Birth defects: from molecules to mechanisms

ABSTRACT—Birth defects remain a major clinical problem and, although much progress has been made in prenatal diagnosis, few measures are available for primary prevention. This is due, in large part, to our rudimentary understanding of the embryonic mechanisms of birth defects. Until recently it was customary to concentrate on defining teratogenic factors that may be active in humans. Now, with the rapid expansion in molecular biological technology, it has become possible to identify and isolate the genes that determine heritable predisposition to birth defects. The most productive strategy appears to be the genetic analysis of animal, principally mouse, models in which particular classes of birth defects develop owing to known genetic mutations. Gene targeting techniques allow mutations to be induced in previously cloned genes, permitting their potential as birth defect-inducing genes to be evaluated. Gene cloning alone, however, cannot reveal the entire pathogenetic sequence for any birth defect, since the most downstream events can be elucidated only by experimental embryological analysis. Culture methods are now available in which intact mouse and rat embryos undergo normal development for limited periods in vitro. Studies of this type have revealed several steps in the embryonic development of genetically determined spina bifida. The combination of gene centred and embryo centred research promises to advance our understanding of the pathogenesis of major birth defects.

Birth defects remain an important cause of death in utero, at birth and in the neonatal period, and continue to cause considerable morbidity and handicap in childhood. Despite progress in prenatal diagnosis, there has been less success in developing methods for primary prevention of birth defects. One important reason for this lack of progress is our limited understanding of the embryonic mechanisms that underlie the early abnormalities of organ formation that lead to birth defects.

It is 20 years since Wilson [1] stated the principles of teratology that govern the susceptibility of embryos to teratogenic insults and Carter [2] outlined the pivotal role of interactions between multiple genetic and environmental factors which determine susceptibility to major birth defects. Although these principles have long been familiar to scientists and clinicians concerned with studies of birth defects, it is only recently that we have begun to discover the mechanisms that may produce specific birth defects.

What has been missing is an understanding of the embryonic processes that intervene between the aetiological factors and the gross manifestations of disturbed development. The pathogenetic mechanisms of birth defects have hitherto been regarded as a mysterious 'black box' in which cells and molecules go about their business in vaguely imagined ways (Fig 1). We are now in the process of opening the black box of embryonic mechanisms. The keys that have permitted this access are, first, the technology for isolating and manipulating genes and, second, the ability to study early-stage mammalian embryos outside the maternal uterus.

Genes and embryos: tools and approaches

The study of birth defects (teratology) has long been synonymous with environmentally induced defects. Their genetic causes have had much less attention but it is here that the embryonic events may ultimately be most readily understood. In the case of single-gene conditions the defects can ultimately be traced back to the absence or malfunction of one species of protein, but the same cannot be assumed for teratogen related birth defects; multiple mechanisms seem likely to operate for many single teratogens. There are good reasons for placing more emphasis on the genetic aetiology of birth defects. According to most estimates, genetic causation or predisposition is present in more than 90% of human birth defects.

The progress in molecular genetics has made it possible to clone almost any gene. Determining the nucleotide sequence of a gene can provide information on the nature of the mRNA and protein gene products through computerised comparison of the sequence with that of other genes whose functions are known. The process of cloning also generates tools, such as cDNA probes, that can be used for the analysis of 'downstream events', i.e., the events that are set in train by the normal or abnormal expression of the gene. Among the downstream events that can be studied in this way are the embryonic mechanisms that lead to genetically determined birth defects. Thus a gene centred approach to understanding birth defects aims to clone the genes responsible and work towards an understanding of the embryonic mechanisms (Fig 2, bottom).

However, cloning genes that cause birth defects
Aetiologial factors

Cellular and molecular mechanisms

Birth defects

Fig 1. A prevalent view of birth defects. Much attention has been paid to the aetiological factors, genetic and/or environmental, and to the morphological description of the birth defects. Relatively little research has been performed on the cellular and molecular mechanisms of pathogenesis which have tended to be consigned to a ‘black box’.

cannot completely elucidate the pathogenetic sequence. The reason for this is that events progressively further downstream involve cell interactions and morphogenetic cell movements (eg tissue folding, cell migration). These types of event cannot be analysed by molecular strategies alone and require an experimental embryo centred approach (Fig 2, top). Although experimental analysis of organogenesis-stage embryos is routinely possible in lower vertebrates, this has not yet been extensively carried out in mammals. The importance of being able to study mammalian embryos experimentally is particularly evident for birth defects where extrapolation to human embryonic development is a desirable goal. The events of mammalian embryogenesis following implantation in utero of the blastocyst are hidden from view, making experimental studies difficult. In the 1960s and 1970s new culture methods for organogenesis stage rat and mouse embryos [3,4] offered to free the mammalian embryologist from the constraints of the maternal uterus, but this technique has only fairly recently begun to be used for experimental studies. This is in contrast to the large number of studies employing rodent embryo culture to evaluate potential teratogenic agents [5].

The ultimate challenge is to bring both the gene-centred and embryo-centred approaches to bear on the problem of the pathogenesis of birth defects.

Linking genes with birth defects

A typical approach to determine the genetic basis of disease is to perform a linkage analysis in order to identify DNA sequences that are genetically associated with the disease and thereby progressively work towards identifying the disease locus itself. Major birth defects such as neural tube defects, congenital heart defects and cleft lip/palate rarely occur in large families that facilitate such an analysis, so an alternative approach is needed. Currently the most promising strategy appears to be the detailed analysis of animal models of birth defects, with later extrapolation to humans. Evolutionary conservation of gene structure and function is so extensive that it seems almost certain that a locus identified in the mouse will prove to have a human homologue, but there is no guarantee that the homologous loci will control precisely the same biological functions in human and mouse.

Cloning mouse genes that cause birth defects (Fig 3)

To date the most successful approach to cloning mutant genes in the mouse has involved the search for ‘candidate genes’. Mutant loci are mapped as precisely as possible to a chromosomal location; then previously cloned genes, mapping to the same region of the genome, are evaluated for allelism with the mutant locus. Two examples where this approach has been successful are the mutations splotch (Sp) and extra-toes (Ex). Both mutations cause major birth defects in the mouse. Splotch homozygotes have severe neural tube
defects, anencephaly and spina bifida (Fig 4), together with neural crest related abnormalities, including persistent truncus arteriosus. Homozygotes do not survive beyond birth, but heterozygotes are viable and show only neural crest related pigmentation defects. Molecular genetic studies recently identified the gene Pax-3 as being mutated in splotch mice [6] and led to the subsequent discovery that Waardenburg type I syndrome is the human counterpart of the mouse mutation [7,8]. In Waardenburg syndrome, patches of hypopigmentation, characteristically a white forelock, are associated with craniofacial abnormalities and sensorineural hearing deficit. Most patients are heterozygotes and so would not be expected to exhibit the severe neural tube and cardiac defects characteristic of splotch homozygotes. Recent reports describe an increased incidence of meningomyelocele in Waardenburg families.

Extra-toes produces preaxial polydactyly in both heterozygotes and homozygotes, and severe craniofacial defects and exencephaly in homozygotes. During the course of a transgenic mouse study, a particular integration (named add) was found to give rise to a phenotype resembling extra-toes. A molecular genetic approach subsequently showed that the two mutations are allelic [9]. The gene that is disrupted in extra-toes and add is named Gli3 [10]; this gene has recently been shown to be mutated in cases of human Greig cephalopolysyndactyly syndrome [11], a rare congenital condition in which craniofacial defects and polydactyly coexist.

In both these examples, therefore, the availability of mouse mutations that cause major birth defects, together with the relative ease of performing molecular genetic analysis in mice, has led to the discovery of the responsible mouse genes and has helped to identify the homologous human diseases. Research is now in progress to determine the pathogenetic mechanisms underlying the birth defects in splotch and extra-toes. Both Pax-3 and Gli3 are transcription factors; that is, they control the expression of other genes via the binding of their gene products to DNA control regions in the regulated genes. Both seem likely to be key genes controlling cascades of embryonic events. The expression of each gene has been described during normal embryonic development; in each case specific mRNA is synthesised in tissues that become abnormal in the mutant mice. The challenge is now to determine which genes are regulated by Pax-3 and Gli3, and so move towards an understanding of the downstream events that precede the development of these birth defects.

Creating mouse models of birth defects by gene targeting

So far we have considered the use of gene cloning to link genes with birth defects. This approach begins with the birth defect phenotype and, by a process of molecular genetic analysis, identifies the causative gene. A powerful alternative approach developed in recent years is 'gene targeting'. With this strategy a cloned gene is the starting point, and mutations are introduced with the aim of defining the mutant phenotype (Fig 3). The development of embryonic stem (ES) cells, tissue culture cell lines derived from preimplantation mouse embryos, has made it possible to select for rare recombination events between an endogenous gene and an artificially mutated form of the same gene that can be introduced into the ES cells. Homologous recombination events lead to replacement of the normal endogenous gene by the mutant form, resulting in the production of mutant ES cells. These mutant cells can be selectively grown in culture and then introduced into preimplantation embryos. There they will participate in development, forming all embryonic tissues including the germ cells of the resulting chimaeric mice. The offspring of the chimaeras, if derived from mutant germ cells, are heterozygous for the mutation, and matings between such heterozygotes produce homozygous mutant offspring that can be expected to express the mutant phenotype.

This strategy is used to create null (ie loss of function) mutations in a wide range of previously cloned genes. A surprising finding is that some genes expressed in the tissues of early embryos can be inactivated without apparent deleterious effects on embryonic development. For instance, the extracellular matrix glycoprotein tenascin has an interesting pattern of expression at various stages of development, for instance in gut and tooth development [12,13], suggesting that this molecule may play a significant role in developmental processes. However, homozygous null mutant mice not only develop normally but appear to function and breed normally as adults [14]. A widely accepted explanation for this apparent
redundancy of gene function is that related genes, perhaps other extracellular glycoproteins in the case of tenascin, can compensate for loss of a particular molecular species. An alternative view, suggested by Erickson [15], is that there may be superfluous gene expression during embryonic development and that only a subset of the observed gene activity is actually vital for development.

A small number of the genes targeted to date have yielded phenotypes that include embryonic birth defects. For instance, the gene WT1 which is mutated in patients with Wilms' tumour appears to be indispensable for normal embryonic development: mice with null mutations in WT1 fail to develop kidneys [16]. WT1 is known to be expressed during the early development of embryonic mesodermal tissues and, according to Erickson's hypothesis, clearly must fall into the category of essential embryonic genes. Further analysis of mutant mice produced by gene targeting promises to yield new model systems for the analysis of birth defects and to provide important insights into their molecular development.

**Embryological analysis of birth defects: experiments in vitro**

We have seen the power of modern molecular genetic approaches for identifying and modifying genes important in the development of birth defects. We now turn to the embryo centred approach as illustrated in Fig 2, to see to what extent the experimental analysis of mammalian embryos can shed light on the downstream events that culminate in the occurrence of birth defects. An example is the development of spina bifida in the mouse mutation curly tail (ct). The curly tail gene has not yet been cloned, so our understanding of the developmental origins of spina bifida in homozygotes derives entirely from the experimental embryological studies. Spina bifida results from failure of the neural tube to complete closure at the posterior neuropore in the future lumbar and sacral regions of the developing spinal cord. The defect in the mouse closely resembles the corresponding human malformation at this embryonic stage, suggesting that the pathogenetic mechanisms may be similar in both species.

An important finding to emerge from the experimental analysis of curly tail embryos is that the basic defect resides not in the neural plate of the embryo but in ventrally located tissues, the notochord and hindgut. Incomplete closure of the posterior neuropore occurs in curly tail embryos when they are maintained in culture, whereas control embryos close their neuropores normally in vitro. Significantly, however, neural plates, microdissected from the neuropore region and cultured in isolation from other tissues,
were found to be capable of normal neural tube closure in both curly tail and control embryos [17]. Thus the mutant neural plate fails to form a closed neural tube only when it is attached to mutant notochord and hindgut.

Further experimental studies have revealed that the curly tail defect involves a significantly reduced rate of cell proliferation in notochord and hindgut in embryos undergoing delayed neuropore closure compared with normally developing control embryos [18]. What controls the rate of cell proliferation in these tissues during normal development, disturbance of which may be implicated in curly tail embryos? One possibility relates to the extracellular matrix component hyaluronan, a high molecular weight carbohydrate which is particularly abundant around the notochord at the stage of posterior neuropore closure and accumulates in smaller amounts in curly tail embryos undergoing delayed neuropore closure [19]. Hyaluronan synthesis correlates with, and may mediate, rapid proliferation in a variety of cell types, probably via interaction with its cell surface receptor CD44 [20]. It is possible, therefore, that the subnormal proliferation of notochord and hindgut in affected curly tail embryos results from the decreased accumulation of hyaluronan (Fig 5). An alternative, though not mutually exclusive mechanism has arisen from studies of the pattern of protein expression in mouse embryos undergoing neuropore closure. The iron-binding growth factor transferrin localises specifically within the lumen of the hindgut immediately beneath the posterior neuropore of normal embryos [21]. Experimental labelling studies in embryo culture showed that transferrin in the hindgut originates outside the embryo in the culture medium. It seems likely that the embryo developing in utero takes up transferrin from the maternal serum exudate that surrounds the early postimplantation embryo prior to the onset of placental function. Preliminary studies with curly tail mutant embryos indicate that the pattern of localisation and uptake of transferrin in the hindgut is disturbed in embryos undergoing delayed closure of the posterior neuropore (Hoyle and Copp, unpublished), raising the possibility that the hindgut of curly tail embryos receives a suboptimal growth stimulus from exogenous transferrin (Fig 5). Either of these mechanisms, or others as yet undiscovered, may ultimately prove to be responsible for the cellular defect in the curly tail mutation.

Experimental studies of mouse embryos in vitro have provided a further important piece of the spina bifida jigsaw puzzle. It is not immediately obvious why a reduced rate of cell proliferation in the notochord and hindgut should prevent the neuropore from closing in curly tail embryos. The caudal region of these embryos is ventrally curved to a greater extent than in normal embryos. Experiments in which a splint, the terminal 0.5 mm of a human eyelash, was inserted into the lumen of the hindgut to keep the caudal region

**Fig 5. Schematic representation of the defect that underlies development of spina bifida in the mutant curly tail mouse embryo.** The embryo on the left (aged approximately 10 days post coitum) has been sectioned through the posterior neuropore region, shown by the vertical line, producing the transverse section shown enlarged on the right. The notochord (black) and hindgut endoderm (grey) are known to proliferate abnormally slowly in curly tail embryos developing spina bifida, whereas the overlying neural plate is unaffected. This cell proliferation defect correlates with reduced accumulation of newly synthesised hyaluronan in the extracellular matrix around the notochord and altered uptake of the growth factor transferrin from the lumen of the hindgut.

straight (Fig 6) showed that the enhanced curvature is directly responsible for the delayed closure of the neuropore in curly tail embryos [22]. It seems very likely that the curvature is a direct result of the reduced proliferation of the ventral tissues, the notochord and hindgut. Thus the sequence of pathogenic steps leading from the genetically determined cell proliferation defect via enhanced ventral curvature to delayed closure of the posterior neuropore has been worked out for the curly tail mutation entirely by experimental embryological analysis. However, analysis of the further upstream events may need to await the cloning of the gene.

**Genes and environment: understanding how they interact**

This review has concentrated on elucidating the pathogenesis of birth defects with primarily genetic aetiology but it would not be fair to ignore the importance of environmental factors. In particular, the interactions between environmental teratogens and a predisposing genetic constitution deserve detailed study since they may provide a paradigm to represent a great many apparently spontaneous birth defects in humans. It is unfortunate that the vast majority of reproductive toxicological and teratological studies have been performed without reference to the genetic status of the experimental animals, usually employing outbred heterogeneous stocks of rats and mice. The reasoning
behind this approach is that outbred animals are more likely to mimic the outbred human population than are homogeneous inbred strains. The counterargument is that valuable information could be lost from the studies since genetic differences in susceptibility to a teratogen will be obscured when each animal is unique in its genetic constitution. Particularly susceptible or resistant genotypes will occur only sporadically in the studies, as in the human population, and are unlikely to be recognised.

A few studies have now begun to compare the susceptibility to teratogens of different inbred strains of mice, while other studies have compared the severity of expression of genes that produce birth defects on different genetic backgrounds. Certain strains appear especially prone to birth defects while others appear resistant. Thus the C57BL/6 inbred strain is highly susceptible to birth defects, for instance with a high incidence of cortisol induced cleft palate and a predisposition to neural tube defects when the curly tail mutation is bred on to this background [23]. On the other hand, the DBA/2 inbred strain shows a reduced propensity to develop neural tube defects, both when exposed to high temperatures in utero [24] and as a result of expression of the curly tail mutation. It seems likely that genetic differences between the inbred strains account for these differences in susceptibility and raise the possibility that ‘professional modifier genes’ may exist to govern susceptibility to a wide range of environmental and genetic factors that cause birth defects. Genetic mapping technology using recombination inbred mouse strains [25] offers the possibility of mapping and ultimately identifying these modifying genes in the near future.

The interaction between retinoic acid and mouse mutations that cause neural tube defects has been studied in detail. Retinoic acid is a powerful teratogen that can induce a variety of birth defects in both humans [26] and rodents [27]. It increases the incidence of exencephaly in mutant curly tail embryos but when administered a day later in gestation at the critical time for closure of the posterior neuropore, low doses of retinoic acid cause a reduction in the incidence of spinal neural tube defects [28,29]. Two families of nuclear receptors interact with retinoic acid and related molecules: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Upon binding of their ligands, both types of receptor can act as transcription factors to control the expression of other genes [30]. Recent studies have shown that RAR isoforms β and γ are downregulated in tissues of the posterior neuropore region in curly tail embryos (Chen, Morriss-Kay and Copp, unpublished), raising the possibility that modulation of RAR expression may be the mechanism whereby retinoic acid can reduce the incidence of spinal neural tube defects in this mutant.

A clinically relevant question in terms of environment–gene interactions is: what is the mechanism whereby folic acid can reduce the recurrence risk of neural tube defects in fetuses of mothers who have already had an affected child? This preventive measure has been established through a series of clinical trials with little consideration, to date, of the embryonic mechanisms that may underlie it. The next step must be to determine whether an inherited folate-related defect is responsible for this category of birth defects, or whether the action of folate is purely pharmacological in correcting a defect that does not primarily involve folate. Epidemiological studies indicate that neural tube defects are likely to result from genetically determined abnormalities in the embryo rather than in the mother. This is supported by a recent study that found no abnormalities of folate uptake or metabolism in mothers of affected fetuses [31]. Thus an embryo-centred experimental analysis of the role of folate metabolism and function in relation to the
development of neural tube defects is urgently required.

Conclusion

Although we are not yet in a position completely to explain the pathogenesis of any single birth defect, the technology is now in place for a major advance in our understanding. A full appreciation of the mechanisms will require a multidisciplinary approach with molecular biologists and toxicologists to identify the responsible genes and teratogens, biochemists and cell biologists to unravel the complex molecular pathways that underlie organ formation, and experimental embryologists to probe the most 'downstream' events involving cell and tissue interactions that comprise the changing shape of the embryo. Equally significant is the recent alteration in our thinking about birth defects. Con-signing the pathogenetic mechanisms to a 'black box' is no longer acceptable.

Acknowledgements

I am most grateful to Dr J Chan for critical reading of the manuscript and to Dr S Conway for providing Fig 4. The author's research is supported by a Wellcome Trust Senior Clinical Research Fellowship and is part of a Multicentre Agreement for Studying Neural Tube Defects in Mutant Mice funded by the National Institute for Child Health and Human Development, NIH, USA, through Cooperative Agreement HD28882.

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