INTRODUCTION

Cadherins are cell surface glycoproteins that are responsible for calcium-dependent cell-cell adhesion. Cadherin molecules appear to have an intimate spatial relationship with the actin-based cytoskeleton in various types of cells and their interaction is required for cell adhesion (Takeichi, 1988, 1991). The cell-to-cell adherens junction (AJ) is a specialized cell-to-cell contact region where cadherins act as adhesion molecules and actin filaments (AF) are densely associated with the plasma membrane (Geiger and Ginsberg, 1991; Tsukita et al., 1992).

In intestinal epithelial cells, which are well polarized, E-cadherin is concentrated at the most apical part of the lateral membrane together with AF, forming the belt-like AJ (zonula adherens) associated with the circumferential AF bundle (Farquhar and Palade, 1963; Boller et al., 1985).

Although the process of tight junction (zonula occludens) formation during polarization of MDCK cells has been thoroughly investigated (Gumbiner and Simons, 1986; Nelson and Veshnock, 1986; Gumbiner et al., 1988; Bacallao et al., 1989; Wang et al., 1990), knowledge about AJ formation in polarized epithelial cells remains fragmentary. To approach this problem, McNeill et al. (1993) recently investigated the binding ability of E-cadherin to the actin-based cytoskeleton during an early stage of cell-to-cell contact of MDCK cells. Several unsolved issues remain with regard to AJ formation, such as how the AJ/AF relationship changes during the polarization of epithelial cells, the role of AF organization in the process of AJ formation and the origin of the circumferential AF bundles.

In cardiac muscle cells, N-cadherin is concentrated at the intercalated discs where AF of myofibrils terminate at the plasma membrane in a perpendicular fashion (Geiger et al., 1980; Volk and Geiger, 1984). In fibroblasts, however, the structural aspects of AJ have not been fully investigated, although they have a Ca^2+-dependent adhesion system (Takeichi, 1988).

In rat 3Y1 fibroblasts, P-cadherin is reported to be concentrated at cell-cell adhesion sites (Itoh et al., 1991, 1993). Chick heart fibroblasts in primary culture reportedly have AJ-like structures at the electron microscopic level (Heaysman and Pegrum, 1973). However, there is no convincing evidence at

SUMMARY

Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells

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Summary

Cadherin has an intimate spatial relationship with actin filaments (AF) in various types of cells, forming the cell-to-cell adherens junction (AJ). We compared the AJ/AF relationship between non-polarized fibroblasts (NRK cells) and polarized epithelial cells (MTD-1A cells). E/P-cadherin, α-catenin, ZO-1 and vinculin were localized with reference to AF in these cells using laser scan microscopy as well as conventional light and electron microscopy. NRK cells adhered to each other at the tips of thin cellular processes, where spot-like AJ were formed, where P-cadherin, α-catenin, ZO-1 and vinculin were concentrated. Some stress-fiber-like AF bundles ran axially in these processes and terminated at spot-like AJ on their tips. At the electron microscopic level these spot-like AJ were seen as aggregates of small ‘units’ of AJ, where AF were densely and perpen-

Key words: adherens junction, actin filament, polarized epithelial cell, laser scanning microscopy, cadherin
present to show whether they are recognized as AJ, and our understanding of the differences and similarities between these cadherin-based cell-cell adhesion sites in fibroblasts and the typical AJ in polarized epithelial cells and cardiac muscles cells is limited.

Some proteins that may be responsible for the interaction between cadherins and actin-based cytoskeletons have been identified and analyzed. α and β-catenins with molecular masses of 102 and 94 kDa, respectively, are associated directly with the cytoplasmic domain of cadherin molecules. ZO-1, with a molecular mass of 220 kDa is coconcentrated at cell-to-cell adhesion sites with the cytoskeleton-bound cadherins in fibroblasts. In epithelial cells, this molecule is localized at tight junctions. Vinculin, with a molecular mass of 106 kDa, is thought to be indirectly associated with cadherins as well as integrins, and to be involved in cadherin/actin and integrin/actin interactions (Geiger and Ginsberg, 1991; Tsukita et al., 1992). Specific antibodies for these proteins can help identify the cadherin/AF interaction sites by immunofluorescence microscopy.

Recent progress in laser scan microscopy (LSM) enables simultaneous fluorescence images from one doubly labeled specimen to be obtained and precisely compared. Furthermore, the digitized data of images from serial optical sections in LSM offer three-dimensional information. Thus the relationship between AJ formation and AF organization can be analyzed in detail. In this study, we compared the AJ/AF relationship between non-polarized fibroblasts and polarized epithelial cells. Localization of E-/P-cadherins, α-catenin, ZO-1 and vinculin was correlated with AF using LSM, as well as conventional light and electron microscopy. The results are discussed, with special reference to the similarity and difference of the AJ/AF relationship between non-polarized fibroblasts and polarized epithelial cells.

PHASE-CONTRAST AND IMMUNOFLOUORESCENCE MICROSCOPY

All procedures were performed at room temperature. For phase-contrast microscopy, cells grown on coverslips were fixed with 2% fresh formaldehyde and 2.5% glutaraldehyde in 0.1 M HEPES buffer (pH 7.5) for 2 hours. They were washed with PBS, and mounted on glass slides.

For immunofluorescence microscopy, cells grown on coverslips were fixed with 1% fresh formaldehyde in 0.1 M HEPES buffer (pH 7.5) for 15 minutes. After washing with PBS containing 30 mM glycine (G-PBS), the cells were exposed to 0.2% Triton X-100 in G-PBS for 5-15 minutes. After a brief wash, they were soaked in blocking solution (G-PBS containing 2% normal goat serum) for 10 minutes. The cells were incubated with the first antibodies for 30-60 minutes, washed with G-PBS, then incubated with the secondary antibodies and/or rhodamine-phalloidin for 30-60 minutes. All antibodies were diluted in blocking solution, and the following secondary antibodies were used: FITC-conjugated goat anti-rat Ig (TAGO, Inc., Burlingame, CA), FITC-conjugated sheep anti-mouse IgG (CHEMICON, Temecula, CA). After washing, specimens were mounted in 90% glycerol-PBS containing 0.1% para-phenylenediamine on glass slides. Specimens were observed using a Zeiss Axioskop Photomicroscope with objective lenses, Plan APOCHROMAT ×40 (NA 1.0), Plan NEOFLUAR ×63 (NA 1.25) and Plan NEOFLUAR ×100 (NA 1.30) (Carl Zeiss, Oberkochen, Germany). Photographs were taken on Neopan F film (Fuji Photo Film Co., Ltd, Tokyo, Japan) for phase-contrast images and T-Max 400 film (Eastman Kodak Co., Rochester, NY) for fluorescence images.

LASER SCAN MICROSCOPY

For LSM, strips of coverslips were inserted between glass slides and specimens as spacers to avoid sample compression after the specimens were prepared as described above. Cells were analyzed using a laser scan microscope, Zeiss LSM 410 invert equipped with Ar (488 nm) and HeNe (543 nm) lasers. LSM analysis using the confocal overlay mode allowed simultaneous recording and display of different fluorophores within the same focal plane. The microscope output yielded an overlaid double-labeled image showing either en face or computer-generated cross-sectioned views. Conventional overlaid images were composed of 512 by 512 pixels. For three-dimensional reconstruction of images of serial optical sections, 256×256 pixel images were recorded. Usually, the number of optical sections was 30 and the interval was 0.3-0.4 µm. For printing, photographs were taken on the color reversal film, Fujichrome RDP 135 (Fuji Photo Film Co., Ltd, Tokyo, Japan). Throughout the LSM analysis, Plan APOCHROMAT ×63 (NA 1.4) was used as the objective lens.

ELECTRON MICROSCOPY

For conventional electron microscopy, cells were fixed with 2% fresh formaldehyde and 2.5% glutaraldehyde in 0.1 M HEPES buffer (pH 7.5) for 1 hour followed by postfixation with 1% OsO4 in the same buffer for 1 hour on ice. The samples were then dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a diamond knife, doubly stained with uranyl acetate and lead citrate, then examined using an electron microscope, JEM 1200 EX (JEOL, Tokyo, Japan), at an accelerating voltage of 100 kV. For immunoelectron microscopy using the mAb ECCD-2, 4 hours after replating, MTD-1A cells were incubated in ECCD-2-containing culture medium for 3 hours, then fixed and processed for electron microscopy as described above.

REPLATING AND WOUNDING OF MTD-1A CELLS

For replating, subconfluent MTD-1A cells were treated with PBS containing 2 mM EDTA followed by trypsin digestion, then replated at 128 S. Yonemura and others

MATERIALS AND METHODS

Cell culture

Fibroblastic cell lines, NRK-49F from rat kidney (De-Larco and Todaro, 1978), CV-1 from monkey kidney (Jensen et al., 1964) and NIH/3T3 clone 5611 from a mouse embryo (Jainchill et al., 1969), were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Rat 3Y1 fibroblasts (Kimura et al., 1975) were a generous gift from Dr Masatoshi Takeichi (Kyoto University, Kyoto, Japan). The cells were cultured on clean coverslips (14 mm in diameter) in DME supplemented with 10% FBS.

Antibodies

The following monoclonal and polyclonal antibodies (mAb and pAb, respectively) were used: mouse anti-rat ZO-1 mAb (T8-754; Itoh et al., 1991), rabbit anti-rat ZO-1 pAb (Itoh et al., 1993), rat anti-mouse E-cadherin mAb (ECCD-2; Shirayoshi et al., 1986), rabbit anti-mouse P-cadherin pAb (Nose et al., 1987), rat anti-α-catenin mAb (6x-18; Nagafuchi and Tsukita, 1994); mouse anti-chicken vinculin mAb (V115; Sigma Chemical Co., St Louis, MO); rabbit anti-rat vinculin pAb (Tsukita and Tsukita, 1989). AF was stained with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR).
the cell density necessary to obtain a nearly confluent monolayer. Cells grown on coverslips were examined at 4, 7, 12 and 24-36 hours after replating.

To analyze wound healing, confluent MTD-1A cells were wounded manually (i.e. by scratching) with the tips of a pair of forceps. Six linear wounds were made on each coverslip. The culture medium was exchanged with fresh medium and the wounds were allowed to heal for 6 hours.

RESULTS

AJ formation and AF organization in non-polarized cultured fibroblasts

Our preliminary studies showed that among various types of fibroblasts, NRK (normal rat kidney) cells were the most appropriate for analyzing and demonstrating the AJ/AF relationship, mainly due to the simple organization of their actin-based cytoskeletons. The AJ/AF relationship observed in NRK cells was fundamentally the same as that in other types of fibroblasts (CV-1, NIH/3T3, and 3Y1).

Phase-contrast microscopy revealed that under semi-confluent culture conditions, NRK cells adhered to each other through thin cellular processes, and that the tip of each process, a possible adhesion site, was seen as a phase-dense dot (Fig. 1A). AF stained by rhodamine-phalloidin were observed as stress-fiber-like bundles, most of which were arranged in the cytoplasm in a parallel fashion (Fig. 1B). These parallel bundles originated or terminated at rhodamine-phalloidin-positive dots at the cell boundaries, which corresponded to the phase-dense dots in phase-contrast images. These dots were specifically stained with anti-ZO-1 mAb (Fig. 1C). The ZO-1 signal was detected at the cell boundaries as dots. NIH/3T3 cells also had rhodamine-phalloidin- (Fig. 1D) and ZO-1- (Fig. 1E) positive dots at the tips of stress-fiber-like bundles although these bundles were not more developed than those in NRK cells.

**Fig. 1.** Light microscopic images of NRK and NIH/3T3 fibroblasts. Phase-contrast (A), and immunofluorescence micrographs of NRK cells doubly stained with rhodamine-phalloidin (B) and anti-ZO-1 mAb (C). Thin processes connecting adjacent cells have phase-dense dots in their tips (arrowheads). These dots were stained with rhodamine-phalloidin and anti-ZO-1 mAb (C). Through these dots, parallel stress-fiber-like bundles appear to have penetrated several cells (B). NIH/3T3 cells doubly stained with rhodamine-phalloidin (D) and anti-ZO-1 mAb (E). Bar, 10 μm.
The precise localization of ZO-1 to AF was analyzed by LSM. As shown in Fig. 2A, when AF and ZO-1 were stained with red and green, respectively, all the rhodamine-phalloidin-positive dots were seen in yellow, indicating that ZO-1 is highly concentrated at these dots. Then, the distribution of P-cadherin, α-catenin and vinculin was analyzed by LSM. Both P-cadherin and α-catenin were also concentrated at the rhodamine-phalloidin-positive dots (Fig. 2B,C). Vinculin was concentrated at the dots, when the plane of focus was fixed at the cell-to-cell adhesion level (Fig. 2D). At the level of the substratum, an intense vinculin signal was detected at the so-called focal contacts (cell-to-substratum AJ), where stress-fiber-like

![Fig. 2. Overlaid LSM images of NRK cells doubly stained with anti-ZO-1 mAb (green)/rhodamine-phalloidin (red) (A), anti-P-cadherin pAb (green)/rhodamine-phalloidin (red) (B), anti-α-catenin mAb (green)/rhodamine-phalloidin (red) (C), anti-vinculin pAb (green)/rhodamine-phalloidin (red) (D,E), or anti-vinculin pAb (green)/anti-ZO-1 mAb (red) (F,G). The plane of focus was fixed at the cell-cell adhesion (A-D,G) or the cell-substratum adhesion level (E,F). ZO-1, P-cadherin, α-catenin and vinculin were colocalized at rhodamine-phalloidin-positive dots (yellow in A-D), where AF bundles terminated, indicating that these dots are spot-like AJ. At the level of cell-substratum adhesion, vinculin was concentrated at the ends of stress-fiber-like bundles, i.e. focal contacts (E), which lacked ZO-1 (F). In F, dots in red represented cell-cell adhesion sites (AJ) close to the substratum. As shown in G, the relative amounts of vinculin and ZO-1 in AJ were variable in each AJ. Bar, 10 µm.]
Fig. 3. Electron microscopic images of spot AJ in NRK cells. (A) Cells adhered to each other through thin cellular processes containing AF bundles (arrows) in their axis. (B) At higher magnification, the cell-cell adhesion regions on thin processes looked like a staircase, which was an aggregate of small units of AJ (arrows). (C,D) Small units of AJ. This unit was characterized by the perpendicularly associated AF (arrowheads) and the intercellular bridges (arrows). Bars: 1 µm (A); 0.2 µm (B-D).

Fig. 4. LSM images of fully polarized MTD-1A epithelial cells (24-36 hours after replating). (A,B) Overlaid computer-generated cross-sectional images of the cells doubly stained with anti-ZO-1 mAb(green)/rhodamine-phalloidin(red) (A) or anti-ZO-1 pAb(green)/anti-vinculin mAb(red) (B). The configuration of each cuboidal cell was revealed by rhodamine-phalloidin staining, and the position of the substratum is indicated by arrowheads. Vinculin was localized slightly nearer the substratum than ZO-1, indicating that vinculin and ZO-1 were concentrated at AJ and tight junctions, respectively. Vinculin was also localized at the substratum level. (C,D) The 21st and 23rd optical sections from the substratum level, respectively, which were used to generate the cross-sectional view of B. Comparison of these images clearly revealed that vinculin (red) is concentrated more at the basal side on lateral membranes than ZO-1 (green). Bar, 10 µm.
bundles terminated (Fig. 2E). These bundles were distinct from those associated with the rhodamine-phalloidin-positive dots. Therefore, we concluded that ZO-1, α-catenin and P-cadherin were concentrated in fibroblasts at the rhodamine-phalloidin-positive dots, but not at the focal contacts, while vinculin was concentrated at both the rhodamine-phalloidin-positive dots and the focal contacts. To confirm this conclusion further, we compared the distribution of vinculin with that of ZO-1 by LSM (Fig. 2F,G). Taking these data together, the rhodamine-phalloidin-positive dots were recognized as AJ at the light microscopic level.

Next, we analyzed the ultrastructure of this AJ region by ultrathin section electron microscopy. The cellular processes at the cell-cell boundaries contained AF bundles in their axes, and adjacent cells adhered preferentially around the tips of these processes (Fig. 3A). At higher magnification, the AJ looked like a staircase, and appeared to consist of two types of membrane domains (Fig. 3B-D). The first domain was characterized by the plasma membrane where AF were bound perpendicularly. In the extracellular space of this domain, thin filamentous structures were observed as intercellular bridges. No membrane fusion like that in tight junctions was detected. The second domain lacked the intercellular bridge where AF ran parallel to the plasma membrane.

**MTD-1A cells as polarized epithelial cells**

Since they are polarized epithelial cells, the MDCK cell line has often been used in various analyses. In this study, however, to clarify the AJ/AF relationship, we used MTD-1A cells. The reasons are as follows: (1) AF bundles in MDCK cells were thinner than those in MTD-1A cells. (2) In MDCK cells, the frequent overriding of cell peripheries during the establishment of cell polarity interfered with detailed analyses of the formation of cell-cell contact regions. (3) The formation of contact regions is more synchronous in MTD-1A cells.

To confirm that MTD-1A consists of highly polarized epithelial cells, like MDCK, a series of optical sections were obtained by LSM, and computer-generated cross-sections were demonstrated. AF were stained in red to show the cell configuration, and ZO-1 was revealed in green (Fig. 4A). Under confluent culture, each MTD-1A cell appeared cuboidal, and ZO-1 was concentrated at the most apical region of lateral membranes. Next, we compared the localization of vinculin (red) with that of ZO-1 (green), precisely (Fig. 4B-D). As shown in Fig. 4B, a cross-sectional view, ZO-1 signals were detected more at the apical side on lateral membranes than vinculin signals. This difference in the level
of localization between ZO-1 and vinculin was more obvious when the 21st optical section from the substratum (Fig. 4C) was compared with the 23rd section (Fig. 4D). These findings indicate that MTD-1A cells are well-polarized epithelial cells with typical junctional complexes where ZO-1 and vinculin are localized at tight junctions and AJ, respectively.

AJ formation and AF organization in MTD-1A cells during the establishment of cell polarity after replating

Light microscopy
MTD-1A cells were dissociated with trypsin in the absence of Ca^{2+}, replated on coverslips, then examined by phase-contrast microscopy. At the early stage of polarization (6 hours after replating...
replating), adjacent cells adhered through thin cellular processes, just like NRK cells (Fig. 5A). The possible cell-cell contact regions at the tips of processes were phase-dense. At the middle stage (10 hours after replating), the cells were characterized by bright regions at the cell periphery, where the thin processes were virtually undetectable (Fig. 5B). At the late stage (36 hours after replating), cells showed a typical epithelial appearance (Fig. 5C).

To pursue the process of the AJ formation at each stage of cell polarization after replating, samples were doubly stained with anti-E-cadherin mAb and rhodamine-phalloidin and observed by conventional fluorescence microscopy. At the early stage of polarization (4 hours after replating), an intense E-cadherin signal was detected at the cell boundaries as dotted lines, indicating that the spot-like cadherin-based cell-cell contact regions were formed just as they are in NRK cells (Fig. 6A). The cells exhibited numerous stress-fiber-like bundles as well as fine AF by rhodamine-phalloidin staining, and some of the bundles originated (or terminated) at the spot-like cadherin-based cell-cell contact regions, where the stained stress-fiber-like bundles appeared thicker (Fig. 6A,B,D,E). These bundles originating at the contact regions ran towards the cell center, and associated to form thicker bundles running circularly along the cell boundary. In addition to these stress-fiber-like bundles associated with contact regions, AF were observed at both the more dorsal and the basal levels. At the more dorsal level, AF occurred as fine filaments, whereas at the more basal level they formed stress-fiber-like bundles that were associated with the focal contacts. The organization of AF at these levels appeared to be constant throughout the polarization process. At 7 hours after replating, the E-cadherin staining profile was a mixture of dotted and continuous lines, indicating that the spot-like cadherin-based cell-cell contact regions began to fuse side by side to form a ‘belt-like’ cell-cell contact region (Fig. 6G,H,I,K). At this stage, the circular AF bundles described above became thicker and expanded toward the cell periphery with the concomitant shortening of stress-fiber-like bundles originating at the spot-like cadherin-based cell-cell contact regions. Up to this stage, the localization of concentrated E-cadherin precisely coincided with that of ZO-1 (Fig. 6C,F,I,L).

At the middle stage of cell polarization (12 hours after replating), the E-cadherin staining profile at the cell periphery became almost continuous (Fig. 7A). Concomitantly, the circular AF bundles expanding toward the cell periphery reached the cell-cell boundary to form so-called ‘circumferential AF bundles’ at the belt-like cadherin-based cell-cell contact regions (Fig. 7B). The stress-fiber-like bundles remained associated with them only at the spot-like contact regions that had not yet fused. Around this stage, the lateral membrane domain appeared to be established. Since these domains were oriented in a fairly oblique direction to the substratum at this stage, they were observable as an en face view by conventional fluorescence microscopy. In addition to the concentrated type at the most apical region, E-cadherin was distributed rather diffusely at the newly formed lateral membranes, where AF also occurred, although their distributions appeared to be different in detail (asterisks in Fig. 7A,B). As shown in Fig. 7C, a ZO-1 signal was detected around the belt-like, cadherin-based, cell-cell contact region, but not from the lateral membrane domains.

At the late stage (24-36 hours after replating), cells became cuboidal and fully polarized as shown in Fig. 4. Conventional fluorescence microscopy showed that ZO-1 was concentrated at the level of the junctional complex (Fig. 7D), which is completely lined with the circumferential AF bundles (Fig. 7E). As shown by LSM in Fig. 4B, however, the level of ZO-1 concentration was distinct from that of AJ where vinculin is highly concentrated. By contrast, E-cadherin was distributed diffusely at the lateral membrane including its upper (junctional complex level, Fig. 7F), middle (Fig. 7G) and bottom levels (Fig. 7H). At the basal membrane, E-cadherin-positive dots were often present (Fig. 7H), where AF was concentrated (Fig. 7I). This AF concentration appeared to be independent of the stress-fiber-like bundles running along the basal membranes.

The polarization process was investigated again by LSM to obtain three-dimensional information. The cross-sectional view of a cell monolayer at the early stage (7 hours after

Fig. 8. LSM images of MTD-1A epithelial cells during the establishment of cell polarity. (A,B) Early stage of cell polarization (7 hours after replating). Overlaid computer-generated cross-sectional images of the cells doubly stained with anti-ZO-1 mAb(green)/rhodamine-phalloidin (red) (A) and anti-E-cadherin mAb (green)/rhodamine-phalloidin (red) (B). Both ZO-1 and E-cadherin were concentrated at spot AJ located very close to the substratum (arrowheads). (C) Late stage of cell polarization (24-36 hours after replating). Cross-sectional images of the cells singly stained with anti-E-cadherin mAb (green). E-cadherin was distributed evenly along the lateral and occasionally basal membranes. Bar, 10 µm.
replating) was wavy in contour; cells were thickest at the cell center and thinnest at the cell periphery where ZO-1- and E-cadherin-rich cell-to-cell contact regions (yellow) were formed (Fig. 8A,B). In fully polarized cells (24-36 hours after replating), E-cadherin (shown in green) was distributed almost evenly along the lateral membrane, and occasionally at the basal membranes (Fig. 8C).

Electron microscopy
At the early and middle stages of cell polarization, MTD-1A cells were fixed, dehydrated and embedded. Thereafter, ultrathin sections were cut parallel to the substratum, and the cadherin-based cell-cell contact regions were analyzed by conventional electron microscopy. At 4 hours after replating (corresponding to Fig. 6A-F), the cell-cell contact region was found at the tip of cellular processes, and it consisted of two distinct domains with a staircase-like appearance, and their ultrastructural aspects were similar to those of NRK fibroblasts (Fig. 9A; see Fig. 3). At 7 hours after replating (corresponding to Fig. 6G-L), the cell-cell contact region increased in area, and its overall appearance was still staircase-like (Fig. 9B). At around this stage, the typical desmosomes associated with intermediate-sized filaments began to be identified in the contact regions.

At the middle stage (12 hours after replating; corresponding to Fig. 7A-C), the contact region-associated stress-fiber-like bundles were hardly evident, and instead, circumferential AF bundle-like structures appeared (Fig. 9C). The distinction between the above two domains became unclear, although the undercoats and intercellular bridges were not evenly distributed along the plasma membrane. At this stage, occasionally, there was a considerable gap between the circumferential bundle and the plasma membrane, and in the gap stress-fiber-like bundles terminated perpendicularly at the plasma

Fig. 9. Electron microscopic images of AJ of MTD-1A cells during cell polarization. At 4 hours after replating (A), a staircase-like spot AJ was formed at the tip of a thin cellular process, through which two adjacent cells adhered. This AJ was also composed of small unit structures (see Fig. 3). At 7 hours after replating (B), AJ on the tip of processes increased in size by an increase in the number of unit structures. At the middle stage of polarization (C, 12 hours after replating), circumferential AF bundles were located close to AJ (i). AF bundles terminating perpendicularly at AJ were obscure but still recognizable (arrowheads). Thin intercellular bridges (arrows) occurred along AJ but were not evenly distributed. Bar, 0.2 μm.
membrane. At this stage, tight junctions as well as desmosomes were numerous.

Finally, we examined the nature of the intercellular bridges. For this purpose, at 4 hours after replating, living MTD-1A cells were incubated for 3 hours in culture medium containing rat anti-mouse E-cadherin mAb, ECCD-2. This mAb interfered with neither the cadherin-based cell adhesion (Shirayoshi et al., 1986) nor the distribution of E-cadherin (data not shown). These labeled cells were then processed for thin-section electron microscopy without using any second antibodies. When compared with the intercellular bridges in non-treated cells (Fig. 10A), in the antibody-treated cells clear electron density was detected at the midpoint of each intercellular bridge, showing the single line between apposed membranes (Fig. 10B). This appearance was different from that of desmosomes, which were characterized by thicker undercoats and intermediate filaments (Fig. 10C). We concluded that the intercellular bridges are the morphological counterparts of the extracellular domain of E-cadherin.

**AJ formation and AF organization in MTD-1A cells during wound healing**

Since the replating procedure containing low-Ca\(^{2+}\) and trypsin is not physiological, we investigated the process of cell polarization after wounding, a more-physiological treatment. Confluent cultures of well-polarized MTD-1A cells were manually scratched with sharp forceps. Six hours later, the AF organization was markedly changed in the cells at the front row of the wound (Fig. 11A). A high concentration of AF was seen along the free border, and some AF bundles were aligned parallel to the free border as if they had penetrated several cells in the front row (Fig. 11C). This was previously reported as ‘arcs’ or ‘rings’ of AF in other types of cells bordering the wound (Martin and Lewis, 1992; Bement et al., 1993).

The ZO-1 distribution was analyzed in these cells. As shown in Fig. 11B, the front free border lacked ZO-1 staining. The cell-cell borders between adjacent cells in the front row were mostly characterized by the continuous belt-like distribution of ZO-1, but at the front end of these borders, ZO-1 was detected as broken lines or aligned dots (Fig. 11D). A comparison of Fig. 11C and D revealed that the AF bundles along the free border terminated at these ZO-1 dots. This is why these AF bundles appeared to have penetrated several cells.

When wound healing proceeded, cells from opposite sides of the wounded area met and adhered to each other through thin cellular processes. In these processes, newly formed stress-fiber-like bundles extended from the ‘arcs’ (Fig. 12A,C,E), and at the tip of each process a spot-like cell-cell contact region was formed where ZO-1 was highly concentrated (Fig. 12B,D). When wound healing proceeded further, these spot-like contact regions fused side by side, the bundles shortened, and the ‘arc’ expanded toward the wound border (Fig. 12F,G). Finally, these ‘arcs’ were transformed into typical circumferential AF bundles, when wound healing was complete. Therefore, we can conclude that the AJ/AF rela-

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**Fig. 10.** Immunolabeling of the intercellular bridges with anti-E-cadherin mAbs. (A) The intercellular bridges of spot AJ on the tips of processes in MTD-1A cells at 7 hours after replating, which were not treated with antibodies. (B) Intercellular bridges immunolabeled with anti-E-cadherin mAb. Note that the midpoints of the intercellular bridges were specifically labeled. (C) Desmosomes in non-treated MTD-1A cells. Their structural appearance was similar to that of the decorated AJ in B, but desmosomes had thicker plaque structures associated with intermediate filaments. Bar, 0.1 µm.
tionship during cell polarization after wounding is very similar to that after replating.

DISCUSSION

We compared the AJ/AF relationship between non-polarized fibroblasts (NRK cells) and polarized epithelial cells (MTD-1A cells) using laser scan microscopy (LSM) as well as conventional immunofluorescence microscopy and electron microscopy. Our observations are summarized in a schematic drawing (Fig. 13). There are two distinct AJ/AF relationships: namely, 'fibroblastic' and 'polarized epithelial' types. During the establishment of cell polarity after replating or wounding, epithelial cells first exhibited the former type of relationship, which was gradually converted to the 'polarized epithelial' type.

The use of anti-P-cadherin, anti-α-catenin, anti-ZO-1 and anti-vinculin antibodies together with rhodamine-phalloidin allowed the precise identification of AJ in fibroblasts and epithelial cells at the early stage of polarization. In fibroblasts at the light microscopic level, AJ were found at the tip of cellular processes, where a subset of stress-fiber-like bundles is associated at their end. In NRK cells, this type of bundle is rather more developed and aligned in a parallel fashion. Although cadherin-based cell-cell contact regions in fibroblasts have been noticed, this type of bundle has not been described in detail so far. There seem to be several reasons. (1) This type of bundle was partly confused with the bundles associated with focal contacts (cell-to-substratum AJ). (2) In some fibroblasts (for example, NIH/3T3 cells), well-developed bundles associated with focal contacts interfered with the identification of this type of bundle. (3) Some fibroblasts such as mouse L cells lack the expression of cadherins, so also lack this type of bundle. (4) Antibodies specific for the cadherin-based cell-cell contact regions in fibroblasts, such as anti-P-cadherin, anti-α-catenin, and anti-ZO-1 antibodies, have not been used previously to identify this type of bundle.

Close observation of these AJs by electron microscopy revealed that these regions consisted of two distinct membrane domains. In the first domain, AF were associated perpendicularly with membranes. This domain was connected to that with the same characteristics on the apposed membrane through fine strands of intercellular bridges. The second domain lacked both perpendicularly associated AF and intercellular bridges. These distinct types of domains alternated, giving the cell-cell contact regions a staircase-like appearance. Considering that the intercellular bridges were immunolabeled with anti-cadherin mAbs, the first domain can be recognized as a 'unit' of AJ: α-catenin, vinculin and ZO-1 can be localized in the plaque structure in

Fig. 11. The AJ/AF relationship in MTD-1A cells at the early stage of wound healing. Lower (A,B) and higher (C,D) power images of the cells in the front row of the wound (*), which were doubly stained with rhodamine-phalloidin (A,C) and anti-ZO-1 mAb (B,D). Thick AF bundles ('arcs') were observed along the free border of the cells (arrows). At both ends of these arcs, ZO-1 was concentrated in a dotted manner along the cell-cell border of adjacent cells (arrowheads). Bars, 20 µm (A,B); 10 µm (C,D).
Adherens junction formation and actin

this domain. Therefore, we concluded that in fibroblasts and in epithelial cells at the early stage of polarization, AJ on the tip of cellular processes was an aggregate of unit structures ~40-200 nm in diameter. This feature appears to be quite common among various types of cells such as chick heart fibroblasts (Heaysman and Pegrum, 1973), chick lens epithelial cells (Volk and Geiger, 1986), human keratinocytes (Green et al., 1987) and chick retinal pigmented epithelial cells (Kodama et al., 1991). From an ultrastructural perspective, including the manner of the AF association (end-to-membrane fashion), this unit structure is very similar to that of AJ in the intercalated discs of cardiac myocytes.

At early stages of cell polarization after replating as well as after wounding, the AJ/AF relationship in epithelial cells was...
very similar to that in fibroblasts (see Fig. 13). The difference is that, in epithelial cells, the AJ-associated stress-fiber-like bundles are radially arranged and associated to form a circular bundle, whereas in fibroblasts such as NRK cells they are aligned in parallel. This type of AJ/AF relationship has been identified in various types of epithelial and endothelial cells such as rat hepatocytes in primary culture (Miettinen et al., 1978), PtK1 cells (Albrecht-Buchler, 1979), PtK2 cells (Sanger and Sanger, 1980), human umbilical vein endothelial cells (Hormia et al., 1985; Kolodney and Wyssolmerski, 1992; Salmon et al., 1992), chick lens epithelial cells (Geiger et al., 1985), human keratinocytes (Green et al., 1987), and chicken spinal cord glial cells in primary culture (Hirano et al., 1987). This similarity between fibroblasts and epithelial cells indicates that similar underlying mechanisms are involved in the organization of AJ in these cells.

At the middle stage of cell polarization, each cell-to-cell AJ increased in area at the immunofluorescence microscope level. Electron microscopy revealed that this is due to the increase in number of the ‘units’ of AJ per contact region, not to the increase in area of each unit. As a result of the expansion of each contact region, the cellular processes became thicker and shorter, and finally the contact regions fused side by side. Around this stage, the AJ/AF relationship appeared to undergo a qualitative change: the unit structure of the AJ became unclear and circumferential AF bundles were established from the circular bundles with the concomitant disappearance of the stress-fiber-like bundles associated perpendicularly with the membranes.

The manner of AF/plasma membrane association can be classified into two types in general, end-to-membrane and side-to-membrane. In well-polarized epithelial cells, the circumferential AF bundles run just beneath the plasma membrane of the belt-like AJ. Therefore, we speculate that in the established belt-like AJ, the manner of AF/plasma membrane association is side-to-membrane. However, this study revealed that in the process of AJ formation in epithelial cells, the end-to-membrane fashion is predominant, suggesting that this type of association is primarily required for AJ formation. In the qualitative change in the AJ/AF relationship at the late stage of cell polarization, this end-to-membrane association may be replaced by the side-to-membrane type. Alternatively, does the end-to-membrane association still remain in the established belt-like AJ? This point remains to be elucidated.

Finally, we should discuss the peculiar behavior of ZO-1 and E-cadherin during the establishment of cell polarity. Itoh et al. (1991, 1993) have reported that ZO-1 is precisely colocalized with cadherins in non-epithelial cells, whereas in epithelial cells it is concentrated at tight junctions (Stevenson et al., 1986; Anderson et al., 1988) but not at AJ. Using LSM, we confirmed that in NRK cells ZO-1 is concentrated exclusively at AJ, and that in polarized epithelial cells, such as MTD-1A cells, ZO-1 and E-cadherin are segregated into the tight junction and AJ, respectively. At the early and middle stages of cell polarization, even in epithelial cells, ZO-1 was localized precisely at AJ where the tight junction-like membrane fusion was hardly visible by electron microscopy. As the junctional complex as well as apical and basolateral membrane domains were being established, ZO-1 appeared to move from the AJ to the newly developed tight junctions. By contrast, E-cadherin spread to the whole lateral, and occasionally to the basal, membranes. This profile of E-cadherin distribution has been identified in both MDCK (Gumbiner and Simons, 1986; Nelson et al., 1990; Wang et al., 1990) and Caco-2 cells (Zahrnau et al., 1994). Further analysis of the molecular basis for this behavior of ZO-1 and E-cadherin should clarify the molecular mechanism of cell polarization.

In this study, we closely analyzed the AJ/AF relationship in non-polarized fibroblasts and polarized epithelial cells, and identified the similarities and differences between these two cell types. During AJ formation, both types of cells first form the unit structure of AJ, where AF are associated perpendicularly with membranes, then the AJ/AF relationship differentiates, becoming cell type-specific. The molecular mechanism

Fig. 13. A schematic drawing of the AJ/AF relationship in fibroblasts (A) and polarized epithelial cells (B, early; C, middle; D, late stages of polarization). See details in the text. N, nucleus; CAFB, circumferential AF bundles.
REFERENCES


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