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THE PROCESS OF CELL ADHESION AMONG DISSOCIATED SINGLE CELLS OF *HYDRA*: MORPHOLOGICAL OBSERVATIONS

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Summary

Ultrastructural observations were made on the initial adhesion process at the adherent region of *Hydra* endodermal cell pairs brought into contact (following dissociation) using a three-dimensional laser manipulator. Total contact length across the diameter of the adherent region decreased during the period 10–60 min after initial adhesion. However, the mean numbers of closest (<4 nm) and medium (5–25 nm) separation distances between membranes (thought to be important in total cell adhesion) were not significantly different. These data indicate that adherent cell pairs maintain a constant adhesiveness during the first 60 min of the adhesion process, despite membrane rearrangements. The relative length of each separation distance in adherent cell pairs approached that reported previously for intact *Hydra*. The sums of lengths in both the closest and medium categories (as a proportion of total contact length) increased because the length of cleavages (distances >25 nm) decreased significantly during the same time period. These results suggest that adherent cell pairs undergo rapid, active membrane changes in the adherent region, which might be associated with cell sorting. The possible significance of these changes for active rearrangement are discussed.

Key words: cell adhesion, cell contact, cell junction, *Hydra vulgaris*.

Introduction

The freshwater polyp *Hydra* has a diploblastic body plan (i.e. consisting of two layers of cells: ectodermal and endodermal epithelial cells; e.g. Campbell and Bode, 1983). The two layers form a hollow cylindrical body column with a head and a foot. *Hydra* has a strong regenerative capacity and can regenerate into a complete adult form, either from a small tissue piece excised from the body column (Bode and Bode, 1984; Shimizu et al., 1993), or from a cell aggregate produced from dissociated single cells (Noda, 1971; Gierer et al., 1972).

Study of the early stages of the regeneration process reveals important information not only about rearrangement of differentiated cells (Townes and Holtfreter, 1955) but also about the whole process of animal development (Spemann and Mangold, 1924). In *Hydra*, cell–cell interactions and cell rearrangements have been examined extensively in the process of epithelial sheet formation during regeneration from a cell aggregate. The cell sorting process begins in the initially irregular cell mass (reviewed by Armstrong, 1989), and the cells form a firm aggregate with a smooth surface within 6 h (Gierer et al., 1972). The phenomenon of cell sorting in animals involves the transformation of an initially disordered array of cells into one in which the cells are organized into homogeneous tissue domains. The driving force for the cell sorting process in *Hydra* appears to be a differential adhesiveness (Steinberg, 1970; Technau and Holstein, 1992), which explains how cells can rearrange themselves.

Cell aggregation is a phenomenon that can be produced artificially *in vitro* but each cellular interaction step is also observed during normal *in vivo* development. Cell–cell recognition (Takaku et al., 2000), cell–cell adhesion (Sato-Maeda et al., 1994), cell sorting or alignment (Wolpert, 1969) and pattern formation (Berking, 1997), are fundamental and occur during both aggregation and *in vivo* development. Cell–cell recognition and adhesion are the first steps of the early stages of the regeneration process. It has been confirmed that contact of an aggregated pair with additional homotypic cells of *Hydra* facilitates the occurrence of homotypic adhesions; heterotypic adhesions are discouraged (Takaku et al., 2000). This suggests that adhesion of homotypic cells contributes to a state of increased readiness for subsequent homotypic cells to adhere. Sato-Maeda et al. (Sato-Maeda et al., 1994) showed that, in *Hydra*, adhesive strength is greater between endodermal cell pairs (larger than 50 pN) than between ectodermal cell pairs (30 pN), and hypothesized that

different adhesive forces among single, isolated cells would contribute to the beginning of cell sorting. In our previous ultrastructural study (Takaku et al., 1999), it was found that after only 10 min of adhesion cell pairs had already started to acquire adhesive properties similar to those in intact *Hydra*, since more close membrane contacts were observed between endodermal cells than between ectodermal cells. The cellular interactions of isolated cells, therefore, can be useful for investigating the mechanism of patterning in multicellular organisms, in addition to investigating cell aggregates.

Here we demonstrate ultrastructural changes in the adherent region of endodermal cell pairs (dissociated from intact *Hydra* and allowed to readhere), several minutes after the initial adhesion event.

Materials and methods

Animals and cellular dissociation

Hydra vulgaris (strain K9, provided by Professor T. Sugiyama, National Institute of Genetics, Mishima, Japan) was used for all experiments. Stock cultures of animals were maintained in a *Hydra* culture medium ('M' solution; Sugiyama and Fujisawa, 1977) at a constant temperature of 18 °C. Animals were fed on newly hatched brine shrimp nauplii six times per week. Experimental animals were starved for 24 h prior to dissociation. The middle part of the body excluding the head and foot was dissociated into single cells (Takaku et al., 1999) by repeated pipetting in hyperosmotic medium (Flick and Bode, 1983).

Preparation for scanning and transmission electron microscopy

Cell-contact assays using a three-dimensional laser manipulator (Tashiro et al., 1993) were performed as previously described (Takaku et al., 1999). Briefly, a randomly selected single cell was lifted by laser beam to a glass micropipette, where the cell was immobilized by gentle suction. Another isolated single cell was then transported by laser beam to a position at the side of the first cell and maintained at that position to make good contact. After 30 s of forced contact, the laser beams were switched off and the cell pair was assessed to determine whether the cells remained adherent or separated (Sato-Maeda et al., 1994). 10, 20 and 60 min after the period of forced contact, fixation of the cells for electron microscopy was initiated using the concentric double pipette method (Takaku et al., 1999), which maintains the cells in an aqueous environment (i.e. avoiding exposure of cells to air).

The adherent cells were fixed with 3 % paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) for 30 min, and followed by three washes of 10 min each in 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing 4 % sucrose. The specimens were then dehydrated through a graded series of ethanol, transferred to isoamyl acetate and critical-point-dried (Hitachi HCP-1) using liquid CO_2. The specimens were coated with gold–palladium and observed in a Hitachi S430 scanning electron microscope at an acceleration voltage of 20 kV.

For transmission electron microscopic observations, fixation was for 2 h in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4), followed by three washes of 10 min each in 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing 4 % sucrose. The cells were then postfixed for 30 min in ice-cold 2 % OsO_4 in the same buffer and washed three times for 10 min each in ice-cold distilled water. Dehydration through a graded series of ethanol solutions was followed by embedding in an Epon–Araldite mixture. Ultrathin (approximately 50 nm) sections were cut perpendicular to the plane of attachment of the adhering cells and stained with 2 % uranyl acetate followed by 0.4 % lead citrate for 5 min each. Five serial sections of the widest adhering region were observed for each of five pairs of adhering cells (25 sections in total) using a Hitachi H-300 transmission electron microscope (50 kV). An

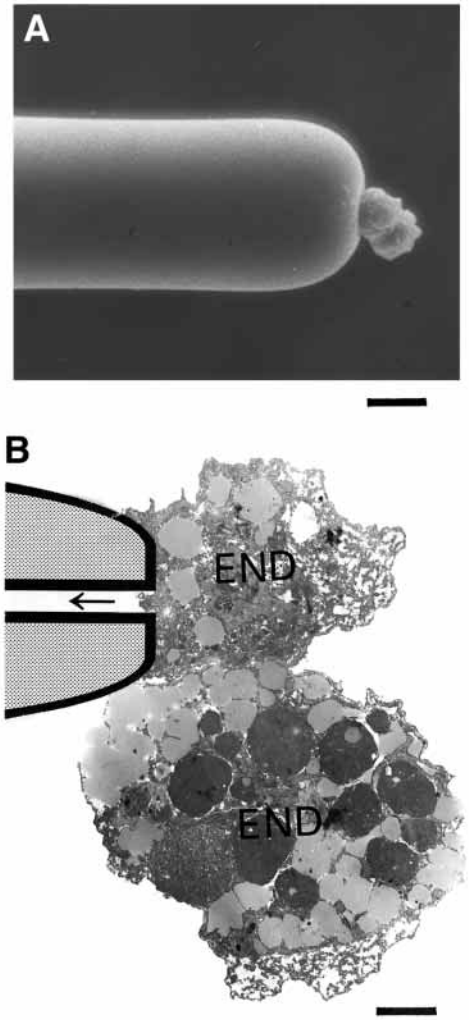

Fig. 1. A pair of *Hydra* endodermal cells (END) attached to the tip of a pipette after 10 min of adhesion. (A) Scanning electron micrograph. (B) Transmission electron micrograph (TEM). The cell was immobilized by gentle suction (arrow) into the pipette (schematic drawing: pipette detached for TEM preparation). Scale bars, 10 μm (A) and 2 μm (B).

ANOVA test was used to analyze the results, with a level of significance set at $P<0.05$.

Results

Observations on the adhering region of cell pairs

Fig. 1 shows low-magnification micrographs of a pair of adhering endodermal epithelial cells, each approximately 10 μm in diameter. The adherent region has a total contact length of approximately 5 μm after 10 min of adhesion. Cells were examined by scanning electron microscopy after 10 min and 60 min of adhesion (Fig. 2), during which their paired cells maintained good contact. However, their overall shapes changed and the area of the adherent region decreased (arrows and schematic drawings in Fig. 2). These observations suggest that the adherent cells are undergoing some form of rearrangement as the time of adhesion progresses.

Fig. 3 shows examples of high-resolution micrographs of the adherent region of endodermal epithelial cells. Cells were fixed 10, 20 and 60 min after being brought into contact. The widest adhering regions were observed in serial sections as follows: (A) 6.3±0.8 μm after 10 min, (B) 5.5±0.2 μm after 20 min and (C) 3.7±0.7 μm after 60 min (mean ± S.E.M.). These results confirm that the width of the adhering region is indeed decreasing during the adhesion process. In the intracellular space of endodermal cell pairs, no filament-like structure (polarized adjacent to mesogloea in intact *Hydra*; West, 1978) was observed.

The region of adhesion between adherent pairs of endodermal cells exhibited several classes of separation distance ('clearance') between membranes (Fig. 3). We recognized three classes (cf. Takaku et al., 1999). (1) The closest (less than 4 nm), includes gap junctions (Wood, 1977); (2) medium (approximately 5–25 nm), includes septate junctions (Wood, 1959), fascial intermediate junctions (Wood, 1977) and interdigitations (West, 1978); and (3) 'cleavages' (clearances greater than 25 nm, with low electron opacity). The latter were considered not to constitute cell junctions.

Quantitative analysis of the separation distance for adhering cell pairs

The three classes of clearance between adherent cell pairs, following the categorization described above, were measured at 10 min, 20 min and 60 min adhesion (Fig. 4A). The total contact lengths (across the diameter of the adherent region) decreased significantly from 10 min to 60 min ($P<0.004$). The mean values for closest and medium clearance, however, did not show any significant differences with time of adhesion (Fig. 4A). In contrast, the length of cleavages (>25 nm) significantly decreased from 3.3±0.6 μm (10 min) to 1.3±0.3 μm (60 min) ($P<0.0002$). Despite the fact that the total contact lengths decreased significantly, these results suggest that cell pairs maintained an adhesive force during the adhesion process, since there were no significant differences in the number of close membrane contacts (<4 nm, 5–25 nm), and this is thought to contribute to total cell adhesion (Takaku et al., 1999).

The proportions of the different categories of clearance for cell pairs are illustrated in Fig. 4B. The sum of clearance in both the closest and medium categories as a proportion of total contact length was not significantly different for 10 and 20 min adhesion times, but that for 60 min was significantly higher ($P<0.01$).

The number of cleavages decreased significantly (Fig. 5A) from 10 min to 60 min ($P<0.004$). However, the mean clearance length of cleavage profiles increased significantly from 20 min to 60 min (Fig. 5B) ($P<0.003$).

Taken together, the results clearly indicate that the adherent region changes morphologically during the first hour of adhesion.

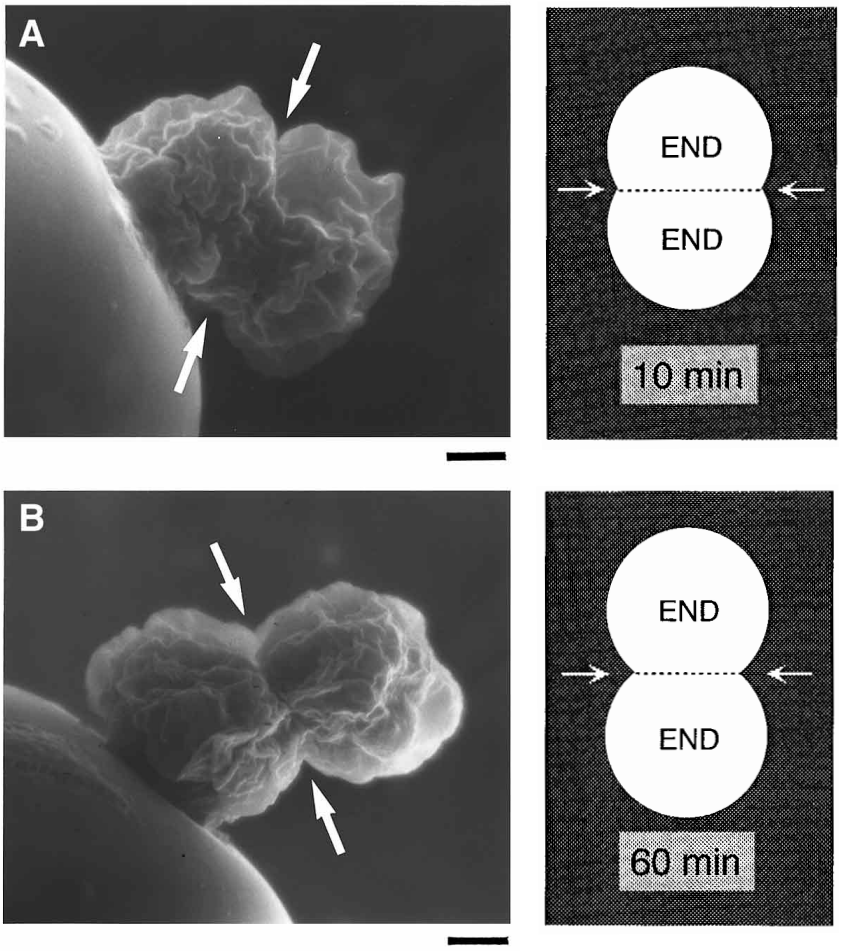

Fig. 2. Scanning electron micrograph of a pair of endodermal cells after 10 min (A) and 60 min (B) of adhesion. Arrows indicate the cell–cell adherent region, which is more constricted at 60 min: a difference emphasized in the accompanying schematic drawings. END, endothelial cells. Scale bars, 3 μm.

Discussion

Using combined chick and mouse cell aggregates, Overton (Overton, 1977) suggested that differences in adhesive forces could control cell sorting, manifested by differences in the density of cell junctions. Technau and Holstein (Technau and Holstein, 1992) invoked the differential adhesion hypothesis to account for the distribution of dissociated cells of *Hydra* spread evenly in flasks using a rotating shaker for 20 min: endodermal cells formed bigger cell clumps than ectodermal cells. In *Hydra*, adhesive forces of dissociated epithelial cells were measured after 3 min of adhesion (Sato-Maeda et al., 1994), using a three-dimensional laser manipulator to position individual cells (Tashiro et al., 1993). The adhesive strength was found to be higher between endodermal cell pairs than between ectodermal cell pairs. In our previous ultrastructural study (Takaku et al., 1999) of *Hydra* cell pairs after 10 min of adhesion, we found that membrane contacts were closer in endodermal than in ectodermal cell pairs. These reports suggest that differences in adhesive strength between tissues of different origins play a role in the early stages of cell sorting. However, there have been few studies on the details of the initial cell adhesion process. In this paper we have focused on the adhesion process of endodermal cell pairs, over precisely controlled steps of adhesion time.

In intact *Hydra*, the contact areas comprising the closest (<4 nm) and medium (5–25 nm) clearances are thought to contribute to total cell adhesion, since both are observed in all cell adhesion complexes (Wood, 1959; Wood, 1977; West, 1978), but cleavages (>25 nm) are not (Takaku et al., 1999). In adherent cell pairs, although total contact lengths decreased (Figs 2, 4A), the mean numbers of closest and medium clearance showed no significant differences over 60 min of the adhesion process (Fig. 4A). This suggests that adherent cell pairs maintain their initial adhesive contacts during this first hour, despite the occurrence of dynamic membrane rearrangements.

We have previously reported on the separation distances (clearances) in intact *Hydra* at the middle part of the body using similar methods to those used in the present paper. The ratio of clearance categories between endodermal cells was about 23 (closest) to 67 (medium) to 11 (cleavage). The combined sum of the closest and medium clearance categories as a proportion of total contact length was approximately 89 %. In adherent cell pairs, the corresponding sums showed no significant differences from 10 min to 20 min (49.2±5 % compared with 48.4±4.5 %; Fig. 4B), but increased significantly from 20 min

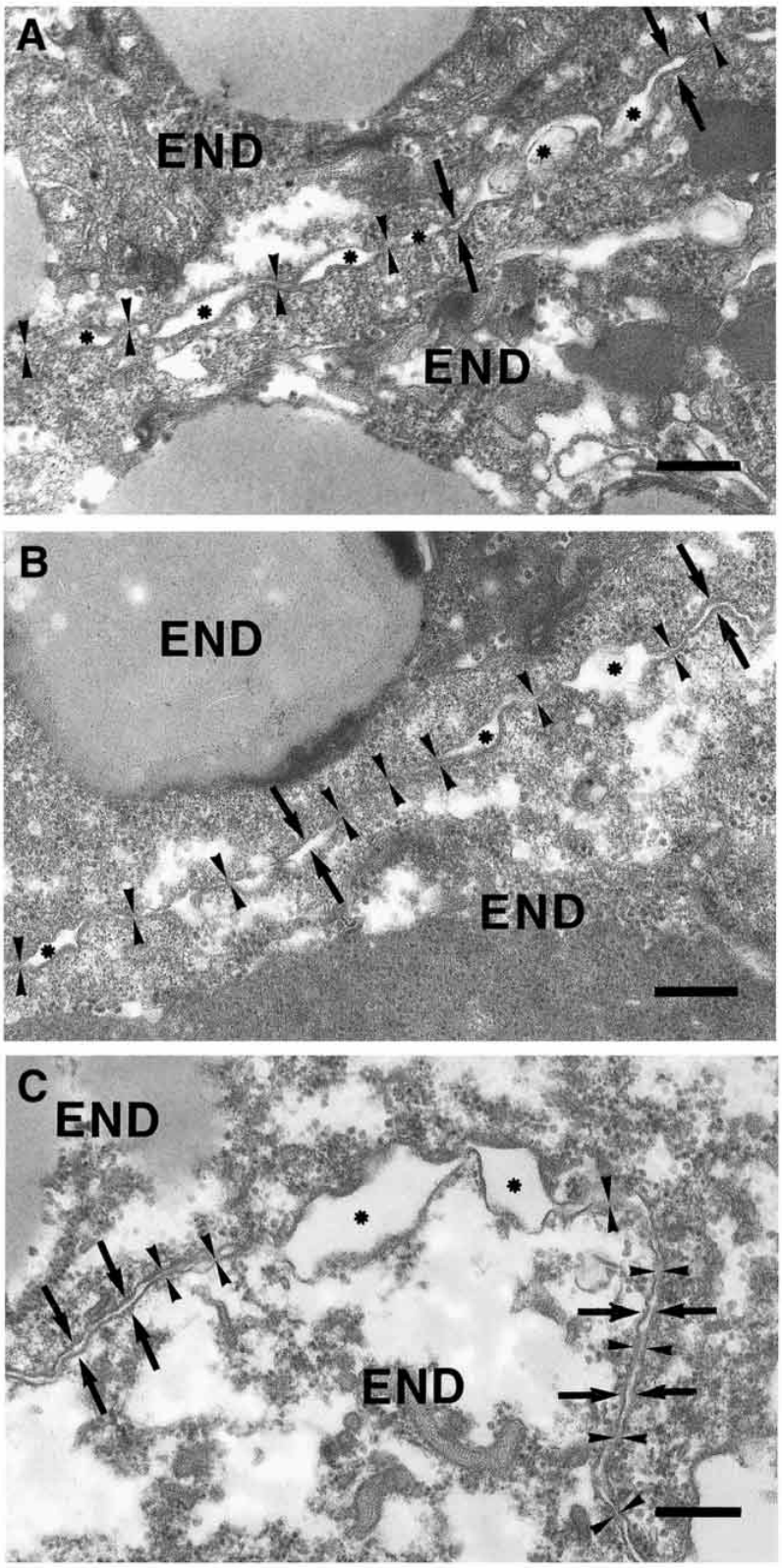

Fig. 3. Transmission electron micrographs of part of the adherent region of a pair of endodermal cells (END) after 10 min (A), 20 min (B) and 60 min (C) of adhesion, showing several levels of clearance between adjacent cell membranes. Arrowheads indicate the closest clearances, less than 4 nm. Arrows indicate medium clearances of 5–25 nm. Spaces much greater than 25 nm (cleavages) are also observed (asterisks). Scale bars, 200 nm.

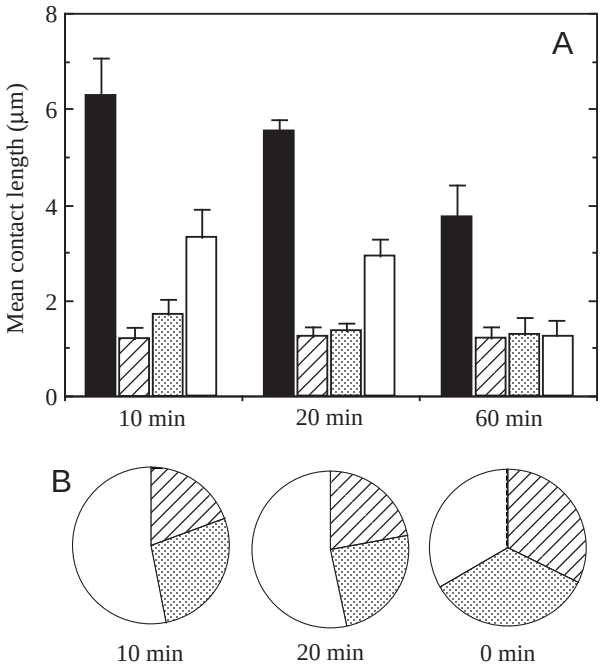

Fig. 4. Analysis of the length of each clearance class in adherent regions of endodermal cells after 10 min, 20 min and 60 min of adhesion. (A) Clearance lengths (mean values ± S.E.M.; *N*=25). Hatched columns, closest clearances (<4 nm); stippled columns, medium clearances (5–25 nm); open columns, cleavages (>25 nm); filled black columns, total contact length. (B) Length of each clearance class expressed as a ratio of total contact length (shading as in A).

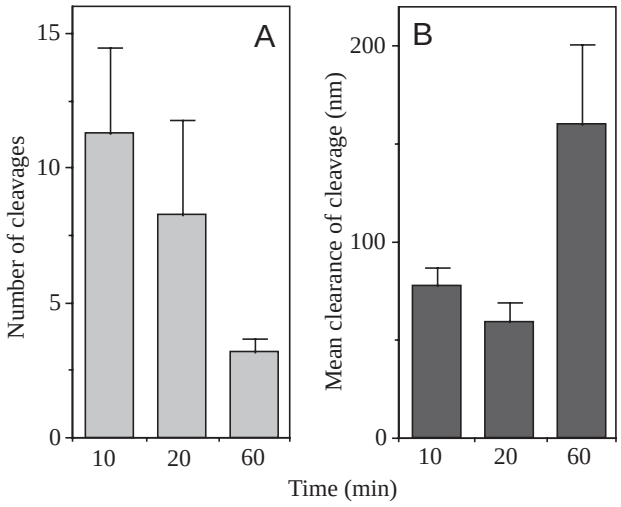

Fig. 5. Number and mean clearance length of cleavages observed in the adherent region (indicated by asterisks in Fig. 3). (A) Number of cleavages within the maximum length of the adherent region. (B) Mean clearance length between the cell membranes in the adherent region comprising cleavages (mean ± S.E.M.).

(48 %) to 60 min (65 %) because the regions of cleavage (>25 nm) significantly decreased (from approximately 52 % to 35 %) during this period. These results indicate that the proportions for adherent cell pairs change with time and approach those for intact *Hydra*.

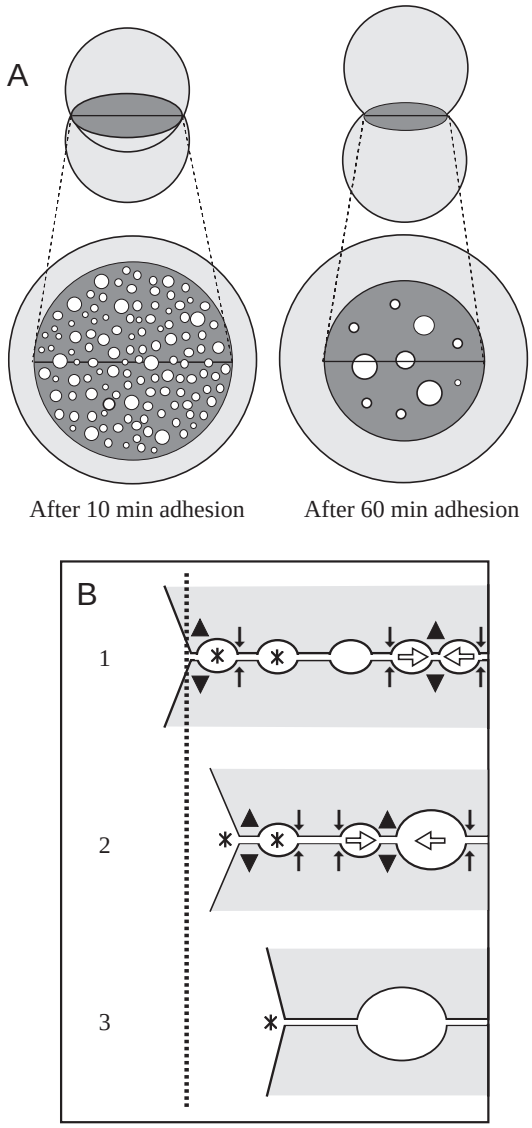

Fig. 6. (A) Schematic drawings of adherent regions between two endodermal cells 10 min and 60 min after adhesion commences. The upper figures show the paired cells as used in the ultrastructural measurements. The lower figures represent sections tangential to both cells, through the adherent region (i.e. perpendicular to the plane of the upper figures). As the time of adhesion increases, scattered small cleavages congregate, and so become larger but fewer in number. Close membrane contacts (<4 nm, 5–25 nm) also congregate during this period. The size of the adherent region decreases during this process. (B) Membrane changes between adherent cell pairs. Congregation (white arrows in cleavages) involves separation of some parts of membrane contact (arrowheads) and readherance of others (arrows), creating excluded peripheral cleavages that result in reduced total contact length of the adherent region (shown reducing from the left: cf. dotted line). The asterisks are explained in the text.

In the initial adhesion process investigated here, one of the main changes in the adherent region, which caused a decrease in the total contact length, was the decreasing number of cleavages (Fig. 4A). Although the number of cleavages

decreased significantly (Fig. 5A), the mean clearance of cleavages increased significantly (Fig. 5B). We suggest that membrane changes are structural rearrangements of cleavages.

Fig. 6 shows schematic drawings that attempt to explain what is happening. Scattered small cleavages (open circles) congregate and then combine to form fewer, larger cleavage regions. Note that close membrane contacts (stippled area, <25 nm) are likewise congregating and combining (Fig. 6A). To effect these changes, some regions of close membrane contact may separate, but the mean length of close contact regions showed no significant difference with time, suggesting that some parts must re-adhere (Fig. 6B). Peripheral cleavage (asterisks in Fig. 6B) is excluded from the adherent region: note that total contact length therefore decreases. The decreasing total contact lengths, which caused increasing free cell surface, may be one of the cell properties in the initial adhesion process and/or it might present other cells with a greater opportunity to form new adhesions.

Sato-Maeda et al. (Sato-Maeda et al., 1994) showed that endodermal epithelial cells in the initial contact could move, actively changing their shape. However, ectodermal epithelial cell pairs showed little evidence of such motility or plasticity of cell shape and remained almost spherical even after adhesion is achieved. Although the driving force for the high motility of endodermal cell adhesions is still unclear, the speed and frequency of the motility changed during 60 min of the initial adhesion process (Y. Takaku, experiments in progress), suggesting that our present observations of membrane rearrangements might be associated with control of the adhesive force, cell motility and cell sorting.

We have demonstrated here the ultrastructural characteristics of the adherent region of endodermal cell pairs during the initial adhesion process. During this 1 h period, adhering cell pairs show morphological changes in cell contact approaching those observed in intact *Hydra*. Our data suggest that cell pairs undergo surprisingly dynamic membrane rearrangements in the adherent region during the initial adhesion process while maintaining adhesiveness.

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