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Effects of retinoic acid exposure during zebrafish retinogenesis

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ABSTRACT

Retinoic acid (RA) is an important morphogen involved in retinal development. Perturbations in its levels cause retinal malformations such as microphthalmia. However, the cellular changes in the retina that lead to this phenotype are little known. We have used the zebrafish to analyse the effects of systemic high RA levels on retinogenesis. For this purpose we exposed zebrafish embryos to 0.1 μM or 1 μM RA from 24 to 48 h post-fertilisation (hpf), the period which corresponds to the time of retinal neurogenesis and initial retinal cell differentiation. We did not find severe alterations in 0.1 μM RA treated animals, but the exposure to 1 μM RA significantly reduced retinal size upon treatment, and this microphthalmia persisted through larval development. We monitored histology and cell death and quantified both the proliferation rate and cell differentiation from 48 hpf onwards, focusing on the retina and optic nerve of normal and 1 μM treated animals. Retinal lamination and initial neurogenesis are not affected by RA exposure, but we found widespread apoptosis after RA treatment that could be the main cause of microphthalmia. Proliferating cells increased their number at 3 days post-fertilisation (dpf) but decreased significantly at 5 dpf maintaining the microphthalmic phenotype. Retinal cell differentiation was affected; some cell markers do not reach normal levels at larval stages and some cell types present an increased number compared to those of control animals. We also found the presence of young axons growing ectopically within the retina. Moreover although the optic axons leave the retina and form the optic chiasm they do not reach the optic tectum. The alterations observed in treated animals become more severe as larvae develop.

1. Introduction

The vertebrate retina develops from a neuroepithelium that gives rise to a highly conserved and layered structure, with specific retinal cell types. The first cells to become post-mitotic in the zebrafish retina are the ganglion cells, followed by cells of the inner nuclear layer (INL) and, finally, by photoreceptors (Raymond et al., 1995; Schmitt and Dowling, 1999; Hu and Easter, 1999). Between 24 and 48 hpf, the ganglion cell layer (GCL) and the INL become organised and the first axons exit the retina to form the optic nerve and reach their target in the optic tectum (Burrill and Easter, 1994).

The development of the vertebrate retina is controlled by intrinsic factors, as well as secreted morphogens like retinoic acid (RA), the active derivative of vitamin A or retinol. During normal development, RA signalling regulates the optic cup morphogenesis and the choroid fissure closure (Mic et al., 2004; Lupo et al., 2011), the expression of photoreceptor specific genes and their differentiation (Hyatt et al., 1996b; Prabhudesai et al., 2005; Stevens et al., 2011), as well as other developmental process. However, there is a little knowledge about the effects of retinoic acid on histology, neurogenesis and cellular differentiation of the retina during embryonic and larval development.

RA is also a teratogen and it is well known that vitamin A or RA exposure during pregnancy causes malformations in newborn animals (Cohlan, 1953; Shenefelt, 1972). Nowadays, RA is used in some treatments of acne and cancer and is found in vitamin supplements. RA exposure during human pregnancy leads to a characteristic pattern of malformations known as retinoic acid embryopathy involving craniofacial and central nervous system structures including retinal and optic nerve alterations (Lammer et al., 1985; Rothman et al., 1995). Zebrafish are an excellent model for embryotoxic and teratological studies (Yang et al., 2009). Previous works in this animal model have reported that exposure of embryos to RA before retinogenesis results in a double retina phenotype (Hyatt et al., 1992), or an expansion of retinal regions with ventral characteristics (Hyatt et al., 1996a), as well as microphthalmic phenotype (Holder and Hill, 1991, Stainier and Fishman, 1992).

In this work, we study the teratogenic effects of 1 μM RA treatment on zebrafish retinogenesis. For this purpose we exposed the zebrafish embryos to RA from 24 to 48 hpf. This developmental time frame corresponds to the retinal neurogenesis and initial retinal cell differentiation. We analysed the histology on semi-thin sections,

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proliferation rate, apoptosis, as well as neurogenesis and cell differentiation. We found that RA exposure results in immediate and persistent microphthalmia mainly due to an increased cell death in the retina. We also found some cellular and neurochemical changes in retinal fate. Our neuroanatomical data might provide valuable information about the teratogenic effects of RA in retinogenesis.

2. Methods

2.1. Animals

AB strain zebrafish embryos were generated by natural pair-wise mating. They were staged and reared according to standard procedures (Westerfield, 1995). In order to inhibit melanin synthesis and keep embryos and larvae transparent for analysis, they were treated with 0.003% phenothiourea (PTU; Sigma) at 24 hpf. Specimens were anaesthetised with ethyl 3-aminobenzoate methanosulphonate salt (Sigma, St Louis, MO, USA) before they were fixed.

All of the following procedures were carried out in control embryos and RA treated embryos. The stages used were 48 hpf, 3 days post-fertilisation (dpf) and 5 dpf.

All procedures and experimental protocols were in accordance with the guidelines of the European Communities Directive (2010/63/UE), the current Spanish legislation for the use and care of animals in research (RD 1201/2005, BOE 252/34367-91, 2005) and conformed to NIH guidelines.

2.2. RA treatment

RA treatment was performed as previously suggested (Hyatt et al., 1992). Briefly, a stock solution of 0.1 M all-*trans* RA (Sigma, St. Louis, MO) was prepared using dimethylsulphoxide (DMSO; Sigma) and was stored in frozen aliquots at −20 °C (Hyatt et al., 1992). The RA was thawed immediately before use and diluted to the appropriate concentration in E3 medium. Embryos were manually dechorionated at 24 hpf and then treated with 0.1 μM or 1 μM RA solution between 24 and 48 hpf in a Petri dish. After treatments, embryos were either fixed at 48 hpf, or were washed three times and maintained in fresh E3 medium (unmodified system water) until their fixation at 3 or 5 dpf.

2.3. Tissue processing

For cell death analysis by terminal dUTP nick-end labelling (TUNEL), in situ hybridisation, and immunohistochemistry, embryos of each stage were anaesthetised and fixed by immersion with 4% paraformaldehyde for 24 h at 4 °C. Embryos were maintained in methanol at −20 °C until their use.

Specific cell markers were analysed by immunohistochemistry in cryosections which were obtained as follows: fixed embryos were washed in 0.1 M phosphate buffer pH 7.4 (PB) and were embedded in 1.5% agar and 10% sucrose. Blocks were cryoprotected in 30% sucrose for 48 h at 4 °C. Coronal sections of 10 μm-thickness were obtained on a cryostat and stored at −20 °C until their use.

2.4. Semi-thin sections

Control and treated specimens were anaesthetised and fixed by immersion with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 24 h at 4 °C, and post-fixed in osmium tetroxide containing 1% potassium ferricyanide for 1 h at 4 °C. Specimens were dehydrated using a graded series of cold ethanol and embedded with Embed-812 (#14120 Electron Microscopy Science, Fort, Washington, PA, U.S.A.). Parasagittal serial sections of 1 μm-thickness were cut on an ultramicrotome Reichert-Jung Ultracut E (Nussloch, Germany). Sections were stained with 1% toluidine blue solution.

2.5. Cell death assay

TUNEL labelling to detect apoptosis in whole-mount embryos was performed using the ApopTag Kit (Chemicon International) and a Cy3-conjugated IgG fraction mouse anti-dig antibody (1:6000, Jackson ImmunoResearch). The embryos were sectioned on a cryostat and viewed using a photomicroscope. Nuclei were counterstained with DAPI (Sigma).

2.6. In situ hybridisation

Pax6a, *ath5* (*ath7*-Zebrafish Information Network), and *islet-1a* probes for ISH were generous gifts from Steve Wilson (University College, London). Whole-mount in situ hybridisation procedures were performed as previously described (Xu et al., 1994). In brief, after rehydration, embryos were treated with a solution of 10 μg/ml of proteinase K (Sigma) for 30 min and hybridised at 68 °C overnight with the probe in a solution containing 50% formamide (Sigma). Visualisation of hybridisation was performed using an anti-dig antibody coupled to alkaline phosphatase (Roche), and colour substrates NBT/BCIP (both from Roche).

2.7. Immunohistochemistry

The immunohistochemistry was performed as previously described (Arenzana et al., 2005, 2006b; Clemente et al., 2004). The following antibodies were used: rabbit anti-phosphohystone H3 (H3P, 1:4000, upstate) rabbit anti-calretinin (CR, 1:10000, Swant, Bellizona, Switzerland), Zn-8 (1:400, ZIRC, University of Oregon, USA), rabbit anti-Pax6 (1:500, Covance), rabbit anti-Sox2 (1:500, Abcam), mouse anti-Glutamine Synthetase (GS, 1:1000, Chemicon), Zpr1 (1:500, ZIRC, University of Oregon, USA) and Zpr3 (1:500, ZIRC, University of Oregon, USA). All the antibodies have been previously used in zebrafish and other teleosts (H3P: Murphey at al., 2006; Sox2: Vitorino et al., 2009; GS: Cid et al., 2010; CR: Castro et al., 2006; Pax6: Sánchez-Simón et al., 2010; Zn-8, Zpr1 and Zpr3 are specific to zebrafish). As the secondary antibody either anti-mouse or anti-rabbit IgG conjugated with Cy2 or Cy3 (both 1:250; Jackson ImmunoResearch) were used. Nuclei were labelled with DAPI (Sigma).

2.8. Image analyses

Digital images of cryosections and whole-mount embryos were obtained with an Olympus OP-70 digital camera (Olympus Corporation, Tokyo, Japan) coupled to an Oympus Provis AX70 photomicroscope. Sharpness, contrast, and brightness were adjusted to reflect the appearance seen through the microscope. The original images were processed digitally with Adobe® Photoshop® 8.0 software (Adobe Systems, San Jose, CA, U.S.A.).

2.9. Retina measurement, cell counts and statistics

The selected sections of retina for the analysis of the different cell specific markers had smaller size in the 1 μM RA treated animals (Fig. 1g). As this difference between control and treated animals could be due to different numbers of cells, we decided to relativise the results of the cell counts as numbers of positive cells per surface units as previously described (Santos-Ledo et al., 2011).

Positive cells for the different markers in the retina were counted in equivalent sections using ImageJ software. The area of the retina was also measured. Counts of cells were performed as previously reported by Kashyap et al. (2007). Briefly, cells were counted for at least three sections per individual embryo, for at least three embryos per count. To avoid double counting, alternate sections were used. Cell counts were carried out as a blind study.