

Teratological Research Using *In Vitro* Systems. III. Embryonic Organs in Culture

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A search of literature published through the spring of 1986 yielded approximately 95 citations for the following embryonic organs in culture: kidney, pancreas, skin, palate, craniofacial tissue, tooth, lens, bones, digits, and liver. However, only the *in vitro* organ culture of the palate and tooth are reviewed in this paper. The other organ culture systems were not reported as teratogenic screens. Although some organs may have the potential for such use, many are currently used for evaluation of the pathogenesis associated with congenital abnormalities, cancer, or transplacental carcinogenesis, or in studies of the structure and function of differentiating organs, and will not be included in this review.

Palatal Shelves Cultured *In Vitro* as a Method of Evaluating Potential Teratogens

Introduction and Methodology

The palatal shelves of rats and mice have been isolated and cultured to evaluate abnormal palatal fusion in response to exposure to potential teratogens. The general methodology is briefly described. Fetal rats of 15 to 16 days of gestation (1-3) and fetal mice at 13 to 14 days of gestation (4-6) are delivered via aseptic cesarean section. The day of finding a vaginal copulatory plug is considered day 0 of gestation. To isolate the organ for culture, the head is severed from the trunk and the mandible and tongue are excised; an incision is made below the level of the orbits parallel to the roof of the mouth. Palatal shelves are placed in close contact as a homotypic pair with the oral side up and the nasal surface down. The organs are placed on a Millipore filter supported by a glass ring (4) or a stainless-steel support (2) and are cultured for 18 to 72 hr in a humidified incubator at 37°C with 5% carbon dioxide using Eagle's Minimal Essential Medium plus 10% fetal bovine serum, a penicillin-streptomycin mixture, and the test agent (1,2). When the organ is removed from culture, it is fixed in Bouin's or Karnovsky's fixative, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The stained sections are evaluated histologically for the adhesion of palatal shelves along the midline with subsequent dissolution of the midline epithelia, which results in the merger of the palatal mesenchymes (4).

Single palatal shelves (4) may also be cultured as an alternative to the culture of homotypic pairs. The pro-

grammed cell death at the medial edge epithelium appears to occur in the culture of both single and paired shelves. However, the use of single shelves permits an internal control: the right shelf may be used for test and the left shelf for control. The dissolution of the epithelia is the normal situation; if the epithelia persist in the presence of the test agent, the test is considered positive for teratogenesis.

More recently, the palatal shelves of mouse (day 13), chick (day 7), and alligator (day 23) were successfully cultured in a chemically defined medium with or without 10% fetal bovine serum (7). Differentiation occurs 1 day earlier with chemically defined, serum-free medium. In comparison to what is observed *in vivo*, general differentiation was similar *in vitro* but was delayed both with and without serum. Palatal differentiation in each species occurs by different mechanisms or processes. Death of the medial edge epithelial (MEE) cells was greater in mice than in alligators (in which MEE cells develop a characteristic cobblestone appearance with numerous microvilli and migrate out of the zone of fusion in a posteronasal direction) and did not appear to be characteristic of chick MEE cells (MEE cells differentiate into keratinised stratified squamous epithelia which contact the opposing shelf but do not adhere, fuse, migrate, or die).

Critical Review

The *in vitro* organ culture of palatal shelves offers several advantages. This technique provides the teratologist with a one-compartment pharmacological model by which physiological doses of test compounds may be examined. Although most organ culture assays have not been designed to provide information on maternal contributions to fetal maldevelopment or toxicity, this system has been modified for this purpose. The amniotic

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fluid of treated or untreated dams may be added to the system to permit investigation of maternal factors contributing to the observed anomaly (2). In contrast, chemically defined media eliminate the variability which is frequently observed with different batches of serum and extract used to supplement media (7), and decreased variability favors reproducible results.

The biosynthesis of macromolecular components by the palatal shelves may be monitored *in vitro* in the presence of a potential teratogen. Alterations in normal cellular biochemical events that lead to adhesion between shelves may be studied (1,3) in a mechanistic approach to explain observed response. Because differentiation of MEE cells occurs by different mechanisms in different species, the option to evaluate various mechanisms of teratogenesis exists.

Because the technique of culturing single palatal shelves involves an internal control, this technique is cost-effective. The technique does not require exhaustive manpower. One person with reasonable manual dexterity performs the microdissections, and another performs and interprets the histological preparations. Thus, under ideal conditions, an investigation could be performed and evaluated in about a week.

Culturing single palatal shelves, however, has a number of disadvantages: (a) The size of the organ culture rudiment should be kept consistent, a result that generally is difficult to accomplish; (b) failure of the nutrient medium to permeate the core of the tissue block may lead to necrosis, and thus a method to determine tissue viability is essential for valid interpretation of the results (histology); (c) methodology for this technique is far from standardized; (d) fusion of palatal shelves is influenced by culture conditions, another variable that makes validation of the assay difficult (8); (e) the age at which palatal shelves become horizontal and thereby eligible for fusion may vary (9); and (f) information on the background frequency of spontaneous cleft palate is needed for each strain of animal being used.

Attempts have been made to devise a grading system for the response of cultured homotypic pairs of palatal shelves, e.g., processes do/do not meet; fusion takes place with complete/incomplete mesenchymal penetration (10). However, the method that uses the single palatal shelf does not permit the grading of the response. The epithelium either does or does not undergo dissolution.

This assay, like most other *in vitro* work, must be performed under aseptic conditions. Standard laboratory equipment and supplies include a laminar flow cabinet, humidified incubators, microdissection instruments, microscopes, organ culture dishes, and media.

A metabolic activating system has not been developed for use with this assay. If such a system is not toxic to the developing organ it would extend the use of the assay. Several reports describe the evaluation of chemicals such as vitamin A (2,3), glucocorticoids (4,5,9), and diazo-oxo-norleucine (DON) (1). More studies need to be done with other chemicals and by other investigators using a standardized technique to make a better as-

essment of the use of this assay as a teratogenic screening system.

Embryonic Tooth Primordia as an *In Vitro* Teratology Screen

Introduction and Methodology

Most *in vitro* teratology studies that evaluate the effects of chemicals on tooth development use embryonic rudiments of the first mandibular molars, and in some instances, the bud of the second molar of mouse embryos. In the publications reviewed, the organs of embryos at 14 to 18 days of gestation are used (detection of a vaginal copulatory plug the morning after mating was consistently considered to indicate day 0 of pregnancy) (11-14).

The primordia of the embryonic molars of the mandible (lower jaw) are isolated by removing the jaw and dissecting the primordia from adjoining tissue before culture. Generally, two methods may be used to culture tooth primordia: (a) The embryonic tooth rudiments may be supported on a screen and grown at an interface between a gaseous atmosphere and a liquid culture medium in BGJb medium supplemented with 10% chick embryo extract, 20% horse serum, and 0.9 mM ascorbic acid (11); or (b) the method of Trowell (15). Alternatively, the tooth rudiments may be grown on solid nutrient medium (11). The test agent is usually incorporated into the culture medium. The cultures are maintained at 37°C in a humidified incubator with 5% CO₂ for 5 to 14 days. The culture medium should be changed every 2 days. The organ explants are then fixed, embedded, sectioned, and stained with Mallory's phosphotungstic acid-hematoxylin. This stains the enamel matrix blue-black and the dentine matrix red. The samples are evaluated under a light microscope for interference in the process of differentiation, retarded growth, or cell death.

Critical Review

A number of end points may be evaluated: retarded growth, abnormally shaped teeth, failure of the second molar tooth germs to appear, dead cells or "ghosts" of tooth germs, or a selective action on either the epithelial or mesenchymal cells. The histological evaluation of the differentiation of cultivated tooth primordia allows direct observation and evaluation of interaction between the epithelial enamel organ, basement membrane, and mesenchymal dental papilla in both normal and anomalous development. Aside from an effect on both differentiating tissues, the observation of any change in the basement membrane or tissue structure may provide clues to interference with transmission of inductive signals mediated by cell contact.

The secretion of the predentine matrix by odontoblasts and enamel by ameloblasts is used as a criterion for differentiation (12), as well as an indicator of biochemical activity (16). Alterations in the distribution of

connective tissue components (i.e., collagen, fibronectin, proteoglycan, and laminin) may be evaluated, as described by Thesleff et al. (14), by use of purified antibodies or antisera specific for these components and detection by indirect immunofluorescence.

Each of the tooth primordia culture methods offers its own advantages. Rudiments cultured in agar, as a solid nutrient medium, do not spread out. Therefore, this method provides the investigator with a better three-dimensional morphological picture (analysis) of the tissue. However, the hard tissue deposition appears to be better in the liquid medium than on agar (E. J. Kollar, personal communication, 1984).

Very few chemicals (the ferrous ion chelator α, α' -dipyridyl, the antibiotic hadacidin, DON, β -aminopropionitrile, and a few amino acid analogs) have been tested, according to the reviewed papers. It appears that the maximal effect of a test agent on this system may be determined after 4 days in culture (E. J. Kollar, personnel communication, 1984). In general, cells grown *in vitro* may be more sensitive than organs to solvents used to solubilize insoluble chemicals in the test medium. Nonetheless, it would be good practice to use a solvent control when appropriate to differentiate solvent from compound-specific effects. Although not yet attempted, the introduction of gaseous agents to the system via the gas-liquid interface may be possible.

This organ culture system may provide a dose-response relationship to test compounds and may be measured by the spectrum of effects discussed earlier. Cultures in which mitosis and/or development has been arrested by the test agent may recover if the organ is placed in control medium (11). At least 10 cultures should be read per measured parameter. The length of time required to process the samples of tissue is the determining factor for the time required to perform this assay. As with the other organ culture methods, aseptic technique is essential for successful cultivation. The standard organ culture and histological equipment must also be considered in the cost analysis.

A metabolic activating system to evaluate potential teratogens via odontogenesis *in vitro* has not been developed. This deficiency could be remedied if such a system is not toxic to the developing organ. Inasmuch as so few chemicals have been tested by either of the two methods, the evaluation of more chemicals by a

standardized method is needed for validation of this assay.

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