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The differential adhesion hypothesis: a direct evaluation

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Abstract

The differential adhesion hypothesis (DAH), advanced in the 1960s, proposed that the liquid-like tissue-spreading and cell segregation phenomena of development arise from tissue surface tensions that in turn arise from differences in intercellular adhesiveness. Our earlier measurements of liquid-like cell aggregate surface tensions have shown that, without exception, a cell aggregate of lower surface tension tends to envelop one of higher surface tension to which it adheres. We here measure the surface tensions of L cell aggregates transfected to express N-, P- or E-cadherin in varied, measured amounts. We report that in these aggregates, in which cadherins are essentially the only cell–cell adhesion molecules, the aggregate surface tensions are a direct, linear function of cadherin expression level. Taken together with our earlier results, the conclusion follows that the liquid-like morphogenetic cell and tissue rearrangements of cell sorting, tissue spreading and segregation represent self-assembly processes guided by the diminution of adhesive-free energy as cells tend to maximize their mutual binding. This conclusion relates to the physics governing these morphogenetic phenomena and applies independently of issues such as the specificities of intercellular adhesives.

Keywords: Cadherins; Cell–cell adhesion; Cell binding energy; Tissue affinities; Differential adhesion; Sorting-out; Cell sorting; Tissue spreading; Surface tension

Introduction

The differential adhesion hypothesis (DAH; reviewed in (Foty and Steinberg, 2004; Steinberg, 1970, 1978) was formulated as a physical explanation of the spontaneous liquid-like tissue segregation, mutual envelopment, and sorting-out behaviors of embryonic tissues and cells, described earlier in terms of "tissue affinities" (Holtfreter, 1939; Townes and Holtfreter, 1955). The DAH explains these behaviors and the rounding-up of irregular embryonic tissue fragments as cell rearrangements guided by the diminution of a cell population's adhesive-free energy as the totality of cell–cell bonding increases. We were led to this thermodynamic hypothesis by experiments that compared several aspects of the behavior of cell populations during cell sorting and mutual tissue spreading with expectations based upon each of the hypotheses then extant to explain these processes (Steinberg, 1962a,b,c, 1963, 1964, 1970). Only the DAH made the correct predictions. Further alternatives have subsequently been proposed by others but have not until now been evaluated experimentally. Our analysis indicated that embryonic tissues capable of the morphogenetic behavior at issue could be physically characterized macroscopically as elasticoviscous liquids whose elemental components are motile, mutually adhesive cells. This was confirmed by ultrastructural and mechanical studies of rearranging cell aggregates (Forgacs et al., 1998; Gordon et al., 1972; Phillips and Steinberg, 1978; Phillips et al., 1977; Steinberg and Poole, 1982).

Relative surface tensions specify which of a pair of immiscible liquids will tend to envelop the other. The conclusion of the DAH that tissues' mutual spreading

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preferences (Davis, 1984; Davis et al., 1997; Foty et al., 1994, 1996; Phillips and Davis, 1978) are similarly specified by their relative surface tensions (Steinberg, 1970) was confirmed by the development and application of tissue surface tensiometers. Through the use of these devices, it was demonstrated, in every mutually adhesive tissue pair tested, that it is always the tissue of lower surface tension that tends to envelop its partner. This rule is independent of the identities of the adhesion molecules utilized by the interacting cells (Duguay et al., 2003; Foty et al., 1994, 1996). It has remained only to evaluate the single element of the DAH that has until now remained unconfirmed: the postulate that the tissue surface tensions that underlie mutual tissue segregation, spreading, and cell sorting are generated in turn purely from the intensities of adhesion between the cells comprising these tissues. The present studies were undertaken as the final, direct test of the DAH.

Materials and methods

Generation of cadherin-expressing L cell lines

All cells were grown in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS), 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 10 μg/ml gentamicin in a humidified 5% CO_2 atmosphere. P-cadherin- (P-cad-) and E-cad-expressing L cells were produced as described previously (Duguay et al., 2003). N-cad-expressing L cell lines were produced by electroporation. 4×10^6 cells in 300 μl of RPMI 1640 without FCS/10 mM dextrose/0.1 mM dithiothreitol were transferred to a 0.4-cm electroporation cuvette. Cells were transfected with 40 μg of pMiwcN chicken N-cad expression vector (Fujimori et al., 1990) along with 4 μg of pZeoSV (Invitrogen, Carlsbad, CA) for Zeocin selection using a Bio-Rad Gene Pulser II gene transfer apparatus at 0.350 kV and 500 μF. Transfected cells were diluted 1/100 and plated into medium containing 300 μg/ml Zeocin. Resistant cells were grown to confluence, detached by 0.05% trypsin/5 mM Ca^{2+} (TC) treatment and stained with an antibody against chicken N-cad (NCD-2, Zymed, CA) on ice for 45 min. After several washes in Hanks' balanced salt solution (HBSS), cells were mixed with a fluorescein isothiocyanate-conjugated (FITC) secondary antibody and placed on ice for 30 min. N-cad-expressing cells were autocloned into 96-well plates using the CloneCyt Integrated Deposition System (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Positive clones were re-analyzed by FACS. N-cad lines designated LN1, LN2a, LN3, and LN4, expressing progressively increasing levels of N-cad; P-cad lines designated LP1a and LP2, expressing increasing levels of P-cad; and the E-cad line designated LE1 were used for all subsequent studies.

Immunoblot analysis of N-cadherin expression by transfected L cells

Lysates of control or N-cad-expressing L cells were prepared as follows: near-confluent 10 cm tissue culture plates were washed twice in ice-cold Tris-buffered saline containing 5 mM $CaCl_2$ and 1 mM PMSF (TBS+ Ca^{2+}). Cell monolayers were washed twice with ice-cold HBSS then lysed by the addition of 500 μl RIPA lysis buffer (150 mM NaCl, 50 mM TRIS pH 7.5, 1% NP40, 0.25% DOC) containing a protease inhibitor cocktail (Calbiochem, CA). The lysates were transferred to microcentrifuge tubes, rotated at 4°C for 1 h, then passed through a Qia-shredder (Qiagen, CA), and centrifuged at $14 \times g$ for 15 min at 4°C. Protein concentration was determined by the Markwell modification of the Lowry method (Markwell et al., 1981). Protein was separated on a 7% SDS-PAGE gel and blotted to a polyvinylidene fluoride (PVDF) membrane using standard protocols. Blots were blocked in Blotto (5% nonfat dry milk, 0.02% Na azide in PBS) for 1 h, then incubated at room temperature (RT) for 1 h in a solution containing 10 μg/ml mouse monoclonal antibody 6B3 directed against chicken N-cad (Knudsen et al., 1995) and 10 μg/ml rabbit polyclonal anti-actin antibody (used here as an internal standard). Blots were rinsed three times in TBS-0.2% Tween 20, then incubated at RT for 1 h in the appropriate secondary antibodies conjugated to horseradish peroxidase. After three more rinses, blots were developed using enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ).

Quantification of cell surface cadherin expression

Cadherin-transfected L cells were treated with 0.05% trypsin in HBSS with 2 mM $CaCl_2$ until they were released from the plate. Ca^{2+} in the medium protects the exposed cadherin molecules from digestion by trypsin (Takeichi, 1977). Absolute cadherin surface expression levels were determined by a quantitative flow cytometric assay (Brockhoff et al., 1994; Zagursky et al., 1995) using Quantum Simply Cellular® (QSC) microbeads (Bangs Laboratories, Fishers, IN) following the manufacturer's protocol. This is the most accurate method for making such determinations of which we are aware. The QSC kit contains five populations of 8.8-μm microbeads, a blank (negative control), and four populations with different calibrated binding capacities for mouse or rat IgG monoclonal antibodies. For N-cad-expressing cells, we employed Fab fragments of the 6B3 N-cad antibody generated using the Immunopure Fab Preparation Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. Two milligrams of Fab was coupled to NHS Sulfo Biotin (Pierce). Biotinylated Fab fragments were further purified by FPLC. QSC microbeads and transfected cells were incubated in 20 μg/ml biotinylated 6B3 Fab for 1 h at 4°C, washed several times, then resuspended in 10 μg/ml