during this stage causes proliferation of cells in the ventral region of the eye and results in a duplication of the retina (Fig. 2A). This duplication occurs along the dorsoventral axis of the eye. The RA-induced hyperproliferation of cells within the ventral segment of the eyefield generates a new layer of neural epithelium, separated from the original
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This process is observed approximately 7.5 hours after the application of RA, and each of the neural retinal epithelia subsequently develops independently. Invagination of the dorsal retina occurs before invagination of the ventral retina, and cell differentiation within the dorsal retina precedes cellular differentiation in the ventral retina by 4 to 8 hours. In addition, separate fields of ganglion cell axons exit each of the retinas independently.

The ventral enzyme in the zebrafish that synthesizes retinoic acid from retinaldehyde is inhibited preferentially by citral, and the dorsal enzyme is inhibited by disulfiram. These drugs, when applied in vivo, decrease endogenous RA levels. The inhibition of endogenous RA synthesis by citral during the early stages of zebrafish eye development (when only the ventral enzyme is present) results in half-eyes that lack ventral retinas (see Figs. 2B, 2C); this is the opposite effect of excess retinoic acid. The vitamin A deficiency studies conducted by Warkany and Schraffenberger during the 1940s reported similar malformations of developing eyes, in which the ventral retina—along with other ocular malformations, such as coloboma, and lens defects—was preferentially reduced. When citral treatment in the zebrafish is combined with exposure to high exogenous RA, the fish embryos are severely deformed overall because of exposure to two teratogens. However, the eyes are more normal, suggesting that RA is an important factor for ventral retina development. These data demonstrate the role of RA for the specification and proper development of the ventral eye segment, and they identify an RA-responsive cell type within the ventral region of the retina.

The genetic obliteration or knockout of the RA receptors in the mouse has been generated for most of the RA receptors. These RXRa null mice mutants also display an alteration of the ventral retina, as do RAR knockout mice with at least a single RXRa allele. The severity of the ventral retina defect is increased by adding RAR defects to an RXR-defective background, indicative of a role for RAR/RXR heterodimeric mediation of ventral retinal morpho-
genesis. A similar ventral eye defect is observed in
Drosophila ultraspracle (usp) mutants. Ultraspracle, the
Drosophila RXR homolog, functions as a heterodimeric
partner for the ecdysone receptor. This receptor can
heterodimerize with the vertebrate RXR partners, but
to date it cannot be activated by retinoids. The genera-
tion of mutant genetic mosaics revealed abnormal
rhabdomeres and ommatidia in Drosophila in which
the size of the ventral portion of the eye was reduced.
These data may mirror a conserved function for usp/
RXR in dorsoventral patterning of eyes.2

To test further the hypothesis that RA plays a sig-
nificant role in the establishment of ventral retinal
characteristics and in the maintenance of the dorso-
ventral axis of the eye, dorsal and ventral ocular mark-
ers in RA-treated zebrafish were examined.11 The optic
stalk represents the ventralmost region of the early
eye field; later, it decreases in width, becomes obliter-
ated, and gradually is replaced by components of the
optic nerve. High systemic RA levels cause changes in
the stalk—increased cell content and a patent lu-
men—that resemble persistence of its early ventral
character. Expression of the transcription factor
pax[b], normally confined to the ventral retina, ex-
pands into the dorsal retina after RA treatment,
whereas msh[c], normally expressed in the dorsal re-
tinal pole, disappears. Activity of an aldehyde dehydro-
genase that normally occupies the dorsal third of the
retina is reduced or abolished after high systemic RA.
When a localized RA source, an RA-soaked bead, is
placed in the eye field, a fissure resembling the ventral
choroid fissure can be induced at any location around
the eye. These observations indicate that RA is in-
volved in the determination of ventral retinal charac-
teristics and directly in dorsoventral patterning of the
retina.

THE ROLE OF RETINOIC ACID IN
PHOTORECEPTOR AND RETINAL CELL
DIFFERENTIATION

Stenkamp et al12 first showed that retinoids, including
all-trans RA, support the survival of cells dissociated
from embryonic chick retinas and cause a dose-depen-
dent increase in the number of differentiated photore-
ceptors in cell cultures. Kelley and colleagues13
showed later that retinoic acid mediates a dose-depen-
dent increase of rod photoreceptor differentiation in
dissociated retinal cell cultures from embryonic and
neonatal rats. In the same cultures, RA caused a dose-
dependent decrease in the number of cells that de-
velop as amacrine cells, suggesting that the primary
effect of RA is to influence progenitor cells to develop
as newly generated rod photoreceptors.

To determine whether RA is involved in cellular pat-
terning at later stages of retinal development in vivo, ze-
brafish embryos were exposed to RA or to inhibitors of
endogenous RA synthesis during the onset of photorecep-
tor histogenesis.14 Application of exogenous RA to zebra-
dish during the initial stages of photoreceptor differen-
tiation results in a precocious development of rod photo-
ceptors (Figs. 2D, 2E) and an inhibition of cone photo-
receptor maturation. The acceleration of rod differentia-
tion was observed initially within the ventral retina 3 days
after fertilization, after 24 hours of RA application, and
within the dorsal retina 4 days after fertilization, after 48
hours of RA application. The differentiation of rods was
impeded significantly when the synthesis of endogenous
RA was inhibited by citral before the initial stage of rod
differentiation. Retinoic acid-treated embryos labeled for
bromodeoxyuridine uptake revealed that RA exerts its
effects on a postmitotic cell population within the devel-
oping retina that is specified to become rod photorecep-
tors. This conclusion was supported by the finding that
other retinal cell populations (ganglion and amacrine
cells) were not affected by RA treatments—that is, precur-
sors of these cells were not recruited by RA to become
rod cells, as was the case in rat cell cultures.14 A number
of factors, in addition to RA, have been shown to promote
rod photoreceptor cell differentiation in retinal cell cul-
tures, among them ciliary neurotrophic factor, acidic fi-
broblast growth factor, basic fibroblast growth factor, and
taurine. The expression of several of these factors may
be enhanced by RA in the retina because RA is known to
induce the expression of several growth factors in various
tissues. During normal development in zebrafish, rod and
cone differentiation is most robust within the ventral ret-
ina, a region rich in RA. These data suggest therefore,
that the RA signaling pathway is involved in the differen-
tiation and maturation of rod and cone photoreceptors,
as well as in early eye development.

FUTURE PERSPECTIVES

What genes does RA influence during early eye and pho-
toreceptor development? This is a major question and
one barely touched on thus far. It is known that RAREs
exist in the promotor regions of many of the visual pig-
ment genes, and it is through such response elements
that RA may exert effects on visual pigment expression.
In the developing mouse eye, for example, blue cones
are restricted to the ventral retina, whereas green cones
are found in the dorsal retina. Retinoic acid-regulated
gene products may be involved in this dorsoventral seg-
mentation of these visual pigments and cone types. Var-
ious other genes certainly are influenced as well. As noted,
RA significantly affects the expression patterns of a paired
box gene (pax-2) and a homeobox gene (msh-c) during
early zebrafish eye development, and these genes may
play a role in eye development along the dorsoventral
axis of the eye, perhaps contributing to the projection of
ganglion cell axons to appropriate target locations in the
optic tectum.

Sonic hedgehog (shh), or a homolog, has been impli-
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cated in early eye development. Like excess RA, the over-
expression of shh expands the optic stalk and the expres-
sion of the pax-2 gene. However, whereas RA expands
the retina ventrally, causing a duplication of the neural
epithelium, shh reduces retinal size. Other differences
have been noted between the effects of RA and shh on re-
tinal markers, and RA does not alter shh expression.
Thus, these two agents appear to act independently in
early eye development.

Determining all the genes involved in eye and retinal
development, as well as how the various genes relate to
one another, is of paramount importance. Discovering
RA’s role in regulating these genes and in their cascades
represents another major challenge. These questions
have piqued much interest among investigators. Un-
doubtedly, enormous progress will be made toward an-
swering them during the next several years.

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