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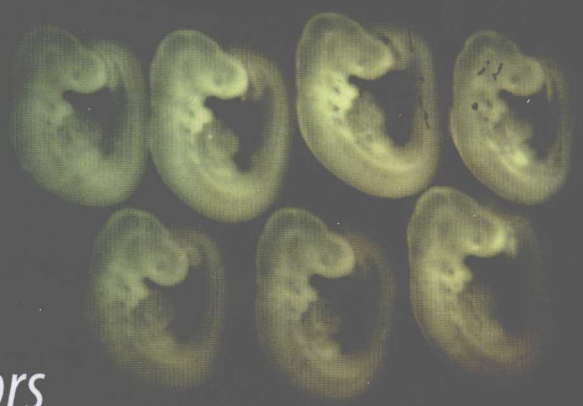

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Developmental Toxicology

Methods and Protocols

Humana Press

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Preface

The discipline of developmental toxicology is, at its core, an integration of concepts, models, and methodologies based most heavily on the superimposition of toxicology principles upon the science of developmental biology. The science of developmental toxicology also borrows heavily from other research areas that are concerned with regulation of cell growth, migration, differentiation, and cell death, as such are central to the study of stem cells, cancer, and chronic diseases. Several methodological approaches used to investigate these aspects of developmental toxicology need to be modified and adapted to meet the unique restraints inherent in developing organisms. This volume seeks to illustrate some of these adaptations and to highlight the evolution of methods from classical teratology approaches to the dynamic, state-of-the-art molecular methods, systems biology, and next-generation models and procedures. We regret not being able to represent all emerging technologies and applications in this volume, but hope that the sections we have included will pique the interest of those less familiar with developmental toxicology. This work is primarily intended for basic scientists, academics, and industrial toxicologists whose research and interests include references to the period of life between fertilization and parturition, although isolated events during gestation are known to have profound consequences across the entire lifespan. This work should provide a valuable resource to those planning experiments to investigate consequences of environmental, nutritional, or chemical effects caused during development.

The chapters and topic areas are organized in order of descending biological complexity, beginning with whole animal or in vivo study models proceeding to the more focused in vitro models. The in vivo and in vitro sections are each prefaced with a brief overview. Subsequent chapters focus on specific areas of toxicology or developmental biology principles, such as biotransformation of chemicals, induction and regulation of antioxidant and protective pathways, assessment of specific diseases, and focused assessment of biological processes. Much is yet to be learned about the modes of action of environmental factors and chemicals during the critical growth and highly vulnerable stages of embryogenesis and fetogenesis. We look forward with great anticipation toward the creation and application of many new methods and models for developmental toxicology that can begin to answer many of the enigmatic questions that have puzzled researchers for decades.

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Contents

Chapter 1

Volume Overview: Introduction

Craig Harris

Abstract

The origins and history of the study of teratology and developmental toxicology span centuries of human experience. Anatomical malformations observed at birth have been described across many generations but the root causes of these events have been enigmatic and difficult to understand. Many factors may contribute to the lack of mechanistic understanding, but the miniscule size, inaccessibility, and the consequences of ethical concerns contribute heavily to the unique restrictions on developmental toxicology research. Classic teratogens, such as vitamin A, Rubella virus, thalidomide, and methyl mercury, have provided many insights into understanding the modes of chemical action that are responsible for causing defects but the specific mechanisms remain unclear. Developmental toxicology research has benefitted greatly in the past decades from discoveries made in related fields of study, including those from cancer research, stem cell biology, and developmental biology. New methods created and adapted for studies in developmental toxicology have taken on greater importance as gestational lesions can now be shown to have developmental and health consequences across the entire lifespan.

Key words: Developmental toxicology, Teratology, Birth defects, Thalidomide, Methyl mercury, Rubella virus, Vitamin A

Anatomical malformations have been observed and described in humans and other animal species for thousands of years. Clinical descriptions of anatomical defects have been cataloged under the rubric of "teratology" which has become a part of our modern lexicon and the terminology traces its origins to the ancient Greek language associated with the meanings "*...of or pertaining to monsters*". People of many cultures have recorded descriptions of and maintained a fascination with congenital malformations ranging from dicephalic and craniopagus twinning, as well as the limb, craniofacial, midline, and other various defects seen in singleton births. The considerable superstition and folklore surrounding the origin of these individuals has been slow to dissipate. Many of the most common and most severe developmentally relevant defects have been overlooked because they affect critical internal organs,

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such as the heart, brain, kidney, and gut. Physicians and philosophers of the middle ages were the first to apply scientific reasoning and experimental methodologies toward understanding the causes of developmental defects and were instrumental in laying the foundation for the modern clinical science of teratology. Still, even in the beginning of the twentieth century little experimental evidence was being generated to elucidate the causes and consequences of developmental defects. Much of the clinical description of defects and malformations as a part of the science of teratology has now given way to a more mechanistic and molecular approach toward gaining an understanding of how environmental, nutritional, chemical, and other exposures and factors combine to elicit anatomical and functional birth defects. This ever evolving and emerging science is now identified as "developmental toxicology," which more accurately captures the cause-and-effect relationships on both the biochemical and molecular levels that can result in the disturbance of developmental mechanisms and pathways that lead to defects and abnormalities. Experiments designed to help understand and elucidate the importance of nutrients and xenobiotic chemicals in their effects on the developing conceptus have been conducted for the better part of a hundred years. Developmental biologists of the nineteenth century provided remarkable insights into potential mechanisms of vertebrate development as well as the role of nutrients and environmental factors in altering the normal course of development. Significantly focused experimental work, however, did not commence until the early part of the twentieth century when it was shown that pregnant swine administered an excess of vitamin A gave birth to offspring that had an unusually high frequency of anatomical defects. The German Measles (Rubella virus) epidemic of the late 1940s showed the potential of infectious agents to produce a spectrum of very different malformations and was especially instructive in demonstrating the now accepted canon that a single agent or insult is capable of causing vastly different malformations and functional deficits depending on the age/stage of the conceptus at exposure and its duration. Other well-documented cases of exposure to environmental conditions and chemical agents were important in ushering in the age of modern developmental toxicology. The confirmation of a chemical, nutritional, or environmental factor as being responsible for producing a specific defect in humans has historically required that a significant number of pregnancies were affected. Widespread exposure to toxins, such as methyl mercury (MeHg) as a waste product of industrial processes in Minamata Bay, Japan or the treatment of pregnant women prone to miscarriage with the artificial estrogen diethylstilbestrol (DES) are just a couple of examples where clear associations between exposure and altered developmental outcome can be confirmed.

None of the many and important incidences that set the stage for the modern study of development toxicology, however, had greater impact that the thalidomide tragedy of the early 1960s. Manufactured and marked as a mild sedative hypnotic and antiemetic, thalidomide was sold over the counter and recommended for use by pregnant women for the symptomatic treatment of morning sickness. Thalidomide was extolled for its lack of observable side effects and safety in human adults. At the time of its introduction, scientific dogma suggested that most, if not all, therapeutic agents were safe during pregnancy in women where no overt maternal toxicity was observed. The notion that the placenta is an effective and impermeable barrier to chemical toxicants remained entrenched in the scientific literature for many years even though recent research has shown that most chemical agents do cross the placental barrier. Several therapeutic agents are also known to accumulate at higher concentrations within the conceptus, possibly through mechanisms such as ion trapping, because many of the known human teratogens are weak acids, likely to ionize within the relatively alkaline environment of the conceptus. The study of thalidomide and its effects have now been ongoing for the better part of 50 years and even though significant progress has been made, a clear consensus regarding thalidomide's molecular mechanism of action has yet to be reached. The considerable obstacles encountered in this search, and its unfulfilled conclusion, can be used to illustrate the complexities and challenges often encountered by the developmental toxicologist.

Several distinct hypotheses have been generated to explain the mechanisms of thalidomide teratogenesis. They range from disturbances of biochemical and metabolic processes, such as glutamate utilization, energy production and utilization, defective chondrogenesis on toward the disturbance of molecular pathways, and misregulation of molecular signaling, such as affects cell proliferation, differentiation, migration, angiogenesis, nerve outgrowth, and the ability of transcription factors and second messenger systems to properly regulate gene expression and pattern formation. One of the more recent contributions to add to our understanding of the mechanisms of thalidomide teratogenicity has come from the observations that thalidomide significantly increases the production of reactive oxygen species in biological organisms. The study of thalidomide, in terms of its biotransformation and disposition is complicated by its initial nonenzymatic hydrolysis in biological fluids to a myriad of breakdown products. It is still not clear how, or even if, these metabolites undergo further metabolism and whether a metabolite per se is responsible for the increased generation of reactive oxygen species. This observation has implications for the types of toxicity that result from exposure to the chemical agent as well as its mechanism of action in current therapeutic applications. The overall challenge in clearly defining the mechanism

of action for a developmental toxicant includes the difficulty of aligning the genetic program with dynamic environmental conditions that inform molecular signaling and control, with the myriad of factors that are responsible for biotransformation and disposition of the offending chemical agent.

Unique challenges are encountered in attempts to study the biology and toxicity of the developing conceptus. One is the considerable lack of accessibility within the womb. It is for this reason that so many developmental toxicology studies have relied on species, such as amphibians, avians, and fish, as model experimental systems for experimental studies in developmental toxicology. These systems have been useful because of the ability to exercise direct control on the dose of chemicals administered and the ability to directly observe effects and outcomes. Another challenge, which has been overcome to a large degree by advances in the molecular methodologies, is the small size of the developing embryo. Historically, it was often necessary to pool large numbers of early gestation conceptuses to provide enough tissue for any type of biochemical or molecular analysis. Current experimental methodologies capable of specificity, amplification, and visualization at the molecular level have opened doors to new investigations that were impossible in a previous generation. Other methodological challenges are closely related to the dynamic and context-specific nature of the developing conceptus. Rapid growth, migration, and differentiation during embryogenesis combine to produce a complex three-dimensional structure that is in a constant state of change. The spatial and temporal susceptibility to chemical agents can change in individual cells and tissues over a time frame measured in minutes and hours. We know relatively little about the mechanisms through which these changes are initiated and the ways through which they can be altered to elicit defects of structure and/or function.

Recent contributions from the field of developmental biology have provided a wealth of knowledge aiding our improved understanding of molecular signaling, growth regulation, pattern formation, and structural and functional morphogenesis. Upon this framework, we have begun to superimpose the complexities of biotransformation, bioactivation, and all of the resultant consequences of toxicity. Many factors and truths related to that complete understanding of mechanisms are yet to be discovered. These needs include a better understanding of the biotransformation capabilities of the conceptus and how inductive and inhibitory factors are perceived and regulated. The developmental toxicologist seeks to borrow from all relevant sciences and to apply the respective methodologies in order to understand how chemicals affect and are affected by the developing conceptus and fetus. Many answers may come from the considerable body of cancer biology literature, as it is now well accepted that the mechanisms of cellular

transformation that cause cells to lose their ability to properly regulate growth within an living organism, thus leading to cancer, is associated with a reversion from normal mature differentiated cell morphology and gene expression to the embryonic phenotype. The transformation to a cancerous phenotype was once thought to be purely a gene mutation-driven consequence. It is becoming abundantly clear that a wide variety of environmental, chemical, nutritional, and genetic factors can combine to drive the transformation processes via more epigenetic mechanisms. This correlation has been instructive and very helpful for the improved study of developmental toxicology because epigenetic controls, such as DNA methylation, histone protein methylation, and acetylation, and small RNA regulation are precisely those also believed to be responsible for regulating and signaling the forms and functions of normal development. In addition, these events are known to be prone and receptive to the subtle environmental changes and chemical exposures that could alter the normal course of embryo and fetogenesis. As a result of the compelling parallels between cancer cells and embryonic tissues, the emerging methods, technologies, and contemporary ideas from cancer research can easily be transferred to studies of the developing conceptus.

The methods applied to the study of teratology and developmental toxicology represent a broad spectrum of experimental models and designs that range from the mere observation of gross developmental abnormalities to the focused analysis of specific molecules, macromolecules, and the molecular pathways in which they act. Although the macroscopic and histologic methods may seem, to some, antiquated and of little value, they are included in this volume because they are still used and of considerable value in determining the overall developmental outcome.