Morphogenetic Properties of Human Embryonic Cells: Aggregation of Dissociated Cells and Histogenesis in Cultured Aggregates

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Extract

To test the hypothesis that the morphogenetic behavior of human embryonic cells can be characterized *in vitro*, human and mouse embryonic cells were compared by several variables which are thought to reflect morphogenetic cellular behavior. Trypsin-prepared cell suspensions of human (8-12 weeks) and mouse (13-18 days) embryonic organs were analyzed for (1) initial rate of aggregation, (2) aggregate size, (3) histogenesis in aggregates derived from a single organ, and (4) segregation of cells according to tissue type within mixed aggregates derived from different organs.

The initial rate of aggregation followed strict first order kinetics for 40 min at 37°. The rate was identical ($P > 0.05$) for different cell types, for different ages of the same tissue, in the presence or absence of serum, and for mouse or human tissues. Aggregate size varied widely, regardless of the species, tissue type, or embryonic age. Thus, under the conditions employed, these variables are not worthwhile indices of morphogenetic cell behavior.

On the other hand, histogenesis in human or mouse aggregates derived from a single organ was tissue specific. Mixed aggregates of human and mouse cells of the same organ behaved identically as aggregates derived from a single species, which indicates that species specificity is expressed less than tissue specificity. Regardless of species, cells from different organs within a single aggregate segregated from one another according to tissue type, which demonstrates that human embryonic cells show tissue-specific morphogenetic behavior.

These findings confirm, for human embryonic cells, observations made with other species, that morphogenetic cell properties can be analyzed *in vitro*.

Speculation

Demonstration that human embryonic cells maintain morphogenetic properties capable of analysis in a defined system *in vitro* opens the way for the direct study of human morphogenetic mechanisms and of possible genetic and environmental influences. Readily available normal or abnormal mouse embryonic cells could be used in combination with human embryonic cells to experimentally examine the pathogenesis of human developmental defects.

Introduction

Embryonic tissues can be dissociated into suspensions of single cells which can be made to reaggregate under appropriate conditions *in vitro*, reconstructing the dissociated tissue. The histologic architecture of the reconstructed tissue often reproduces with high fidelity the *in situ* organization of the cells, their specializations, and extracellular products [6, 18, 19]. Although this process of *in vitro* tissue reconstruction does not duplicate *in vivo* organogenesis, reaggregation of dissociated cells has been used in studies of morphogenesis since Wilson [22] observed that a complete sponge could be reconstructed from a single cell suspension. Recently, it has been shown that cells from tissues expressing genetically determined developmental defects reassemble in a manner which reflects the morphologic abnormality [4, 5]. Thus, *in vitro* tissue reconstruction may allow normal and abnormal morphogenetic cellular behavior to be assessed in an indifferent environment under controlled, replicable conditions.

In vitro tissue reconstruction, as developed by Moscona [11], involves trypsin dissociation of the embryonic tissue, followed by incubation of the cell suspension in a swirling flask during which the cells collide and adhere, forming cell aggregates. During culture of the aggregates, the cells rearrange, yielding tissue-specific histologic patterns in isotypic aggregates (cells derived from a single tissue), or segregation of cell types called "sorting-out" in aggregates of mixed tissues (heterotypic aggregates). Cells of the same tissue from different species do not segregate from one another [10, 11], which demonstrates that species specificity is expressed less than tissue specificity.

The morphogenetic properties involved in cell aggregation and tissue reconstruction include adhesion, specific cell recognition, mobility, growth, change in cell shape, production of extracellular materials, and cell death [1]. Several methods have been developed for measurement of various components of the overall process [13], but it is unclear whether these are accurate assessments of morphogenetic cellular properties. To test the hypothesis that the morphogenetic behavior of human embryonic cells can be characterized *in vitro*, human and mouse embryonic cells were compared through several variables previously suggested to reflect morphogenetic cellular behavior. The variables examined were (1) the initial rate of aggregation, (2) aggregate size, (3) histogenesis in isotypic aggregates, and (4) sorting-out in heterotypic aggregates. The experimen-

tal results show that initial rate of aggregation and aggregate size are either insensitive or nonspecific indices of morphogenetic cell behavior, that histogenetic and sorting-out behavior of human embryonic cells are indistinguishable from that of mouse embryonic cells and that this behavior is identical in mixed combinations of homologous human and mouse cells. These findings demonstrate that the morphogenetic properties of human embryonic cells can be analyzed *in vitro* and that readily available normal or abnormal mouse cells can be used in combination with human cells to experimentally examine the pathogenesis of human developmental abnormalities.

Materials and Methods

Source of Embryonic Tissue

Human embryonic tissues were obtained from therapeutic abortions performed by vacuum aspiration between the 7th and the 14th week of pregnancy. Embryo age was estimated from the development of the extremities and the face and was correlated with the date of the last menstrual period and the size of the uterus at the time of the procedure. By these assessments, over 90% of the organs studied were from embryos between 8 and 10 weeks of age. Mouse embryonic tissues were obtained by dissection of DBA/2 $\times$ C57BL/6 hybrid embryos [23]. Embryo age was determined by the day of discovery of the vaginal seminal plug (day 0) and confirmed in embryos 14 days and younger by examination of external morphologic features and salivary gland development.

Media and Solutions

Hanks' balanced salt solution [24] was supplemented with 100 U penicillin and 50 μ g streptomycin/ml. Basal medium eagle (BME) with Hanks' salts [24] was supplemented with 2 mM L-glutamine, 100 U penicillin, and 50 μ g streptomycin/ml, and modified by replacement of NaHCO_3 with 15 mM N tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) and 15 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) buffered at pH 7.5 [25].

For aggregation kinetics and for *in vitro* culture, the BME medium was supplemented with 10 μ g/ml deoxyribonuclease I [26] and 0.4 g/100 ml glucose. Calcium-magnesium-free Hanks' solution (CMF-Hanks') was buffered with TES and HEPES as in BME. Cells were trypsinized with a solution which contained 0.5% two times crystallized trypsin [27] in CMF-Hanks'.

Preparation of Cell Suspensions

Immediately upon removal from the human uterus, the aspirate was washed with sterile normal saline. In no instance was the embryo intact, but in most instances unmacerated embryonic organs were readily identified. These were placed in chilled Hanks' solution containing 50% fetal calf serum (FCS) and kept on ice until processing later the same day. Mouse embryos obtained by direct removal from the uterus were processed identically. Embryonic organs were transferred to BME supplemented with 50% FCS and dissected free of extraneous tissue. The tissues were rinsed in CMF-Hanks', minced into small pieces, transferred to 12 × 35 mm plastic tubes, and incubated in CMF-Hanks' for 10 min at 37°. The CMF-Hanks' was removed, 1 ml trypsin solution added, and the incubation continued for another 10 min. The trypsin solution was carefully removed and the fragments rinsed three times with 2 ml CMF-Hanks' before adding 1 ml BME (with or without 10% FCS) containing 10 µg/ml DNase. The fragments were disrupted into a cell suspension by frequent pipetting through small bore Pasteur pipettes. Further procedures were done at 4°. The cells were centrifuged (400 g for 5 min), resuspended in 2 ml cold BME, filtered through a 20-µ pore size nylon screen [28], and stored at 4° before aggregation.

The cell suspension thus obtained consisted essentially of single cells. Cell viability, measured by trypan blue exclusion, was greater than 95% for mouse tissues and varied between 60% and 90% for human tissues.

Aggregation Kinetics

Quantitative aggregation studies were performed in plastic dishes (35 × 10 mm) layered previously with 2 ml 1.5% noble agar. The agar prevents the adherence of cells to the substratum, a significant source of error, especially in serum-free medium. The cell suspension was diluted to 1×10^5 cells/ml in serum-free BME, and 3 ml were added to the dishes which were placed on a gyrotory shaker with a 3/4-inch orbital motion [29] in a dry incubator at 36° and rotated at 74 rpm. At 5- or 10-min intervals, the contents of a dish were diluted with 17 ml Isoton solution [30] and the single cells and very small clumps counted with a Coulter counter model A[30] equipped with a 100-µm aperture and a lower threshold set at 6 µm. The mean of two counts was taken for each dish, and duplicate dishes were counted for each time point.

The best straight line, which related logarithm of the percentage of single cells to time of aggregation,

was obtained by computer analysis. The linearity of the regression line was tested by F test at $P = 0.05$, and the slope and SE were compared by Student's t test at $P = 0.05$.

Histology

For histologic studies, 3 ml cell suspension diluted to $1-1.5 \times 10^6$ cells/ml with BME containing 10% FCS was added to siliconized glass dishes (30 × 12 mm) [31], which were rotated and incubated as described for the kinetic studies. Fresh medium (2 ml) was exchanged at 48-hr intervals, and the aggregates were rotated for up to 2 weeks before fixing in Carnoy's solution. When cells from two different tissues were aggregated together, equal numbers of cells were mixed up to a total of $1-1.5 \times 10^6$ cells/ml and the aggregates rotated for 4 days.

Results

Initial rate of aggregation, aggregate size, histogenesis in isotypic aggregates, and sorting-out in mixed aggregates were assessed in different tissues of 60 human embryos. All studies, except for the sorting-out experiments which required a large number of cells, were conducted on organs from single embryos.

Initial Rate of Aggregation

The number of single cells in embryonic cell suspensions decreases with time of rotation incubation. This reduction in single cells can occur by disruption, adhesion to the substratum, or incorporation into an aggregate. The decrease in single cell number was due exclusively to aggregate formation inasmuch as total cell number (free cells plus cells mechanically dissociated from formed aggregates) did not decrease during the 40-min period of aggregation and since by microscopy, no cells were adherent to the substratum at the completion of the aggregation period.

Single cell suspensions prepared from human embryonic organs (brain, spinal cord, lung, kidney; 8-10-week gestation) and from mouse embryos were assessed for their initial rates of aggregation. Best-fit regression lines for the number of single cells as a function of incubation time were computed for each cell suspension. Plots of log percentage of single cells *versus* time for the first 40 min of aggregation were linear (F test, $P > 0.05$ for each point), which indicates that the aggregation rate followed strict first order kinetics for 40 min. Comparison of the mean slopes in the absence of serum for 10 human brains, 5 human spinal cords, and brains

from 5 litters of 13-day-old mice by Student's *t* test showed no significant differences ($P > 0.05$) in aggregation rate between the same organs from different individuals or litters, which allowed results to be pooled for further comparisons. These comparisons revealed that the presence or absence of serum does not affect the initial rate of aggregation and that human and mouse cells of similar type aggregate at identical rates (Table I, Fig. 1). Results too limited for accurate statistical analysis on human lung and kidney suggest no difference in aggregation rate between organs.

Aggregate Size

Densely packed aggregates were observed within 2–3 hr of rotation incubation in serum-containing medium. After 18–24 hr, duplicate dishes were examined for aggregate number and for size with a calibrated reticule. The number of aggregates per dish varied between 1 and 10 for brain cells and between 10 and 20 for the other cells; no difference was noted between mouse and human tissues. Aggregate size varied widely; in the same dish or in duplicate dishes aggregate diameter ranged between 0.2 and 1 mm, regardless of the tissue type or embryonic age, with the exception that brain cells formed larger aggregates (0.5–1.0 mm). Aggregates formed in agar-layered dishes tended to be smaller and more uniform in size than those formed in glass dishes.

Table I. Rate of aggregation for different embryonic tissues

Embryonic tissue ¹	Aggregation rate ² mean slope $\pm$ SE $\times 10^3$ /min
Serum free	
Human brain (10)	4.0 ± 0.4^3
Human spinal cord (5)	5.2 ± 0.7
13-Day mouse brain (5)	6.5 ± 1.1
17-Day mouse brain (2)	5.5 ± 0.7
13-Day mouse limb (2)	3.3 ± 0.3
Plus serum	
Human brain (10)	4.1 ± 0.3^3
14-Day mouse brain (2)	5.4 ± 0.9
18-Day mouse brain (2)	4.3 ± 0.2

¹ The number of human embryos or mouse litters examined for each organ is indicated in parentheses. Each organ was run in duplicate.

² Aggregation rate is expressed as the mean slope, which is defined as the logarithm of the percentage of free cells removed from the suspension per minute. These first order kinetics were maintained for 40 min. The best-fit regression lines for each organ were linear ($P > 0.05$).

³ Comparison of the mean slopes for each organ and age to that for any other organ and age, and for serum free to plus serum by Student's *t* test showed no differences ($P > 0.05$).

Fig. 1. Aggregation rate of human embryonic brain cells. The disappearance of single cells with time is expressed as the $\log_{10}$ percentage of free cells remaining in suspension at 10-min intervals over the first 40 min of rotation-incubation. The best-fit regression line for 10 human brains in serum-free medium ($\circ$) and for 10 human brains in serum-containing medium ($\blacktriangle$) is represented with its 95% confidence limits (---). Similar lines computed for other human or mouse embryonic tissues fall within these 95% confidence limits and have a similar slope ($P > 0.05$).

Histogenesis

Human brain (30 embryos). To compare histologic pattern formation in aggregates with the *in situ* pattern, pieces (about 1 cu mm) of human embryonic (8 weeks) telencephalon taken perpendicular to the neural canal were incubated as aggregates for 14 days. Histologic organization in these fragments was very similar to that seen in 14-day aggregates derived from dissociated whole brains of similar embryonic age (Figs. 2, 3, 4, and 5). In the lower power views (Figs. 2 and 4) groups of cell bodies are seen predominantly at the periphery. These groupings are separated by an extensive fibrillar network. At higher magnification, occasional mitoses can be observed and two cell types can be distinguished: one with high nuclear-cytoplasmic ratio and one with a low ratio. The fibrillar network and cellular organization in aggregates is not as extensive as in the control fragment. The histologic pattern in aggregates derived from 13-day mouse brain cultured for 14 days was virtually identical with that of the human brain aggregates.

Human spinal cord (15 embryos). Histologic organi-

Fig. 2. Section through an intact fragment of an 8-week-old human embryonic telencephalon cultured for 14 days on the gyrotory shaker ($\times 64$). Note the symmetry in this fragment.

Fig. 3. High magnification ($\times 252$) of fragment in Figure 2, which shows ependymal and neuronal cells surrounded by an extensive fibrillar network.

Fig. 4. Representative section of an aggregate derived from an 8-week-old human brain cultured for 14 days ($\times 128$). Cell bodies concentrated at the periphery are separated from those in the center by a fibrillar network.

Fig. 5. Representative section at high power magnification of an aggregate derived from a 9-week-old human brain cultured for 14 days ($\times 252$). Cells with different nuclear-cytoplasmic ratios of neuronal, glial, and ependymal types are present. The cellular morphology and fibrillar network strongly resemble those in Figure 3, although there is no apparent orientation of the cells.

zation in spinal cord aggregates closely resembled the organization in brain cell aggregates. Cellular organization in both brain and spinal cord aggregates is clearly distinct from the organization of cells in aggregates from any other tissue.

Human lung (5 embryos). Aggregates of lung cells cultured for 4 days–1 week formed typical glandular structures which duplicated those seen in analogous mouse lung aggregates (Fig. 6). Loose mesenchymal cells surround groups of columnar epithelial cells,

Table II. Histologic organization in aggregates derived from mixtures of paired cell suspensions¹

Fig. 8. Representative section through a 4-day-old aggregate obtained by mixing equal numbers of human 8-week embryonic brain and 9-week limb cells ($\times 252$). The organization resembles that found in limb aggregates; multinucleated cells are clearly identifiable in clusters. Neural epithelial cells are scattered throughout the aggregate.

Fig. 9. Representative section through a 4-day-old aggregate obtained by mixing an equal number of human 9-week embryonic brain and mouse 13-day lung cells ($\times 252$). The histotypic organization of this aggregate is typical for lung tissue. Typical brain aggregates were found in the same dish.

Fig. 10. Representative section through a 4-day-old aggregate obtained by mixing equal numbers of mouse 13-day embryonic brain and liver cells ($\times 400$). Large liver cells form clusters which are surrounded by neural epithelial cells.

two species did not segregate from one another: *e.g.*, human brain and mouse brain; human lung and mouse lung. (2) Combinations of neural epithelial cells (brain with spinal cord) did not result in patterns that could be distinguished from brain or spinal cord aggregates. (3) Mixing of brain cells with lung cells resulted in extreme sorting-out in that only typical lung and brain aggregates were found in the same dish (Fig. 9). Similar results were obtained for other heterotypic mixtures of human mouse cells. (4) Aggregates of human or mouse liver cells with human or mouse brain cells showed clear evidence of sorting-out within an aggregate. The liver cells grouped in the center of the aggregates, and formed multiple small foci of liver tissue, which were easily distinguished from the neural epithelial cells which surrounded them (Fig. 10).

Discussion

The present study establishes that cells dissociated from 8- to 12-week-old human embryonic tissues reaggregate, undergo histogenesis in isotypic aggregates, and sort out in mixed aggregates in a fashion identical with mouse embryonic cells. Further, combinations of human and mouse cells of the same tissue type form "mosaic" aggregates which undergo histogenesis characteristic of the original tissue. Although this confirmation, for human embryonic cells, of prior observations made with embryonic cells of other species is not surprising, it provides a basis for the direct study of human mor-

phogenetic cell behavior and of possible genetic and environmental influences on this behavior. Mouse cells of defined morphogenetic phenotype are readily available. Because human cells apparently share similar morphogenetic mechanisms, combinations of these cells with human cells may serve as a test system for detecting abnormalities in human morphogenetic cell behavior.

Measurements of the initial rate of cell aggregation and aggregate size reflect the degree of damage and recovery of the cells from tissue dissociation, the probability of adhesion after collision and the formation and strength of intercellular bonds, as well as experimental variables not intrinsic to the cells. In the present study, extrinsic factors were minimized by use of organic buffers to provide rigorous pH control, use of chemically defined media, inclusion of DNase to prevent interference by viscous glue-like DNA, and the use of agar-coated dishes to eliminate cell adhesion to the substratum. Under these conditions, aggregation reproducibly followed first order kinetics for 40 min at 37° and rates were identical in the presence or absence of serum, which confirms the results of Orr and Roseman [14] on chick neural retina cells. However, initial aggregation rate and aggregate size were not worthwhile parameters for distinguishing morphogenetic cell properties. Identical aggregation rates were found for different cell types and for different ages of the same tissue, and, aggregate size (at 18–24 hr) varied widely even in the same or duplicate dishes. These results conflict with

reports of tissue- and age-dependent differences in rate of aggregation (over several hours [5, 10]), and aggregate size [6]. Possible explanations for the conflicting results include our use of whole organs consisting of various cell types, differing embryonic ages, and different aggregation conditions (time after trypsinization, buffer, agar coating, configuration, of vessel). Cell viability and variation in cell type are unlikely explanations since mouse cell suspensions had uniformly high degrees of viability and similar variation in cell type as in the prior studies.

Adhesion involves trypsin-susceptible surface components, which makes trypsin treatment and repair after trypsinization the most likely explanation. Our studies of initial rates were done before repair of trypsin-susceptible components. Proteolytic enzymes cannot be avoided, because chelators, such as EDTA (ethylene dinitrilotetraacetate, or Versene) will not completely dissociate these embryonic tissues [13]. The initial rate of cell aggregation and aggregate size may be useful indices of adhesivity, but under the conditions employed, these parameters are either too nonspecific or too insensitive to characterize cell properties responsible for determining final tissue architecture and organ morphology.

Although histogenesis and sorting-out in aggregates do not duplicate the process by which organs are formed *in situ*, the same morphogenetic mechanisms are involved [1, 10, 19, 20]. All of these mechanisms probably occur on or adjacent to the cell surface. Assays for components of the overall process have been reported [14–16], but the molecular events assessed in each method are unclear. On the other hand, several molecular phenomena have been proposed to account for cellular recognition, including tissue-specific aggregation enhancement factors [7–9, 12], surface glycosyl transferases and acceptors [17], genetically variable surface antigens [2, 3], and extracellular matrix materials [9, 12], but the role of these entities in morphogenesis is ill defined. Better defined methods for analyzing morphogenetic cell behavior and for relating diverse biochemical events to this behavior are needed. Development of these methods, together with the increasing availability of human embryonic tissues [21], should make possible the direct study of human morphogenetic cell interactions.

Summary

Initial rate of aggregation, aggregate size, the histotypic pattern and ability to sort out in mixed aggregates were assessed during rotation-mediated aggregation of cell

suspensions obtained after brief trypsinization of different organs of 60 human embryos. The aggregation rate followed strict first order kinetics for 40 min. Comparison of the mean slopes for 10 human brains, 5 human spinal cords, and brains from 5 litters of 13-day-old mouse embryos by Student's *t* test indicated that no significant differences in aggregation rate existed between the same organs from different individuals or litters, that the presence or absence of serum does not affect the initial rate of aggregation, and that human and mouse cells of similar tissue type aggregate at identical rates. Aggregate size varied widely, regardless of the species, tissue type, or embryonic age. Histogenesis in aggregates derived from a single organ was tissue specific regardless of species. Although human and mouse cells of the same tissue did not segregate in mixed aggregates, sorting-out occurred in aggregates which contained human or mouse cells derived from different organs.

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