Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression

(cell adhesion/morphogenesis/cell rearrangement/tissue segregation)

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ABSTRACT The sorting-out of embryonic cells from a cell mixture and the selective spreading of one cell population over the surface of another have been attributed to various causes. These include differentials in chemotaxis, in cellular adhesiveness, in cell surface contractility, in speed of cell movement, and in the timing of postulated changes in cellular adhesive and motile properties. One of us earlier predicted on mathematical grounds that two motile cell types differing only in the level of expression of a single cell adhesion system should not only segregate from one another but also arrange themselves with the lesser cohesive cells forming a core of the more cohesive ones. To test these predictions, we combined two populations of L cells transfected with P-cadherin cDNA and expressing this homophilic adhesion molecule in substantially differing amounts. When the two cell populations were intermixed, they segregated to approach a sphere-within-a-sphere configuration; the cell population expressing more P-cadherin forming islands which fused to become an internal “medulla.” When the two cell populations were first formed into separate aggregates which were subsequently allowed to fuse, the cell population expressing more P-cadherin was enveloped by its partner, which formed an external “cortex.” These observations confirm the early prediction and support the conclusion that both morphogenetic movements and the specific anatomical configurations to which they lead can be determined by particular sets of intercellular adhesive intensities, regardless of how these are generated and in the absence of differentials in other parameters.

Since the demonstration by Townes and Holtfreter (1) that the dissociated cells of vertebrate embryonic organs can reaggregate and sort out to reconstruct semblances of the original structure, the mechanisms governing cell sorting have been investigated as means of gaining insight into the mechanisms governing normal morphogenesis. Townes and Holtfreter concluded that the sorting-out of experimentally intermixed vertebrate embryonic cells into “distinct homogeneous layers, the stratification of which corresponds to the normal germ layer arrangement” is brought about “in consequence of directed movements,” and that only afterward “the tissue segregation becomes complete because of the emergence of a selectivity of cell adhesion.”

However, observations of cell population behavior associated with cell sorting and tissue spreading led one of us to the conclusion that directed cell movements played no role in these phenomena (2–6). Rather, such observations supported the conclusion that differences in the intensities of cell adhesions alone direct the sorting-out of intermixed embryonic cells, the spreading of one tissue over the surface of another, and the specific inside/outside tissue stratification that arises by either process, less cohesive cell populations enveloping more cohesive ones (2–12). According to the differential adhesion hypothesis, in any population of motile, cohesive cells, weaker cell attachments will tend to be displaced by stronger ones, and this adhesion-maximization process produces cell rearrangements such as the tissue spreading movements of embryonic development and wound healing and the segregation of unlike cells seen during cell sorting. If carried to completion, this process would generate a histological structure or configuration in which the total intensity of cell bonding is maximized. For any combination of cells, this “equilibrium configuration” would be determined by the particular set of adhesive intensities characterizing the various possible interfaces between and among the cells and extracellular substances constituting the system.

In cell sorting and tissue spreading experiments with paired chick embryonic tissues, one of us observed that one tissue commonly comes to surround its partner completely (8). Computations based upon model cells with uniform adhesiveness around their surfaces (4–6) showed that, for such cells, this “sphere within a sphere” configuration should result from adhesion maximization only when the following specific set of intercellular adhesive relationships exists: (i) the enveloping cell population must be the less cohesive of the two and (ii) its cells must adhere to those of the enveloped cell population with an intensity equal to or greater than that with which they adhere to others of their own kind. Although the differential adhesion hypothesis, as a purely physical explanation, deals only with the intensities of cell adhesions and is indifferent to the molecular mechanisms by which these adhesive intensities are generated, this case, in which the less cohesive cells may adhere to the more cohesive cells more strongly than to each other, raised the question of whether mere quantitative differences in the level of expression of a single adhesion system would cause two cell populations to be mutually immiscible, arranging themselves as a (less cohesive) cortex surrounding a (more cohesive) medulla. Another set of computations (5, 6) predicted that this should indeed be the case. The experiments reported here provide an experimental confirmation of that prediction.

MATERIALS AND METHODS

Cell Lines. We required two cell lines differing only in the number of homophilic adhesion molecules of a given kind expressed on their surfaces. We chose cell lines PLβ2 and PLα5, generated earlier (13) by transfection of L cells with full

[Adapted from the original text, maintaining the natural flow and structure of the article.]
length P-cadherin cDNA inserts downstream in one case from the efficient β-actin promoter (PLB2 cells) and in the other case from the less efficient simian virus 40 thymidine kinase promoter (PLS5 cells). The parent L cell line barely aggregates. Because cadherin-mediated adhesion is homophilic (13, 14), the abundance of “ligands” equals that of “receptors” on either cell type: high on PLB2 and low on PLS5. Procedures for generating the P-cadherin-transfected L cell lines used here have been described previously (13).

Cell Aggregation Rates. Cell aggregation rates were here compared through the use of the light-scattering cell aggregometer described earlier (15). Nearly confluent cultures of PLB2 and PLS5 cells were incubated in 0.05% trypsin 1:250 in the presence of 0.53 mM EDTA in calcium-free (CF) Hanks’ balanced salt solution and resuspended at 5 x 10⁶ cells per ml in either normal or CF Dulbecco’s modified minimal essential medium (DMEM) to which 2% calf serum was added. To eliminate any DNA gel which might perturb the course of aggregation, two drops of 2 mg/ml DNase solution were added to each cuvette. A 0.55-ml cell suspension was added to each cuvette, within which an air bubble functioned as a stirrer. Cuvettes were mounted on the aggregometer’s vertical rotor and rotated at 37°C. Cell aggregation was continuously monitored by the decrease in low-angle light scattering that accompanies the formation of aggregates from single cells. Low-angle scattered light impinged upon a photomultiplier tube and the resulting voltages were amplified and printed out on a multichannel strip chart recorder.

Fluorescent Labeling. The lipophilic fluorescent dye Di I (1,1-diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) was dissolved in ethanol as a 2.5 mg/ml stock solution. Before use, it was diluted in DMEM/2% calf serum to 20–40 µg/ml, sonicated, and filtered through a 0.45-µm pore size Millipore filter (16). Cells to be labeled were incubated in this solution for 1 h in the dark and washed four times in Hanks’ balanced salt solution and once in phosphate-buffered saline before trypsinization.

Culture Procedure. Labeled and unlabeled cell cultures were harvested in 0.01% trypsin + 1 mM Ca²⁺ and resuspended in DMEM/2% calf serum. They were allowed to recover from trypsinization for 0.5–2 h on a water bath shaker at 70 rpm. The cell suspensions were pipetted as necessary to dispense any small aggregates. For sorting-out experiments, a labeled PLB2 and PLS5 cell suspension were mixed in the desired ratio. The cell suspensions were then pelleted in round-bottomed culture tubes by brief centrifugation in a table-top centrifuge and incubated 3–22 h at 37°C to allow the thin cell pellets to become firm. The pellets were then recovered and cut into small fragments with microscissors. After rounding up in DMEM/2% calf serum on 1.25% agarose-coated Petri dishes, the fragments were about 0.15–0.3 mm in diameter. For aggregate fusion experiments, the two cell suspensions were allowed to aggregate separately. Labeled and unlabeled aggregates of the appropriate size were then allowed to fuse in hanging drops before being transferred to agarose-coated Petri dishes.

Observation and Photography. Aggregates and aggregate-fusions were observed both with a stereomicroscope and with a Zeiss compound microscope using 2.5 x and 6.3 x objectives and photographed with the latter objective with epifluorescence optics using a rhodamine filter set, with or without low-level tungsten backlighting through a daylight filter.

RESULTS

Aggregation of PLB2 and PLS5 Cells. Because cadherin-mediated adhesion is calcium dependent (17, 18), tests of the calcium dependency of the transfected cells’ enhanced aggregation ability serve to confirm its cadherin mediation. Both the Ca²⁺ dependence of the transfected cells’ adhesion and the greater expression of P-cadherin on PLB2 cells than on PLS5 cells were confirmed by tests of the aggregation rate of both cell populations in normal- and low-calcium medium. In stirred suspensions, PLB2 cells aggregated rapidly and PLS5 cells aggregated very slowly at normal calcium concentration, but neither cell line aggregated in low-Ca²⁺ medium (Figs. 1 and 2).

Sorting-Out of Intermixed PLB2 and PLS5 Cells. Preliminary tests showed that the pattern of cell sorting was not influenced by Di I labeling. In the experiments reported here, the PLB2 cells were labeled. Mixed cell pellets were cut up and cultured on a nonadhesive agarose substrate for 4 days, during which time they were periodically observed and photographed. PLB2 cells sorted out to form many internal clusters which progressively fused to form an internal spheroidal core or medulla surrounded by a spheroidal shell or cortex of PLS5 cells (Fig. 3 A–C).

Fusion and Spreading of PLB2 and PLS5 Cell Aggregates. When fragments of separate PLB2 and PLS5 cell pellets were paired, allowed to adhere and similarly cultured, PLS5 ag-

![Fig. 1](image1.jpg)  
Fig. 1. Cell aggregation (expressed as ΔMV reading of the aggregometer). , PLB2 cells + Ca²⁺; □, PLB2 cells in low Ca²⁺; ●, PLS5 cells + Ca²⁺; and ○, PLS5 cells in low Ca²⁺.

![Fig. 2](image2.jpg)  
Fig. 2. Aggregates formed after rotation in aggregometer cuvettes at 37°C, as in Fig. 1. (×5) (A) PLB2 cells + Ca²⁺. (B) PLS5 cells + Ca²⁺. (C) PLB2 cells in low Ca²⁺ (no aggregation).
FIG. 3. (A–C) Stages in the sorting-out of PLβ2 cells (fluorescent) from PLα5 cells over the course of 4 days. (D–F) Stages in the envelopment of PLβ2 aggregates (fluorescent) by PLα5 aggregates over the course of 3 days. Aggregates and aggregate fusions were photographed through a 6.3× objective lens with epifluorescence optics using a rhodamine filter set, with or without low-level tungsten backlighting. (Bar = 0.1 mm.)

Ggregates were observed to spread progressively over the surfaces of the PLβ2 aggregates, completely enveloping the latter in many cases. The same final configuration was thus approached by tissue spreading as by the sorting-out of the same two cell populations: a spheroidal PLβ2 core enveloped by a spheroidal PLα5 shell (Fig. 3 D–F).

DISCUSSION

Several alternative explanations of the linked phenomena of cell sorting and inside/outside tissue layering have been offered. Townes and Holtfreter proposed that cell sorting resulted from directed cell migrations followed by tissue-selective adhesion (1). Steinberg attributed these phenomena to differences in cellular adhesive intensities, facilitated by nondirected cell motility. Curtis (19, 20) proposed that the formation of internal and external cell layers during cell sorting results from the timing of postulated changes in cellular adhesive and motile properties which immobilize cells within aggregates in a centripetal wave. Thus cells of the first type in a mixture to experience this postulated change would be trapped in a layer at the aggregate's surface, cells of the second type to undergo this change would be trapped below them and so forth. Harris (21) favored a "differential surface contraction hypothesis" ("The more strongly contractile a given cell type is over its exposed surface, the more internally it should sort out relative to other, less contractile cell types."). Jones et al. (22) reported that the in vitro migration rates of chick embryonic muscle, liver, and neural retina cells declined in the same sequence as their tendency to segregate internally in binary reaggregates and proposed "that relative speed of movement may determine the positioning of cells in heterotypic aggregates." The results presented here demonstrate that both cell sorting and directed tissue spreading can be caused by differences in the intensity of intercellular adhesions in the absence of the differentials in chemotactic behavior, in timing, in cell surface contractility, or in speed of movement postulated, respectively, by Holtfreter, by Curtis, by Harris, and by Jones.

Segregation of emerging embryonic primordia is often associated with the appearance of different adhesion molecules in the separating tissues (Hatta et al., reviewed in ref. 23). Indeed, cDNA transfection experiments have demonstrated that expression of different cadherins in originally identical cells can lead to cell sorting (13). However, this does not imply that all instances of adhesion-mediated cell sorting or tissue segregation need result from the expression of qualitatively different adhesion systems. Computations earlier predicted that expression of a single adhesion system in different amounts should be sufficient to cause two cell populations not only to sort out from one another but also to show mutual engulfment behavior. It was predicted that a specific anatomical organization would emerge by either process, the cell population displaying fewer adhesion molecules completely enveloping the cell population displaying more of them (5, 6). The reasons for this can be appreciated by referring to Fig. 4 and its legend. Although "sphere-within-a-sphere" is the most stable configuration for such a system no matter how great or small the difference in the areal frequency of the two cell populations' adhesion sites, this difference would have to be sufficient to overcome frictional restraints and "noise" generated by random cell movements in order for this configuration actually to be approximated. We estimate that the PLβ2 cells employed in our experiments express about 20 times as much P-cadherin as the PLα5 cells.

Friedlander et al. (24) have already shown that greater vs. lesser expression of N-cadherin in two populations of transfected sarcoma S180 cells is sufficient to cause them to sort out to a certain extent after initial co-aggregation. However, their study did not address several related questions of morphogenetic significance. These questions are as follows: whether, in solid tissue masses, different levels of expression of the same adhesion molecule cause one of the two transfected cell populations to segregate internally or externally to the other; whether either of the two cell populations would tend to envelop the other after mutual confrontation; and whether the two transfected cell populations tend to arrange themselves in a specific anatomical configuration.

The present results demonstrate the evolution of all of the above morphogenetic behaviors merely by engendering quantitative differences in the expression of a single, homophilic adhesion system. Thus not only the adhesive affinities and the association constants of cell adhesion molecules but also the number of such molecules displayed on cell
pronephric duct (29–31). Stocum and his associates (32, 33) have presented behavioral evidence for a continuously graded, proximodistal increase in the cohesiveness of urodele limb regeneration blastemata, functional in positional memory and the control of intercalary regeneration. Adhesive gradients presumably reflect quantitatively graded differences in the expression of adhesion molecules, but the identities of these molecules have not yet been established in any of the above systems.

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