Genetic determinants of teratogen-induced abnormal development in mouse and rat embryos *in vitro*

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ABSTRACT The response of an embryo to a teratogenic treatment is often critically dependent on its genetic makeup. However, in conventional *in vivo* studies of gene-teratogen interactions it may be difficult to distinguish between the effects of genes that are carried by the embryo and those that are carried by the mother. It is likewise not easy to determine whether an observed interaction is between a particular gene and the parent compound administered, or whether it is with a metabolite that has been generated by the maternal system. The use of whole rodent embryo culture offers certain advantages in the study of gene-teratogen interactions. Not only can the effects of metabolism and the maternal genotype be more carefully controlled, but the stage of development at which embryos of different genotypes are exposed can be matched. Rodent whole embryo culture has been used to a limited extent to study interactions between single gene mutations and teratogenic treatments, variations in responses of different strains to teratogens, as well as species differences in response to teratogens. These studies point to the need to precisely control the stage of development at the time of treatment in order to be able to make valid comparisons. But, even more important, they highlight the versatility of the whole embryo culture technique, and underscore the need for its wider use in evaluating the relative contribution of genes and environment to abnormal embryonic development.

KEY WORDS: *genes, abnormal development, mouse, rat, embryo culture, in vitro*

Introduction

Over the years there have been numerous studies that describe how an embryo's response to a teratogenic treatment is dependent on its genotype. Initial reports involved strain differences in the frequencies of the rumplessness response to insulin in chicks (Landauer and Bliss, 1946) and the cleft palate response to cortisone in mice (Fraser and Fainstat, 1951). In the case of glucocorticoids and cleft palate, much work has been done to analyze the genetic characteristics of the variation in responses (Vekemans and Biddle, 1984; Liu and Erickson, 1986). Strain differences in the production of numerous developmental anomalies have been described, including neural tube defects induced by cytochalasins (Austin *et al.*, 1982) or hyperthermia (Finnell *et al.*, 1986), and limb defects induced by acetazolamide (Kuczuk and Scott, 1984) or cadmium (Layton and Layton, 1979). Other studies have described apparent heterozygote sensitivity in crosses of wild type females to males heterozygous for a mutation that causes a malformation in homozygous form but no, or only minor, abnormalities in heterozygous form. The response to a teratogen in these litters is compared to litters from wild type males and females. Examples of such studies, which compare the response of wild type embryos to those heterozygous for a mutation, include interactions of the Brachyury gene (T) with actinomycin D (Winfield and Bennett, 1971), trypan blue (Hamburgh *et al.*, 1970), or cycloheximide (Lary *et al.*, 1982); the mutations luxoid and luxate with 5-fluorouracil (Dagg, 1967); crooked and rib fusion with insulin (Cole and Trasler, 1980); and splotch with retinoic acid (Kapron-Brás and Trasler, 1984).

Many of these gene-teratogen interactions can be best understood in the context of the multifactorial-threshold concept (Fraser, 1976). This concept suggests that developmental anomalies may result from the combined action of a number of genetic and environmental factors. Although the underlying developmental processes that lead to normal embryogenesis occur in a manner that is continuously distributed, the abnormality that results from interference with these processes is either present or absent. There is a developmental threshold that separates the continuously distributed variable into two: the expected "normal" condition and a malformation. This can be illustrated in the case of mouse spina bifida (Kapron-Brás, 1987). The closure of the posterior neuropore happens continuously in a zipper-like fashion, until it is completely closed. Factors which delay closure, such as a mutant gene or a teratogen, or the two combined, appear to slow down the

Abbreviations used in this paper: ETU, ethylenethiourea.

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process. If the delay is severe enough, a threshold is reached, after which normal closure can no longer occur. The result is a permanently open posterior neuropore, or spina bifida.

Traditionally, studies of the interactions of genes and environmental agents have not been simple. It may be difficult to conclude whether the observed interactions are with the particular teratogen administered rather than some metabolite, and by extension whether genetically distinct embryos or their mothers are equally able to metabolize the parent compound to its active form. Also, it must be separately determined whether there are any deviations in the development of embryos of different genotypes at the time of treatment that could account for their diverse responses. As demonstrated by Juriloff *et al.* (1991) in a study of anterior neural tube closure, a particular strain or genotype of embryo may have a developmental variation that could alter its reaction to a drug. In the case of apparent heterozygote sensitivity, it may not be possible to establish whether it is actually the heterozygotes, or mutation carriers, which end up with the most abnormalities, since litters will be made up of both heterozygotes and wild types. In the case of strain differences, a common problem is whether or not the maternal genotype is altering embryonic sensitivity. Reciprocal crosses or embryo transplants must be done to dissect out this possible maternal effect (Biddle and Fraser, 1976; Takano *et al.*, 1980). The use of whole embryo culture (New, 1978) has the potential of eliminating some of these problems and facilitating the solution of others. In spite of this, it has not been widely used, although there are a number of good examples of the influence of genotype on teratogen sensitivity. This review will examine the use of whole embryo culture in the investigation of gene-teratogen interactions, as well as explore potential future directions.

Gene-teratogen interactions *in vitro*

Single genes

The embryo culture system can be extremely versatile in demonstrating increased sensitivity of mouse mutants or carriers to a particular teratogenic treatment, as demonstrated by Cockroft *et al.* (1992). Mice of the curly tail strain are susceptible to neural tube defects, in particular of the caudal region, because of the action of a recessive gene, *ct*, in homozygous form (Embury *et al.*, 1979). Embryos of the *ct/ct* genotype were found to be more likely to develop anterior neural tube defects when grown in inositol-deficient serum, compared to an outbred and an inbred strain that do not spontaneously develop neural tube defects (Cockroft *et al.*, 1992). It thus appears that the presence of the *ct* gene increases sensitivity to inositol deficiency, although some genetic variability exists between the inbred strain used, CBA, and *ct/ct* mice, which may in part explain the differences in responses. Because of the nature of the teratogenic treatment, this experiment would be extremely difficult to repeat *in vivo*. Serum was made inositol deficient in varying degrees by dialyzing rat serum and returning amino acids, and vitamins with or without inositol. *In vivo*, if it were possible to obtain completely inositol-deficient pregnant animals, one would expect that there would be major effects on the mother's system which would complicate the results. In this study, the authors were able to vary the time of dissection and use embryos of a very particular stage (2-5 somites), and thus control the differences in stage of development that exist between the strains used.

Embryo culture also proved very useful in an investigation of the control of limb growth in mice heterozygous for the gene hemimelia-extra toe (Hm^x) (Knudsen *et al.*, 1985). Day 12 or 13 embryos with 42 to 47 somites were cultured for 4 h in a 1:1 mixture of Waymouth's medium and fetal bovine serum with the addition of tritiated thymidine. Uptake of the radiolabel was found to be increased in the mutants in the preaxial portion of the limb only. No differences were found in other parts of the limb or in the remainder of the embryo. The period of increased DNA synthesis is correlated with the period in which aberrant outgrowth of this part of the limb bud occurs, which leads to the extra-toe phenotype. The usefulness of labeling developing limbs *in situ* in the embryo culture system is nevertheless limited by the length of time these older embryos can survive in culture. Thus, in the Knudsen *et al.* (1985) paper, organ culture of isolated limbs was used for longer term studies.

It is also important to investigate the effects of teratogens on mutant heterozygotes that do not have an obvious phenotype at the stage they are being examined. In an analysis of the effects of retinoic acid on neural tube closure in splotch heterozygotes (*Sp*/+), a marker was introduced to the strain that would enable heterozygotes to be distinguished from wildtype embryos (Kapron-Brás and Trasler, 1988). Crosses were made between *Sp*/+ mice and ones carrying a Robertsonian fusion of chromosomes 1 and 3. Because such a fusion incorporates the same chromosome as that which the *Sp* locus is on, its presence can be used as a marker for the wildtype allele. The accuracy of identification is reduced by the frequency of crossing over between *Sp* and the centromere, which was about 20% (Kapron-Brás *et al.*, 1986). The use of this marker showed that carriers of the splotch allele were indeed more sensitive to the effects of retinoic acid (Kapron-Brás and Trasler, 1988). Previous studies *in vivo* had demonstrated that embryos from a cross of *Sp*/+ males X SWV females were more sensitive to the spina bifida inducing effects of retinoic acid than were embryos from a +/+ X SWV cross (Kapron-Brás and Trasler, 1984). A similar effect was noted *in vitro* but, because of the limited length of time the embryos could grow in culture, overt spina bifida did not develop. Instead, delays in the closure of the posterior neuropore were observed, and the cumulative effects of the *Sp* gene and retinoic acid led to increased delays in the closure of the embryo's posterior neuropore, compared to those that did not carry the mutant gene (Kapron-Brás and Trasler, 1988). In untreated *Sp*/+ embryos, the posterior neuropore was found to be larger than in wildtype embryos, suggesting a possible basis for the combined action of the gene and the teratogen. It is expected that a more accurate marker, such as the one introduced by Moase and Trasler (1987) that gives virtually 100% identification, would lead to an increase in the differences observed between heterozygotes and wild types.

Strain differences

Many strain differences in the response of mouse embryos to teratogens *in vivo* have been described. As with other genetic differences the question arises whether the observed diversity is caused by intrinsic embryonic factors rather than variation in maternal absorption, metabolism or excretion of the compound. *In vivo*, this problem has been approached using reciprocal crosses that maintain a constant embryonic genotype under varying maternal genotypes (Biddle and Fraser, 1976), or by transplantation

experiments, in which embryos of varying genotypes are exposed to identical environments (Takano *et al.*, 1980). Whole embryo culture adds an additional tool in the study of the relative contribution of maternal and embryonic factors to the teratogenic response.

Naruse *et al.* (1988) examined the response of SWV and C57BL/6J to sodium valproate *in vitro*, confirming the *in vivo* demonstration that the former strain is more sensitive to sodium valproate induction of exencephaly than the latter strain (Finnell and Chernoff, 1985). To study intrinsic embryo sensitivity, Naruse *et al.* (1988) cultured 6 to 8 somite embryos from the two strains in the presence of sodium valproate for 48 h. As *in vivo*, the drug was more likely to cause open cranial neural tubes in SWV embryos than in C57BL/6J embryos. The minimum lethal dose was also 1.5 times lower in SWV embryos. Interestingly, some inhibition of cranial neural tube closure was observed in C57 embryos, although only when they were exposed to 3 mM sodium valproate compared to 1 mM in SWV embryos. This contrasts with the *in vivo* situation where this malformation was rarely induced in C57 embryos by sodium valproate. The authors suggest that, because the teratogenic concentration is close to the lethal concentration in this strain, the survival of the exencephalic embryos may also have been affected and thus the malformation would not have been observed in the *in vivo* studies (Naruse *et al.*, 1988). Since embryos of the two strains developed under identical culture conditions, it can be concluded that there are genetic differences intrinsic to the embryos themselves that result in their distinct responses to the drug. Nevertheless, as the authors point out, there may be additional maternal factors at play *in vivo* that cannot be addressed in culture (Naruse *et al.*, 1988).

In an analysis of 5-azacytidine-induced exencephaly, it was again found that the *in vitro* situation closely mirrored the *in vivo* situation (Matsuda, 1990). MT/hokIdr inbred mice and Slc:ICR random bred mice were exposed to 5-azacytidine *in vivo* on day 7 of gestation, as well as *in vitro* on day 8 of gestation, when the embryos had 4 somites. In both cases the primary malformations observed were open anterior neural tubes, and in both cases it was observed that the MT strain of mice was about 10 times more sensitive than the ICR strain. It was suggested that MT mice are genetically predisposed to exencephaly in general, not just in response to 5-azacytidine, and that the drug was effective in causing malformations of the anterior neural tube in these mice at concentrations to which a strain not genetically predisposed to exencephaly would not respond. It is interesting to note that at the end of the culture period 20% of the untreated control ICR embryos with an average of 23 somite pairs had open cranial neural folds, compared to none in the MT mice, which had about 25 somite pairs. One might expect the opposite to be true, as it has been demonstrated in other strains that mice genetically susceptible to neural tube defects have delays in neural tube closure (Dempsey and Trasler, 1983; Copp, 1985). While the open neural tubes in control ICR embryos could be caused by an overall delay in development, the variability between the strains may also indicate that the predisposition and response of embryos to agents that induce exencephaly are actually fairly complex. The basis for the proposed genetic susceptibility remains to be elucidated.

Strain differences in the response of mouse embryos to both hyperthermia and cadmium have been reported *in vivo* (Layton and Layton, 1979; Finnell *et al.*, 1986). The interaction between these two teratogenic agents was investigated in a study that examined the induction of tolerance to cadmium by a prior heat exposure in two strains of mice, BALB/c and SWV (Kapron-Brás and Hales, 1992). The former mice are relatively sensitive to cadmium-induced embryotoxicity (Layton and Layton, 1979), while the latter are more resistant (Kuczuk and Scott, 1984). Embryos of each strain with 4 to 6 somites were cultured under identical conditions, and exposed to various combinations of a known thermotolerance-inducing heat exposure that does not induce malformations (5 min at 43°C) and 1.75 μM cadmium, which causes embryotoxicity. As expected, BALB/c mice were more sensitive to the effects of cadmium, showing a higher percentage of both deaths and malformations. In addition, the BALB/c embryos did not develop cross-tolerance to the teratogenic effects of cadmium, whereas the SWV embryos did. A prior heat exposure caused only minimal changes to BALB/c in the 80% frequency of malformations induced by cadmium, but in SWV embryos the frequency was reduced significantly from 60% to below 20% (Kapron-Brás and Hales, 1992). Since the induction of heat shock proteins has been implicated in the development of thermotolerance (Landry *et al.*, 1982; Li and Werb, 1982) this was investigated as a possible explanation of the diverse embryonic responses. The induction of the 70 kDa heat shock protein by the heat exposure was similar in the two strains and was not actually higher in SWV embryos, the strain that became tolerant to cadmium (Kapron-Brás and Hales, 1992).

Rat embryos have not traditionally been used to examine strain differences in response to teratogens, perhaps because of a more limited availability of genetically characterized inbred strains. However, in a report by Eriksson (1988), two substrains of Sprague-Dawley rats were compared. *In vivo*, the U substrain (bred in Uppsala) showed an increased malformation rate in the offspring of streptozotocin-diabetic mothers compared to the parental H substrain (bred in Hanover) from which they were derived. Both the maternal and fetal genotypes were found to be important for the malformation response. Only diabetic U mothers produced malformed embryos, regardless of the embryonic genotype, but cross-breeding experiments demonstrated that the numbers of resorptions and malformations were correlated with the relative contribution of the U genome within the embryo. Thus, when the diabetic mother was U or H/U, embryos containing a major U genome (U/U or U/(H/U)) showed more resorptions and malformations than did embryos of the mixed H/U type (Eriksson, 1988). The *in vitro* component of this experiment endeavored to determine whether there were differences in response of H and U embryos to the chemicals D-glucose and B-hydroxybutyrate that are associated with diabetic pregnancies. Various parameters of growth and development were negatively affected by these teratogenic treatments, yet the reactions of the two strains were similar (Eriksson, 1988). This may indicate that the variable responses of embryos of the two genotypes to diabetic pregnancies are not associated with either elevated D-glucose or B-hydroxybutyrate.

Species differences

Several studies have been attempted recently that compare the response of rat and mouse embryos to various chemicals. Species differences studied *in vivo* are complicated by a number of factors. As with other genetic investigations, maternal metabolism of test compounds must be distinguished from intrinsic heterogeneity in the embryo's responses. As well, it can be difficult to treat embryos at corresponding stages of development, and to know the precise

level of test compound the embryo is exposed to as absorption may vary among species.

An interesting series of studies has been done that examine the effects of methanol on rat and mouse embryos in culture. These reports demonstrate the usefulness of the *in vitro* approach in making comparisons, but they also illustrate some of the pitfalls that would be avoidable with carefully controlled studies. *In vivo*, it has been found that mouse embryos are damaged by exposure to this compound at levels which are non-toxic in rats (Nelson *et al.*, 1985; Rogers *et al.*, 1993). However, since there was some concern over whether this was the result of species diversity in the absorption of methanol, or whether there was an intrinsic difference in response, Andrews *et al.* (1993) chose to compare the effects of methanol on Sprague-Dawley rat and CD-1 mouse embryos *in vitro*. Rat embryos were exposed to 0 to 16 mg methanol per ml of medium and mouse embryos were grown in concentrations up to 8 mg/ml. Methanol at 2 mg/ml in the mouse caused reductions and delays in growth and development, and at 6 to 8 mg/ml malformations of the neural tube and embryolethality were observed. In contrast, rat embryos were only sensitive to methanol at concentrations of 8 mg/ml or higher, with significant abnormalities observed only at concentrations of 12 mg/ml or higher. Abnormal brain and neural tube defects were seen, as well as delayed limb bud development. Importantly, the species differences in response to methanol were not likely a result of variability in metabolism, as methanol concentrations did not vary considerably over the 24-hour culture period.

There are some problems with the Andrews *et al.* (1993) study that are worth keeping in mind. The stages of development at the time of treatment in rat and mouse embryos were not identical. Rats were cultured and exposed starting at the late head fold stage (0 somites), and mouse embryos at the early somite stage (3 to 5 somites). Rat embryos were cultured for 48 h, with exposure to methanol during the first 24 h, while mouse embryos were cultured and exposed for 24 h. The number of somites attained by each species at the end of their respective cultures was similar. This suggests that exposure to methanol occurred at very different developmental stages in the two species, and thus that exposure time cannot be eliminated as a possible cause of the variation in response of the two species.

Despite these problems, the Andrews *et al.* (1993) paper was important in pointing out the potential of the whole embryo culture system in examining species differences in response to a chemical exposure. A subsequent study by the same group (Andrews *et al.*, 1995) goes further in using the embryo culture system to examine mechanisms of methanol toxicity, although problems with stage variability are still present. Since formic acid, a metabolite of methanol, has been suggested to cause toxicity in primate species (Clay *et al.*, 1975), Andrews *et al.* (1995) examined the role of formate and formic acid in methanol teratogenicity in rodent embryos. Formate does not accumulate in rodents following methanol exposure *in vivo*, and thus these species are not ideal models for the teratogenic action of methanol in humans, where this does occur. In culture, however, formate and formic acid can be added directly to the growth medium, an approach taken in the Andrews *et al.* (1995) paper. Using methodology that was similar to their previous paper, the group exposed presomite rat embryos to 0 to 29.4 mM sodium formate or 0 to 23.5 mM formic acid. Comparable levels in cultures of 3 to 5 somite mouse embryos were 0 to 44.1 mM and 0 to 44 mM respectively. Growth retardation and developmental delays were again observed. Some of the malformations observed were similar to those produced by methanol. Anomalies of the central nervous system were primarily observed, as were enlarged maxillary processes, enlarged pericardium and delayed heart development at the highest concentrations. In rat embryos, rotational and central nervous system anomalies were seen. A comparison of the two species demonstrated that there were no significant differences in response to formate or formic acid, although there was some suggestion that formic acid may cause less embryolethality when administered to mouse embryos, since some embryos were able to survive at concentrations that were 50% higher than in the rat embryos. This contrasts sharply with the previous investigation (Andrews *et al.*, 1993), in which mouse embryos were found to be more sensitive to methanol.

A further study (Abbott *et al.*, 1995) attempted to examine the mechanism of methanol toxicity in Sprague-Dawley rat and CD-1 mouse embryos *in vitro*, by observing the effect of this chemical on cell death. The major developmental abnormalities in both rat and mouse embryos included reduced forebrains, abnormalities of visceral arches, neural tube defects, and abnormal otic and optic placodes. In both species, cell death was increased in affected regions, except for the neuroepithelium in areas of open neural tubes. The major contrast between the two species was again that mouse embryos responded at a lower concentration than rat embryos and that the latter only demonstrated cell death when the culture was continued for 48 h (with or without methanol for the last 24 h), whereas the effects were observed in mice at the end of a 24-hour culture period. It is again worth noting that the developmental stage at the start of culture was 0 somites for rat embryos, whereas for mouse embryos it was 3 to 5 somites.

A dissimilarity between the response of rat and mouse embryos *in vivo* and *in vitro* was also seen when a very different compound was used (Hansen and Grafton, 1994). Dexamethasone, a glucocorticoid, has been found to decrease body weight to a greater extent in rat embryos than in mouse embryos *in vivo* (Holson *et al.*, 1991). However, the opposite was found to be true when embryos were exposed in whole embryo culture (Hansen and Grafton, 1994). Rat embryos of the CD strain were cultured for 48 h starting at the presomite stage, and CD-1 mouse embryos were cultured for the same time period, but starting from 2 to 5 somites. In mouse embryos there were decreases in morphological score or somite number with as little as 5 or 25 μg/ml dexamethasone, whereas similar effects were not seen in rat embryos exposed to less than 100 μg/ml. There were some qualitative differences in the particular features that were delayed in the two species: in mice development of allantois, optic placode, flexion and caudal neural tube were decreased at the lowest doses, while in rats the olfactory placodes, limbs, branchial bars and somites were first affected. Higher concentrations had more wide-ranging effects in both species. In rat embryos, all concentrations of dexamethasone tested altered closure of the anterior neural tube, an anomaly that was less common in mouse embryos. Variation in dexamethasone uptake was investigated as a possible mechanism for the observed species differences. In fact, it was determined by measuring uptake of radiolabeled dexamethasone that although yolk sac content did not differ between the two species, the uptake by mouse embryos was about 50% higher than for rat embryos. Once again, diversity in the developmental age of the rat and mouse

embryos at the time of treatment cannot be eliminated as a source of the variable responses. Ultimately, studies such as this will likely contribute more to our knowledge about how mice and rat embryos compare in their responses to various compounds when they are cultured using standard or typical procedures, rather than point out particular genetic differences. Conclusions about the latter aspect would necessitate stricter controls on developmental stage at the time of exposure.

When mouse and rat embryos of the same developmental stage, as indicated by number of somites, have been compared, variation in their responses have still been observed. Hansen *et al.* (1990) investigated the effects of lithium carbonate on CD rat and CD-1 mouse embryos, both of which had 10±2 somites. The culture period corresponded to 8 to 10 days of gestation in the mouse and 10 to 12 days in the rat. Embryos of the two species showed comparable growth during the culture period, ending up with a similar number of somites and developmental score. Some very specific differences in response to lithium were observed between the two species. The treatment was found to be embryolethal to rat embryos at 1.8 and 5.0 meq/L, but no mouse embryolethality was observed at comparable concentrations. In contrast, open neural tubes were observed in mouse embryos, with a significant increase occurring at the 5.0 meq/L concentration of lithium. Only one rat embryo in the entire group that was cultured from 10 to 12 days of gestation had an open neural tube. Thus, except for the effect on neural tubes, mouse embryos were somewhat more resistant to the effects of lithium than were rat embryos of the same age. A second aspect of the Hansen *et al.* (1990) study addressed the question of sensitivity at different developmental stages. In a separate group of rat embryos cultured from day 9 to 11, a spontaneously high frequency of open neural tubes was found in the control group and this was increased with lithium treatment. Generally, in the younger treatment group developmental characteristics were affected to a much greater degree than in the older group of rats. This again highlights the stage-dependent effects in teratogenesis, and reinforces the need to compare embryos of the same developmental stage.

Finally, a study by Daston *et al.* (1989) made excellent use of the benefits of the *in vitro* system to investigate an observed *in vivo* difference between mice and rats in their response to ethylenethiourea (ETU). *In vivo*, mouse embryos are considerably more resistant to the teratogenic effects of this chemical, requiring treatments that are 10 to 40 times higher than those used in rats (Ruddick and Khera, 1975; Khera, 1984). Daston *et al.* (1989) exposed rat and mouse embryos from comparable developmental stages to ETU using identical culture conditions. It was determined that the changes brought about by ETU were qualitatively similar in the two species but that twice as much ETU was needed to cause abnormalities in mice as in rats. This was obviously not enough of a difference to account for the *in vivo* discrepancy. It was further discovered that addition of a microsomal (S-9) fraction from mouse but not rat liver was capable of eliminating the typical teratogenic response. Since this fraction contains endoplasmic reticulum-associated metabolizing enzymes, it appeared that both maternal and embryonic factors were contributing to the observed species differences. This investigation was complicated by the fact that, although mouse embryos exposed to both the S-9 fraction and ETU developed fewer of the type of malformations normally caused by ETU, other abnormalities were induced. These malformations, principally open neural tubes, were not seen in rat embryos, nor in mouse embryos *in vivo*, and may have been caused by an unidentified ETU metabolite. Nevertheless, the study is very useful in demonstrating how the determination of the mechanisms of species differences in response to a teratogen can be approached using the whole embryo culture system.

TABLE 1

COMPARISON OF OBSERVED SPECIES DIFFERENCES IN RESPONSE TO VARIOUS TERATOGENIC COMPOUNDS

Teratogenic agent	Strain rat/mouse	No. of somites rat/mouse	Sensitivity *in vivo*	Sensitivity *in vitro*	References
methanol	SD/CD-1	0/3-5	mouse>rat	mouse>rat	Andrews *et al.*, 1993; Abbott *et al.*, 1995
formate	SD/CD-1	0/3-5	?	mouse=rat	Andrews *et al.*, 1995
dexamethasone	CD/CD-1	0/2-5	rat>mouse	mouse>rat	Hansen and Grafton, 1994
lithium	CD/CD-1	10±2/10±2	rat>mouse (variable)	rat>mouse (lethality) mouse>rat (neural tube defects)	Hansen *et al.*, 1990
ethylene-thiourea	CD/CD-1	?/? (day 10.5/ day 8.5)	rat>>mouse	rat>mouse	Daston *et al.*, 1989

Evaluation and future directions

Surprisingly, all of the studies comparing mouse and rat embryo responses used CD-1 mouse embryos and CD or other Sprague-Dawley rat embryos (Table 1). Because of the known heterogeneity among strains within a species, one must speculate that some of the observed responses are particular to these strains and thus the information that can be obtained from them regarding general differences between the two species is limited. The studies described here are most useful for pointing out differences in the response to teratogens of animals that are typically used in teratogen identification and testing. In order to learn more about the genetics of the variation in response, it would be of great interest to extend the comparisons to other strains within each species.

This review has also suggested the need for close control of developmental stage during treatment. Is it absolutely necessary that embryos of different genotypes be treated at the same developmental stage in order for the comparison to be valid? Numerous studies have demonstrated that the response of an embryo to a particular teratogen will vary depending on its age at the time of treatment. In general younger embryos show increased sensitivity to the teratogenic treatment *in vitro*. For example, early head fold (0 somite) ICR mouse embryos are more sensitive than neurulation stage embryos (4 to 6 somites) to both hyperglycemia (Hunter and Sadler, 1992) and hypothermia (Smoak and Sadler, 1991). A decline in sensitivity to chlorobutanol with increasing age was demonstrated in a comparison of neurulating (3 to 6 somite) and early limb bud stage (20 to 25 somite) CD-1 mouse embryos

(Smoak, 1993). Even embryos of very close developmental stages show variability in response to hyperglycemia, as there is a decline is sensitivity of 4 to 5 somite mouse embryos compared to those with 2 to 3, or 0 to 1 somites (Sadler, 1980). These studies would indicate that making comparisons among embryos that are of very close developmental age is critical, unless it can be established that developmental age in a particular case does not influence the embryo's response. One of the most straightforward ways of accomplishing this is through the use of whole embryo culture.

What does the future hold in store for the study of genetic susceptibility to teratogens? One of the most exciting possibilities incorporates the use of antisense oligonucleotides in cultured embryos to study the effects of a particular gene of interest and how elimination of the gene product might alter the embryo's response to a teratogen. A short nucleotide sequence that is complementary to the corresponding RNA sequence is micro-injected into the amniotic cavity, and prevents translation of the protein product. Through this technique, it is possible to downregulate the expression of a specific gene product at a precise stage of development. This technique has been used to explore the effects of altering the expression of several genes in whole embryo culture, including *Wnt*-1 and *Wnt*-3a (Augustine *et al.*, 1993), *engrailed*-1 (Augustine *et al.*, 1994) and E-cadherin (Chen and Hales, 1995). To our knowledge it has not yet been used to examine susceptibility to teratogens, but it has the potential to be extremely useful in this respect. Possibilities include downregulating the expression of particular metabolizing enzymes or of proteins known to be of developmental importance and examining changes in sensitivity to teratogens. The whole embryo culture system should prove ideal for such studies.

Summary and conclusions

The importance of the embryo's genotype to its teratogenic response is well recognized. The advantages of whole embryo culture have been used to a limited extent to sort out the interactions of genes and environmental agents by eliminating maternal effects and allowing comparison of differing genotypes under identical conditions of embryonic stage and culture.

1. Several single gene mouse mutants have been shown to have increased sensitivity to particular teratogens. The curly-tail *(ct/ct)* strain, in an experiment that was not possible *in vivo*, was found to be more sensitive than non-*ct* bearing strains to anterior neural tube defects when grown in inositol deficient serum. The *hemimelia-extra toe* (*Hm*^{x}) mutation was shown in embryo culture to have increased DNA synthesis only in the area and at the time of aberrant outgrowth.

Heterozygous splotch embryos (*Sp*/+) that were distinguished from +/+ embryos by a chromosome marker were shown to be more sensitive to neural tube defects caused by retinoic acid. The posterior neuropore closure delays after retinoic acid in *Sp*/+ cultured embryos were also greater in a cross of *Sp*/+ X SWV compared to that of +/+ x SWV, and this agreed with the increased spina bifida found in a previous *in vivo* study. The fact that untreated *Sp*/+ embryos had larger posterior neuropores than +/+ suggested a possible basis for the gene and teratogen interaction.

2. A number of strain differences in response to teratogens have been studied with whole embryo culture to untangle the relative contributions of maternal and embryonic factors. Sodium valproate induced more exencephaly in cultured SWV embryos compared to those of C57BL/6J with identical developmental stage and treatment. This suggested that the differing response was due to genetic variability intrinsic to the embryos. Exencephaly induced by 5-azacytidine was much higher in the MT strain compared to the ICR strain in culture as *in vivo*. It appeared that the MT strain is genetically predisposed to exencephaly in general, although its basis is still to be elucidated. The interaction between hyperthermia and cadmium was studied by inducing tolerance to cadmium with prior heat exposure in the BALB/c and SWV strains. The BALB/c embryos were more sensitive to cadmium-induced embryotoxicity. The embryos received thermotolerance-inducing heat exposure (non-teratogenic) and embryotoxic doses of cadmium. It was found that BALB/c embryos did not develop cross-tolerance to cadmium, while SWV embryos did. Induction of the 70 kDa heat shock protein in the two strains was shown to be comparable, so that there must be another cause for the cross-tolerance of SWV.

Strain differences in rats have seldom been compared. However, in one experiment the U substrain that was made streptozotocin-diabetic produced more malformed embryos than the H substrain. When embryos in culture were treated with D-glucose and B-hydroxybutyrate (which are associated with diabetic pregnancies) the responses of the two types of embryo were similar, which suggested that the genetic differences are not associated with elevation of either of these chemicals.

3. Comparative analyses of the response of rat and mouse embryos to chemicals are complicated by maternal and stage differences, which can be partly circumvented by embryo culture. The teratogenic effects of methanol on rat and mouse embryos in culture showed mouse embryos to be more sensitive and this appeared not to be related to metabolism. However, some experimental differences were involved along with the genetic differences. A further study used formate, a toxic metabolite of methanol. In this more controlled experiment, the mouse and rat embryos responded similarly to formate or formic acid. Dexamethasone developmental toxicity was found to be lower for rat than for mouse embryos in culture, but the uptake of dexamethasone was shown to be higher for mouse embryos. Very specific, stage-dependent responses to lithium were observed in the two species in culture. Embryolethality was high for rats and low for mice at comparable concentrations, while mouse embryos had open neural tubes and rats had virtually none. Mouse embryos required twice the concentration of ethylenethiourea to cause malformations as did rat embryos, but this did not account for the *in vivo* disparity. It was concluded that both maternal and embryonic factors contribute to the species differences.

Collectively, these studies demonstrate that there are clear advantages to using whole rodent embryo culture to study genetic differences in response to teratogenic treatments. The responses are obviously not straight forward, and often do not result in a clear-cut understanding of the underlying mechanisms. Yet it is only with careful dissection of the multitude of factors involved that we will hope to understand the complexity that in reality is at the base of many human conditions.

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