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Review

An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis

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ABSTRACT

Developmental toxicity can be caused through a multitude of mechanisms and can therefore not be captured through a single simple mechanistic paradigm. However, it may be possible to define a selected group of overarching mechanisms that might allow detection of the vast majority of developmental toxicants. Against this background, we have explored the usefulness of retinoic acid mediated regulation of neural tube and axial patterning as a general mechanism that, when perturbed, may result in manifestations of developmental toxicity that may cover a large part of malformations known to occur in experimental animals and in man. Through a literature survey, we have identified key genes in the regulation of retinoic acid homeostasis, as well as marker genes of neural tube and axial patterning, that may be used to detect developmental toxicants in *in vitro* systems. A retinoic acid–neural tube/axial patterning adverse outcome pathway (RA–NTA AOP) framework was designed. The framework was tested against existing data of flusilazole exposure in the rat whole embryo culture, the zebrafish embryotoxicity test, and the embryonic stem cell test. Flusilazole is known to interact with retinoic acid homeostasis, and induced common and unique NTA marker gene changes in the three test systems. Flusilazole-induced changes were similar in directionality to gene expression responses after retinoic acid exposure. It is suggested that the RA–NTA framework may provide a general tool to define mechanistic pathways and biomarkers of developmental toxicity that may be used in alternative *in vitro* assays for the detection of embryotoxic compounds.

Contents

Abbreviations: AOP, adverse outcome pathway; BMD, benchmark dose; CNS, central nervous system; EST, embryonic stem cell test; ESTn, embryonic stem cell test-neural; GMS, general morphology score; GO, gene ontology; MIE, molecular initiating event; NCBI, National Center for Biotechnology Information; NTA, neural tube/axial; NTD, neural tube defects; OECD, organization for economic cooperation and development; RA, retinoic acid; RARE, retinoic acid responsive element; WEC, rat whole embryo culture; ZET, zebrafish embryotoxicity assays.

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1. Introduction

Neural tube and axial defects of the vertebrate embryo are among the most common developmental malformations in mammalian species including man. They include neural tube defects (NTD), which are among the most prominent birth defects in the human population. Birth defect registries have reported a prevalence of around 35 cases of spina bifida, 20 cases of anencephaly, and 10 cases of encephalocele per 100,000 births [1]. In the US, the total number of new neural tube defect cases annually amounts to 2500. Moreover, disruption of axial development may result in a variety of craniofacial, limb as well as cardiac and other malformations. Also in experimental animal studies for chemical and pharmaceutical hazard assessment, neural tube and axial defects are frequently observed findings [2]. Human teratogens such as anticonvulsants (*e.g.* valproate and carbamazepine), cytostatic agents (*e.g.* cyclophosphamide and methotrexate) and retinoids have been shown to increase the risk for such defects [3–5]. The fetal alcohol syndrome has also been hypothesized to be mediated by changes in retinoic acid homeostasis [6]. Neural tube and axial defects are a consequence of perturbation of rostrocaudal growth and differentiation in the very early stages of vertebrate embryogenesis. It is evidently important to be able to predict a compounds' capacity to induce such malformations before deciding on its targeted use and release on the market.

The assessment of developmental toxic potential of chemicals is classically derived from experimental animal studies according to protocolled, globally harmonized test guidelines [7]. These studies have proven useful in providing overall hazard information based on apical endpoints such as resorptions, prenatal death, growth retardation and malformations. Decades of experience with these studies have shown that the nature of apical endpoints cannot readily be extrapolated between species and not even between strains of the same species [2,8,9]. The relevance of apical animal findings for the human situation is therefore not always clear-cut. However, the basic physiological regulation of embryogenesis is highly conserved among vertebrates and especially among mammalian species. Therefore, it is anticipated that mechanistic information on the interference of chemicals with embryogenesis on the molecular level would provide a more informative background for hazard and risk assessment for man. Such data can be collected either in animal studies or from animal or human cell and tissue culture models. Thus, such approaches not only allow a more detailed insight into mechanisms of dysmorphogenesis in animals, but also facilitate direct comparison with the human situation. The US National Academy of Sciences spread this notion in their report on Toxicity Testing in the 21st century [10]. This report proposes to define a limited number of essential toxicity pathways that could be covered by a limited number of animal-free alternative test systems. Interference at a certain magnitude with these pathways would be indicative of toxicity at the level of the organism. The Organization for Economic Cooperation and Development (OECD) followed up on this proposal by their initiative to define Adverse Outcome Pathways (AOP) [11–13]. These were defined as linear cascades of effects leading from a Molecular Initiating Event (MIE) *via* subsequent consequences at the cellular and organ level to an overt adverse health effect. AOPs are thought to become instrumental in a future of innovated hazard and risk assessment applying molecular-based alternative test models, reducing cost, time and animal use, and enhancing mechanistically based prediction of human hazard and risk. Several preliminary AOPs have been generated for further optimization [13,14]. A linear AOP, as proposed by OECD, may indeed suffice for mechanisms where a clear stepwise linear cascade of events describes all the critical stages of the AOP, such as has been envisaged for genotoxic carcinogens or for sensitizers. However, for complex processes such as embryogenesis, where the internal balance of a wealth of competing factors and interacting cell types changes continuously with time and location in the developing embryo, a simple linear approach would undoubtedly miss major players within the AOP, which would significantly reduce its usefulness.

In the current study, we constructed an AOP framework, linking a variety of possible MIEs to yet another variety of adverse outcomes, and thus containing a combination of possible AOPs. The framework is based on the essential role of retinoic acid (RA) in the formation of, and differentiation within the neural tube and body axis. RA is the active form of vitamin A, and the conversion of vitamin A in the body is carefully limited to the extent that the active form is necessary within time- and place-dependent physiological homeostasis. As its diversity of functions in embryogenesis became apparent, RA has been named a morphogen, indicating the fundamental importance of this molecule in embryonic development [15]. The guiding role of retinoic acid is critically dependent on finely tuned tissue concentrations that vary with the location within the embryo as well as with time during embryogenesis. Subtle disturbances of retinoic acid levels can have grave developmental consequences, as has been shown in extensive animal developmental toxicity studies with retinoids as well as in human subjects after the intake of retinoic acid in multivitamin preparations during pregnancy [16–19]. Conversely, RA depletion has similar untoward effects on embryogenesis. The dysmorphogenic effects of the disturbance of RA balance are not limited to the body axis. In addition, all tissues and organs that receive a contribution from neural crest cells migrating into peripheral tissues are vulnerable to retinoic acid embryopathy. This includes facial structures such as the branchial arches and the ears, the heart, and the limbs. The complexity of RA embryopathy stipulates the central role of RA in embryogenesis. As a consequence, we anticipate that an AOP framework based on MIEs interfering with RA homeostasis and related adverse outcomes regarding (dys)morphogenesis of the body axis could allow the detection of a major subset of developmental toxicants. Some of these embryotoxicants may interfere with components of this RA–neural tube/axial (RA–NTA) framework directly, whereas others may have an MIE outside this domain but show secondary effects mediated by components of the framework and may thus be detected through interference with this AOP framework anyway.

The effectiveness of retinoic acid at a given time and location in the embryo is dependent on a combination of factors, all of which need to be considered when constructing an RA–NTA AOP framework. In addition to time in development and location in the embryo, these factors include the RA concentration, and the presence of other regulators that stimulate or suppress the effect of RA. In the following sections, we have mapped critical molecular components of retinoic acid regulated neural tube and axial morphogenesis, based on existing literature data. The multidimensional cascade starts with retinoic acid balance, the concentration of which is regulated by synthesizing and metabolizing enzymes.