Developmental Biology 325 (2009) 94–105

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The temporal requirement for vitamin A in the developing eye: Mechanism of action in optic fissure closure and new roles for the vitamin in regulating cell proliferation and adhesion in the embryonic retina

Angela Wai-Man See [a], Margaret Clagett-Dame [a,b,c,*]

[a] Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, Madison, WI 53706-1544, USA
[b] Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA
[c] Pharmaceutical Science Division, 777 Highland Ave., University of Wisconsin-Madison, Madison, WI 53705-2222, USA

ARTICLE INFO

Article history:
Received for publication 5 May 2008
Revised 24 September 2008
Accepted 25 September 2008
Available online 14 October 2008

Keywords:
Vitamin A
Retinoic acid
Coloboma
Eye development
Vitamin A deficiency
Retina
Cell adhesion

ABSTRACT

Mammalian eye development requires vitamin A (retinol, ROL). The role of vitamin A at specific times during eye development was studied in rat fetuses made vitamin A deficient (VAD) after embryonic day (E) 10.5 (late VAD). The optic fissure does not close in late VAD embryos, and severe folding and collapse of the retina is observed at E18.5. *Pitx2*, a gene required for normal optic fissure closure, is dramatically downregulated in the periocular mesenchyme in late VAD embryos, and dissolution of the basal lamina does not occur at the optic fissure margin. The addition of ROL to late VAD embryos by E12.5 restores *Pitx2* expression, supports dissolution of the basal lamina, and prevents coloboma, whereas supplementation at E13.5 does not. Surprisingly, ROL given as late as E13.5 completely prevents folding of the retina despite the presence of an open fetal fissure, showing that coloboma and retinal folding represent distinct VAD-dependent defects. Retinal folding due to VAD is preceded by an overall reduction in the percentage of cyclin D1 positive cells in the developing retina, (initially resulting in retinal thinning), as well as a dramatic reduction in the cell adhesion-related molecules, N-cadherin and β-catenin. Reduction of retinal cell number combined with a loss of the normal cell–cell adhesion proteins may contribute to the collapse and folding of the retina that occurs in late VAD fetuses.

Introduction

The importance of vitamin A (retinol) in eye development was first demonstrated in the 20th century in animals subjected to varying degrees of vitamin A deficiency. Pregnant pigs or rats with a borderline deficiency in vitamin A were shown to give rise to fetuses or neonates with congenital eye abnormalities, including anophthalmia, coloboma, eversion or folding of the retina and/or retrolenticular fibroplasia (Hale, 1933, 1935; Warkany and Schraffenberger, 1944, 1946). In these early studies however, the severity of the vitamin A deficiency varied from animal to animal and fetal survival was low, making it difficult to study the basis for the defects in any detail. Thus, the mechanism(s) whereby vitamin A exerts its effects in normal eye development during the latter part of pregnancy remains largely unexplained.

Embryonic eye development is a multi-step process that involves tissue induction, as well as the proliferation and differentiation of cells to form mature tissue. The eye originates from the forebrain neuroectoderm, first forming the optic vesicle, which then invaginates and forms the double-layered optic cup (Harada et al., 2007). A role for vitamin A in optic cup formation has been described (Mic et al., 2004). The optic fissure, which arises later from the invagination of the optic vesicle, is a transient structure in the developing eye; it allows the mesenchyme and hyaloid vasculature to enter into the optic cup and then closes during normal embryonic development (Gregory-Evans et al., 2004; Harada et al., 2007). Coloboma, which means a gap or fissure in the eye, is caused by defective optic fissure closure (Mann, 1957; Onwochei et al., 2000; Gregory-Evans et al., 2004). Persistence of the optic fissure results in penetration of the optic cup by mesenchymal tissue and eversion of the retina in the cleft region (Mann, 1957). Coloboma was reported at an 18% frequency in fetuses from rats with a borderline deficiency of vitamin A. Retinal eversion was reported at a similar frequency (27%; Wilson et al., 1953) and was believed to be related to typical coloboma (Warkany and Schraffenberger, 1946).

Retinol is the primary circulating form of the vitamin and is stored in the liver and other tissues as a retinyl ester; retinol supports all known functions of vitamin A. The vitamin A metabolite, all-*trans* retinoic acid (atRA), is generated though a series of oxidation reactions starting with retinol followed by the generation of all-*trans* retinaldehyde and finally atRA, which functions in cellular growth and differentiation (Clagett-Dame and DeLuca, 2002). atRA acts by binding to the nuclear retinoic acid receptors (RAR). The RAR heterodimerizes

* Corresponding author. Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA. Fax: +1 608 262 7122.
E-mail address: dame@biochem.wisc.edu (M. Clagett-Dame).

0012-1606/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.09.030

with the retinoid-X receptor (RXR) and this complex regulates the transcription of atRA-responsive genes in a ligand-dependent fashion. The importance of RAR signaling in the developing eye is supported by the finding that *RAR*α/γ compound null mutant mice exhibit eye defects similar to those reported in VAD fetuses (Lohnes et al., 1994).

A nutritional model of late embryonic vitamin A deficiency was recently reported (See et al., 2008). Normal fetal development is supported in pregnant vitamin A deficient (VAD) rats by feeding an exogenous source of atRA, and fetal vitamin A deficiency is instituted after embryonic day 10.5 (E10.5) by reducing the level of atRA in the maternal diet. A rapid state of deficiency is induced because atRA has a short biological half-life and is not stored in tissues. Using this approach, vitamin A deficiency-induced eye defects of similar severity are produced in all fetuses making it possible to perform molecular studies aimed at understanding the basis for these developmental defects. The current report focuses on determining the temporal requirement for vitamin A in eye development, and examines the molecular basis whereby the vitamin supports normal closure of the optic fissure. New roles for vitamin A in the regulation of cell proliferation, differentiation and cell–cell adhesion in the developing retina are also reported.

Materials and methods

Generation of rat embryos

Female rats (Harlan Sprague–Dawley, Madison, WI) were depleted of vitamin A and mated as previously described (White et al., 1998; See et al., 2008), and assigned to various dietary treatment groups. Food intake was measured daily from E0.5 to the day of sacrifice and did not differ between the groups. The atRA content of the diets was assessed as previously described (White et al., 1998) and was ±10% of the desired value.

Histological analysis

Embryos were collected and fixed overnight in 4% paraformaldehyde (E14.5 and E16.5) or in Bouin's fluid (E18.5) (VWR Scientific Products, West Chester, PA) for 5–7 days at 4 °C and embedded as described (See et al., 2008). Sections (10 μm) from E14.5 and E16.5 embryos were stained with Gill's Hematoxylin and Eosin (H&E) while sections from E18.5 fetuses were stained with Groat's Hematoxylin and Mallory's Trichrome stain. Eight to thirteen embryos from three to seven independent litters were analyzed at E14.5 and E16.5; nine to twelve fetuses from three to five independent litters were analyzed at E18.5.

Generation of riboprobes, vibratome sectioning and in situ hybridization

Rat embryos (E14.5) were collected and fixed in 4% paraformaldehyde, dehydrated into 100% methanol and stored at −20 °C until use. Embryos were rehydrated to phosphate buffered saline (PBS), embedded in 3% agarose in PBS at 65 °C and stored at 4 °C overnight. Embryos were then sectioned frontally at 200 μm using a vibratome (Leica, Wetzlar, Germany).

The *Pitx2* riboprobe (536 bp) was prepared using methods described previously (Kaiser et al., 2003) using the following primer pair: 5′-ACTCTGCCCGAGAAAGACTGAGAA-3′ and 5′-TCAGAAACAAGG-CATCCACCAGAG-3′. *In situ* hybridization of floating sections was carried out as described previously (Kaiser et al., 2003). The sense probe was evaluated as a negative control and no signal was detected (data not shown). Five to six embryos from at least three independent litters were analyzed per group. Imaging of slides and sections was done using a Spot camera (Spotcam, Diagnostic Instruments Inc., Sterling Heights, MI) and MetaMorph software (Molecular Devices, Downingtown, PA).

Immunofluorescence studies

Dent's fixed (methanol:dimethyl sulfoxide 4:1) embryos were dehydrated to 100% ethanol and embedded in paraffin for microtome sectioning at 10 μm. Sections were deparaffinized and rehydrated in PBS, blocked in 5% normal goat serum (NGS) in PBS containing 0.05% Tween 20 for 30 min, and then incubated in primary antibodies diluted in 5% NGS in PBS at 4 °C overnight. Sections were washed in PBS with 0.05% Tween-20 followed by incubation with Alexa Fluor secondary antibodies (1:100–1000 in PBS, Molecular probes) at room temperature for 1 h. Sections were then incubated with nuclear stain 4′6-diamindino-2-phenylindole (DAPI) (1:1000 in PBS, Invitrogen). The primary antibodies used were mouse anti-βIII-tubulin (clone 5G8, 1:1000, Sigma), mouse anti-islet-1 (clone 40.2D6 supernatant; Developmental Studies Hybridoma Bank); rabbit anti-laminin (directed to both the 220 and 440 kDa chains, 1:100, Serotec), mouse anti-cyclin D1 (clone 72-13G, 1:50, Santa Cruz), mouse anti-N-cadherin (clone 32/N, 1:200, BD Biosciences) and rabbit anti-β-catenin (to a peptide region including serine 37, 1:200, Cell Signaling). A minimum of 6 embryos from at least 3 independent litters were analyzed per group for each antibody.

Imaging of sections was done by Metamorph or QED Capture software. Cyclin D1 quantitation was done by imaging embryonic sections at 40× magnification in the medial/dorsal region of the retina. Cyclin D1 positive cells and DAPI positive cells in an area of 215×173 μm were counted and the number of positive cells was expressed as percentage of the total number of cells. Three retinal sections were counted from each embryo and a total of 6 embryos from 3 independent litters/group at each stage were studied. Statistical analysis was done using a one-way analysis of variance (ANOVA) followed by pairwise comparisons (Tukey HSD, Sheffe test and Fisher's least significant difference test; significance set at $p<0.05$).

Results

The optic fissure fails to close in late VAD embryos

Closure of the optic fissure in the rat embryo begins at E13 (late E11 in mouse) and the process is complete by E15 (E13 in mouse, Hero, 1990). When examined at E14.5, optic fissure closure was nearly complete (except in the optic disk region) in all vitamin A-sufficient (VAS) control embryos (group I; $n=8$ from 3 independent litters; Supplementary Fig. 1A), and by E16.5, the optic fissure was completely closed in 100% of the VAS embryos (Fig. 1A). In contrast, when a state of late embryonic vitamin A deficiency was imposed (group II), the fissure was open in 100% of embryos at E14.5 (8 embryos from 4 independent litters; Supplementary Fig. 1B, arrowhead). The failure of the optic fissure to close was not the result of a developmental delay, as it remained open in all late VAD embryos at E16.5 (Fig. 1B, white arrowhead). In the majority of late VAD embryos (5/7), the fissure was open throughout the entire retina, and extended through the optic disc/nerve. Thus, failure of optic fissure closure is fully penetrant in late VAD embryos.

In order to investigate the temporal requirement for vitamin A, ROL was administered to pregnant rats starting at later times (E11.5 to E15.5), followed by analysis of eye development at E16.5 (Fig. 1). Delaying the addition of ROL by one day (starting at E11.5) supported optic fissure closure in all embryos (Fig. 1C), whereas the addition of ROL at E12.5 supported closure in most (83%, Fig. 1D), and yielded coloboma in only 17% (Fig. 1E, white arrowhead). However, when ROL supplementation was started on or after E13.5 (groups V, VI and VII), the optic fissure failed to close in 100% of embryos (Figs. 1F–H, white arrowheads).

The basement membrane fails to dissolve at the optic fissure in late VAD embryos

Closure of the optic fissure begins as the fissure margins approach each other followed by inversion of the outer layer of the optic cup.