Development of the junctional complex during differentiation of chick pigmented epithelial cells in clonal culture

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The structure and development of junctional complexes during redifferentiation of chick pigmented epithelial cells in clonal culture have been studied with TEM and colloidal lanthanum. The mature junctional complex consists of a zonula adherens which is usually surmounted by one or more macular gap junctions of varying length. Tight junctions (zonulae occludentes) appear to surround the gap junctions and extend into the zonula adherens. Punctate intermediate junctions appear first. As differentiation progresses, these extend to form fasciae and zonulae adherentes. Focal membrane fusions are found both within and above the developing adherens junctions; gap junctions appear to form adjacent to the latter structures. Colloidal lanthanum passes through the junctional regions between cells in the outer zone of the colony but is stopped by those of the differentiated cells, suggesting that these cells are sealed by fasciae or zonulae occludentes. During redifferentiation, groups of cells undergo slow, coordinated contractions which appear to be involved in developing the differentiated cell shape. These begin shortly after the formation of the junctional complexes and are most active during junctional complex maturation. Once cellular junctional complex differentiation is well established, the contractions cease. The possible roles of the different junctions in the development of cellular shape are discussed.

Key words: junctional complex, pigmented epithelium, differentiation, clonal culture

Single cells dissociated from 8-day-old chick pigmented retinal epithelium can be grown under long-term culture conditions (circa 3 weeks) which permit expression of their differentiated characteristics. During this process, the cells form disc-shaped colonies roughly 10 mm across, which can be roughly divided into four zones. From center to outside edge these have been called the
pigmented cuboidal, intermediate, stratified, and squamous zones. The inner two have the characteristic shape of pigmented epithelial cells in vivo and contain pigment granules. The outer two zones are irregular in shape and unpigmented for the most part. These zones appear to represent different stages in the re-expression of differentiation, with the most differentiated cells at the center. One aspect of this process is the development of a distinctive junctional complex.

The junctional complex joining the differentiated cells at the center of the clones has been briefly described. This consists of an adherens junction surmounted by either a gap and/or a tight junction. This is almost identical in structure to those which develop between pigmented epithelial cells in short-term culture. Detailed studies involving mass tracers such as colloidal lanthanum have not been done to determine how closely the junctional complex found in cloned pigmented epithelial cells compares with that described in the adult. The first part of the present paper describes the junctional complex joining the differentiated cells at the center of the clone in more detail and compares these junctions with those found between pigmented epithelial cells in vivo.

One of the major morphological indications of cellular differentiation is the development and maintenance of a characteristic cellular shape. Changes in the organization of contractile microfilaments (4 to 7 nm in diameter) or the appearance of arrays of both microfilaments and microtubules have been correlated with specific changes in cellular shape in numerous systems. These movements appear to be controlled to some extent by the contacts and junctions formed between neighboring cells and between cells and their substrate. Studies correlating the development of the junctions with the observed cellular movements should give a greater insight into the way these movements are controlled, however, such studies are difficult because of the lack of suitable material. If junctional complex formation is studied in intact tissues or embryos, it is virtually impossible to observe the dynamic aspects of differentiation due to the thickness of the material. If the process is studied in culture, the living cells are easily observed, but in most cases the cells do not express their differentiation.

Clones of pigmented epithelial cells have none of these drawbacks. The application of time-lapse cinemicrography to studies of cell differentiation in pigmented epithelial clones has already led to the discovery that groups of
Fig. 2. Cross-section through a differentiated cell in the center of the colony which has been treated with lanthanum. Note that the lanthanum appears to be stopped within the apical gap junction (GJ), suggesting the presence of a zonula occludens within this region at this stage. ZA, Zonula adherens; MF, microfilaments; MV, microvilli.

Fig. 3. Junctional complex of a differentiated cell in the center of a pigmented epithelial clone. This consists of a zonula adherens (ZA) surmounted by a single short gap junction (GJ). An area of intracellular density is found on either side of the membrane, below the main portion of the adherens junction. Although it occupies the position of the desmosome in the junctional complex of other epithelia, it does not have the intercellular dense line characteristic of this structure.

Pigmented epithelial cells undergo coordinated contractions (focal contractions) during differentiation and that these contractions appear to be involved in changing the shape in the cells during the differentiation process. The fact that these cells are capable of coordinated activities suggests that they have developed a form of communication not present at the edge of the colony where coordinated movements are not observed. Electrical communication has been demonstrated between cells of pigmented epithelial clones. Cap junctions are reputed to be the sites of electrical communication between retinal cells in vivo as well as the means of forming electrical metabolic connections in numerous other cell types.

The second part of this paper describes the development of the junctional complex during redifferentiation of chick pigmented epithelial cells in clonal culture, with the use of the techniques listed above. It goes on to relate the development of the different elements of the junctional complex, especially...
Fig. 4. Proposed scheme of junctional complex development in cloned chick pigmented epithelial cells. a, Stage 1. Intracellular dense plaques (DP) and associated microfilaments (MF) are found adjacent to the inner leaflets of the membranes of adjoining cells. b, Stage 2. Density and number of the plaques and associated microfilaments have increased. Both leaflets of the membranes adjacent to the dense plaques have increased in electron density. Either the outer leaflets of the membranes opposite them are fused, or the intercellular space has narrowed and contains a material of intermediate electron density. c, Stage 3. Membranes of adjoining cells are more vertically oriented and both leaflets of the entire junctional region of the membranes have increased in electron density. The intracellular dense material and associated microfilaments are concentrated toward the middle of the nascent junctional complex forming an adherens junction which is now zonular. Focal membrane fusions (TJ) are seen both within, above, and below the main body of the adherens junction. d, Stage 4. Regions of decreased intercellular distance and increased intercellular density are present around and between the focal membrane fusions (TJ) located above the main body of the adherens junction (ZA). Some of these regions have the appearance of short regions of gap junctions (GJ). e, Stage 5. Mature junctional complex consisting of an adherens junction (ZA) surmounted by one or more regions of gap junctions (GJ) and an apical occludens junction (TJ). Focal membrane fusions (TJ), representing a zonular tight junction, are found in the apical portion of the junction as well as within the junction. (Drawings by Chris Irvine.)
Fig. 5. Cell at the outer edge of the colony cut parallel to the surface of the plate, showing short regions of adherens junctions (AJ) which join it to the neighboring cells and the microfilaments associated with them (MF). N, Nucleus. Inset, High-power view of a similar adherens junction.

the gap junction, to the movements involved in the development of the differentiated cellular shape.

Materials and methods

Clones 3 to 4 weeks old of chick pigmented epithelial cells were grown in Hams F10g medium containing 4% fetal calf serum and 1% Sephadex G-25-excluded fraction of embryo extract (F10gH0.1) directly on 100 mm Falcon Plastic tissue culture dishes according to the methods described previously. Before fixation for transmission electron microscopy (TEM), the plates were washed two times in saline G, and the colonies were fixed in situ in the following manner.

Clones for normal studies were fixed in 2.5% glutaraldehyde in 0.133M Sorensen's phosphate buffer, pH 7.4, for 1 hr at room temperature. This was followed by one rinse in 2.5% NaHCO₃ buffer, pH 7.4, and fixation in 2% OsO₄ in 1.25% NaHCO₃, pH 7.4, for 1 hr at room temperature. The colonies were then stained in 2% aqueous uranyl acetate for 1 hr, dehydrated in alcohol, and embedded in Epon 812.

Clones to be studied with the colloidal lanthanum technique were fixed in glutaraldehyde as above, rinsed in 0.2M s-collidine buffer, pH 7.4, and postfixed for 2 hr in 2% OsO₄ in 0.1M s-collidine buffer containing 2% lanthanum nitrate. The tissue was then dehydrated and embedded as above.

Sections were cut on a Porter-Blum MT-1 microtome with a Dupont diamond knife and photographed on a Philips 300 electron microscope.

Results

Structure of the mature junctional complex. Well differentiated cells at the center of epithelial clones were joined by junctional complexes consisting of zonular intermediate junctions, (zonulae adherentes). These often included points of fusion of the outer leaflets of the opposing cell membranes (focal tight
Fig. 6. Section in the same plane as Fig. 5 of cells in the outer edge of the stratified zone, showing the fasciae adherentes (FA) and associated microfilaments (MF). Note that the cells still have some free lateral borders.

junctions) (Fig. 1). The intermediate junctions were surmounted by one or more regions of varying height having the typical appearance of gap junctions by TEM. These were partially or completely filled with tracer when lanthanum was added to the fixative. Focal membrane fusions similar to those described above were often found above and below them. Colloidal lanthanum failed to pass between the majority of cells occupying the center of the colonies (Fig. 2). It was stopped for the most part either within the gap junctions or at the focal membrane fusion below the gap junctions.

In some cases plaques of dense intracellular material similar to those found in desmosomes were found below the adherens junctions (Fig. 3). Although there appeared to be some increase in intercellular electron density between the plaques, the dense line characteristic of the typical desmosome was not found between cells of the clones.

Development of the junctional complex. The stages of development of the junctional complex in cells of the pigmented retinal clone are described in Fig. 4. Development of the apical junctional complex began with the development of intermediate junctions and the focal membrane fusions associated with them. These junctions formed between the undifferentiated cells at the outer edge of the squamous zone. Serial 1 μm horizontal sections and similar thin sections for electron microscopy through the developing junctional regions suggested that these began as a series of isolated points on the lateral aspect of two overlapping cells (Fig. 5). The number of points of junctional contact between cells increased from the outside of the squamous zone to the inside of the stratified zone where first fasciae (Fig. 6) and finally zonulae adherentes (Fig. 7) were observed. The latter were restricted to the better-differentiated cells found toward the center of the colony.
Fig. 7. Section through the region of the adherens junction (ZA) of cells of the intermediate zone in the same plane as Figs. 5 and 6. Note that these now form complete zonulae surrounding each cell. Microfilaments (MF) of the apical web and peripheral bands can also be seen.

Fig. 8. Earliest stage of junctional complex formation. Intracellular dense plaques (arrows) are associated with microfilaments (MF). No focal membrane fusions are present.

Observation of cross-sectioned material from cells at the outer edge of the squamous zone of the colony demonstrated that the first event in the appearance of the adherens junctions appeared to be collections of small plaques of intracellular dense material and associated microfilaments along the apical lateral borders of overlapping cells (Fig. 8). These dense regions were usually opposed by similar regions in the adjoining cell. Usually several plaquelike areas were seen in one cross-section, although on occasion only a single such area was present. There appeared to be little other membrane specialization at this early stage; the distance between the nascent junctional membranes was variable,
Fig. 9. Slightly later stage in the development of the adherens junction joining cells of the stratified zone. The intracellular plaques (arrows) have increased in density, and more microfilaments (MF) are present in both longitudinal and cross-sections. The membranes of neighboring cells adjacent to the plaques show an increase in electron density, and the membranes are either closely opposed or fused in these regions. **Inset**, Higher magnification of closely opposed membranes. Note the increase in density of the intercellular matrix between them.

Fig. 10. Focal membrane fusions in a developing junctional complex (arrows).

and there was no increase in electron density of the membranes themselves.

In cells located in the middle of the squamous zone, the short plaquelike regions were denser, larger, and more obvious (Fig. 9). Both leaflets of the cell membranes in the region adjoining the dense plaques were more electron-dense than those surrounding them, and the intercellular space opposite the plaques was narrowed and contained material of intermediate electron density (Fig. 9, inset). In the majority of sections, at least one of these regions showed a focal membrane fusion. In the more differentiated cells located toward the center of the colony, only a few regions of close membrane apposition were present, but focal membrane fusions were more numerous (Fig. 10).

Microfilaments, of the 4 to 7 and 10 nm classes, appeared to be embedded in the dense regions of the developing adherens junctions. In the outer zones, the cells overlapped one another extensively, and the junctional regions of the membranes lay almost parallel to the apical surfaces in many cases. Where this occurred, the microfilaments paralleled the junction arising from all the plaquelike densities adjacent to the
Fig. 11. Junctional complex in the intermediate zone, showing focal membrane fusions (arrows) located toward the apical (free) surface of the cells above the main body of the adherens junction (ZA). Cross-sections of microfilaments (MF) can be seen in association with the adherens junctions.

Fig. 12. Apical region of the junctional complex of a differentiated cell from the inner zone of the clone. Several short gap junctions (GJ) are present above the adherens junctions (ZA) in the regions occupied exclusively by the focal membrane fusions at an earlier stage (Fig. 11).

membrane (Figs. 8 and 9). As the cell profiles changed from squamous to cuboidal during the redifferentiation process, the main axis of the junction shifted from horizontal to vertical, and the majority of intracellular dense material and microfilaments became localized in what was roughly the middle of the nascent junctional regions (Figs. 1-3 and 11). The majority of junctions in the differentiated cells in the center of these colonies still retained small, plaquelike, junctional regions below the main body of the adherens junction, which sent scattered bundles of microfilaments upward to join those emanating from the main body of the adherens junctions. In some cases these regions appeared to be fused into one extensive region below the main body of the intermediate junction, although as stated above, these did not develop the intercellular dense line characteristic of the classic desmosome (Fig. 3). Focal membrane fusions were also found in these regions.

Several focal membrane fusions with their attendant intracellular dense plaques were found above the main body of the adherens junctions in cells in the intermediate zone and those at the inner edge of the stratified zone (Fig. 11). In these cells, areas of parallel membrane separated by a 5 to 7 nm gap, containing material of intermediate electron density, were located just adjacent to some of the focal membrane fusions. The appearance of these regions was similar to that described in developing tight junctions (see above), but they were more extensive. Other cells in the same or more differentiated zones of the colony were joined by several short regions of
Fig. 13. Cross-section through a cell in the stratified zone of the colony. This shows lanthanum (L) penetrating between the cells of this region, including two gap junctions (GJ), one above and one within the adherens junction (AJ).

Fig. 14. Cell similar to that seen in Fig. 10, but treated with colloidal lanthanum (L). Note that the lanthanum passes around the focal membrane fusions (arrows) and is only excluded from the space immediately between them, showing that they consist of either maculae or fasciae occludentes but do not form occluding zonulae at this stage.

gap junctions at these locations (Fig. 12) or by one or two extensive ones (Figs. 1 and 3). Not all sections through the heavily pigmented cells in the center of the colony contained apical gap junctions, although the number of these junctions was greater in this zone than in any other.

Gap junctions were not restricted to cells of the inner zones of the colony. Scattered gap junctions also joined cells in the squamous and stratified zones. These either were separate from the developing adherens junctions or occurred below or within them. Short gap junctions were also found within extensive adherens junctions in the differentiated zones of the colony.

Colloidal lanthanum passed through the nascent junctional complexes and between cells in the squamous and the outer edge of the stratified zone (Fig. 13) and was excluded only from the points of membrane fusion (Fig. 14). In the inner edge of the stratified zone and in parts of the intermediate zone, lanthanum passed through the gap and adherens junctions but did not penetrate to the base of the cells. In the later stages of differentiation it was stopped within or above the junctions themselves (see above).

Although the stages of junctional complex development were most often found in the zones of the colony discussed above, the distribution of the stages shown in Fig. 4 were variable in position; for example, on occasion, nascent gap junctions could be found above
adherens junctions in the inner regions of the squamous zone or in the stratified zones.

Conclusions and discussion

Structure of the junctional complex. The junctional complexes joining the apical lateral borders of the differentiated cells at the center of the pigmented epithelial clones consist of zonular adherens junctions of varying depth surmounted by macular gap junctions. Focal membrane fusions are located both above and below the gap junctions and throughout the adherens junctions. The appearance of this junctional complex by TEM is very similar to that described in pigmented epithelia from the eyes of a number of adult vertebrates.

Freeze-fracture studies of adult vertebrate pigmented retinal epithelia demonstrate that the focal membrane fusion seen in these cells with TEM represent tight junctions. These form continuous occluding zonules located above the adherens junctions and discontinuous ones within them. The fact that colloidal lanthanum is stopped either above or within the apical portions of the junctions in the majority of cloned differentiated cells suggests that the focal membrane fusions seen in these cells also form an occluding zonule. In some cases, however, lanthanum passes between the cells, suggesting that the differentiation of these junctions is incomplete. Detailed freeze-fracture studies of the junctions in this region should help to determine how complete the occludens junctions are.

Development of the junctional complex. In cloned pigmented epithelial cells the first event in junctional complex formation appears to be the development of short regions of adherens junctions. These begin with the appearance of short areas of increased intracellular density and associated microfilaments which appear opposite to one another in adjacent cells in a manner similar to that described during development of the adherens junctions in other tissues (Fig. 4, a). This appears to be followed by an increase in electron opacity of the cellular membranes. Initially the increase in electron opacity is localized to the areas adjacent to the intracellular densities; later it is found dispersed throughout the entire junctional complex.

During the next stage of adherens junction differentiation, both the number and density of the intracellular dense regions increase, and more microfilaments are associated with them. The short regions of the cell membranes associated with the densities become closely apposed, material in the intercellular area condenses, and finally the outer leaflets of the adjacent membranes fuse (Fig. 4, b).

During the next stage (Fig. 4, c), the lateral cell membranes begin to change from almost horizontal to vertical as the cell shape begins to change from squamous to cuboidal. The majority of the intracellular dense material and associated microfilaments becomes localized toward the center of the developing junctional complex.

Focal membrane fusions with accompanying small plaques of intercellular dense material and a few associated microfilaments are found above and below the adherens junctions. At present, it is not known if these represent new regions of fusion or represent those junctions which were present in earlier development. In general, the junctional regions increase in length during the differentiation process, suggesting that at least some of them are new.

The apical gap junctions are generally the last elements of the junctional complexes to form. They appear to coalesce around and between the focal membrane fusions located above the main body of the adherens junctions (Fig. 4, d and e).

Cells in the outer edge of the squamous zone make contact with their neighbors at only a few points. In the inner portions of this zone and the adjacent portions of the stratified zone, the association between neighboring cells is increased, but spaces are still visible with the phase-contrast microscope. The majority of focal membrane fusions in these regions must represent either focal areas of tight junctions or short segments rather than zonulae.

Colloidal lanthanum is found throughout the nascent junctional complexes and within the intercellular spaces in the regions of the
colony which represent early stages in the redifferentiation process. As redifferentiation progresses, lanthanum is gradually excluded from the intercellular regions. The exclusion process begins in short regions between adjacent focal membrane fusions, progresses to penetration through the junctional complex but not to the base of the cell, and ends with almost complete exclusion from the intercellular spaces. This is almost identical with the pattern seen during differentiation of tight junctions in other tissues. Freeze-fracture studies show that tight junctions develop from a series of rows of particles which form short segments of ridges on one fracture face and a complementary set of grooves on the other. These regions correspond to the focal membrane fusions seen in thin sections and are usually referred to as focal tight junctions. Zonular tight junctions, zonulae occludentes, are produced by extension and rearrangement of these junctional components giving an anastomosing network of ridges and grooves which form a band completely surrounding the cell. The gradual exclusion of lanthanum from the intercellular space during redifferentiation of pigmented epithelial cells in clonal culture suggests that tight junctions probably form in a similar manner in these cells. Freeze-fracture studies of junctional complex development in cells of the clones should allow us to test this hypothesis.

Formation of focal tight junctions (focal membrane fusions) and adherens junctions in the pigmented retinal clone is similar to those described during differentiation of the junctional complex in Fundulus blastoderm. One difference is that during the development of the junctional complex in Fundulus, tight junctions are present before extensive development of the adherens junctions takes place whereas, in the cloned pigmented epithelial cells, focal tight junctions develop simultaneously with the adherens junctions. Detailed studies of the development of the junctional complex in vivo will be required to determine whether this difference is real or is an artifact of culture.

Tight junctions are present prior to the formation of gap junctions during junctional complex development in neurulating amphibian neuroepithelium and during regeneration of the rat liver, where it has been postulated that they may aid gap junction formation by providing either membrane proximity or a scaffolding on which the junctional subunits can be arranged. Focal tight junctions do not appear to be necessary for the formation of gap junctions between cells in culture. It has therefore been suggested that the focal tight junctions are remnants of more extensive pre-existing tight junctions.

In cells of pigmented epithelium clones, gap junctions associated with the apical junctional complex appear to develop in association with tight junctions. In this case the tight junctions are not remnants of pre-existing zonulae occludentes but have developed de novo shortly before development of the gap junctions. This evidence favors the idea that the tight junctions facilitate gap junction formation during differentiation of pigmented epithelial cells in clonal culture. It is still possible, however, that the two elements of the junctional complex develop independently of each other and that their apparent association is coincidental.

Although the major function of tight junctions is thought to be a seal forming a barrier to certain materials, focal membrane fusions such as those found in the outer region of the clones fail to do so. Focal membrane fusions form within the developing adherens junctions at a time when contractile activity is placing a great deal of stress on the junctional complex. It is probable that they also play an important role in cellular adhesion in the re-differentiating cells of the pigmented epithelium.

Relationship to cellular movements. Cellular movements occurring during differentiation of pigmented epithelial cells in clonal culture have been described in detail elsewhere; therefore only a brief description will be given here. Cells which had become separated from the colony moved about over the
surface in the manner described by Middleton. All surfaces of these free cells underwent active movements, and there was no apparent direction to their migration. Cells at the outer edges of the colony showed true ruffled membranes only on their free surfaces, although some undulation of the lateral surfaces was also present in regions of cell contact. Toward the center and in the inner regions of the squamous zone and in the stratified and intermediate zones, groups of cells underwent focal contractions. These consisted of a slow pulling together of 10 to 100 cells about one or a small group of central cells; this was followed by a partial relaxation, the entire sequence of movements requiring roughly 3 min. Central cells often exhibited apical protrusions which extended and retracted. At the beginning of each contraction these extended stiffly from the surface and ceased to move. Movements resumed as relaxation began. Cells in the more central regions of the colony also exhibited extensive retraction of apical protrusions and undulation of lateral membranes. No focal contractions were seen in the well-differentiated cells at the center of the colony nor were they found in cells at the outer edge of the squamous zone.

Since the focal contractions and the changes in apical activity occur simultaneously in small groups of cells within the colony, it is probable that some form of communication exists between them and serves to coordinate this activity. Intercellular electrical communication occurs between cells in both the pigmented and nonpigmented zones of the colony. Over the past decade it has become increasingly apparent that gap junctions are involved in intercellular communication by transmitting both ions and small molecules. It seems likely therefore that the gap junctions provide this communication in pigmented epithelial cells in clonal culture, particularly since they are situated above and sometimes even within the adherens junctions which provide points of attachment for the microfilament bundles responsible for the movements. If communication were the only requirement, one would expect that coordinated activities such as the focal contractions would increase as the surface area involved in gap junctions increased. Initially this appears to be the case, for focal contractions are first noted at the inside edge of the squamous zone and it is in this region that gap junctions are first seen in the apical junctional complexes. Focal contractions increase in number and intensity in the stratified zone in conjunction with an apparent increase in the number of gap junctions. At this point, however, the hypothesis breaks down because, although the number and size of gap junctions found in the intermediate and pigmented epithelial zones of the colony progressively increase, the focal contractions decrease in size and number and eventually cease, a result consistent with that seen in pigmented epithelium in short-term culture where a decrease of cellular movement also occurs concomitantly with the formation of extensive junctional complexes.

Although the focal contractions are no longer in evidence in the differentiated cells in the center of the colony, an apical web of potentially contractile microfilaments is still present. Disruption of this web with cytochalasin B causes relaxation of the apical surface and changes in cell shape. Initially it was thought that these microfilaments acted as passive guy wires to maintain cellular shape. It is possible, however, that this is an active process and that the microfilaments in the apical web of cells in the center of the colony are in a tonically contracted state. If this were the case, it is possible that movement in the form of focal contractions is permitted when there are only a few asymmetrically arrayed gap junctions joining the cells permitting the development of electrical and/or chemical gradients. When the cell is completely surrounded by these junctions, no gradient can exist, and the microfilaments of the apical web are tonically contracted. Studies involving the use of the freeze-etch technique and further TEM of carefully oriented specimens will be required to determine if such an arrangement exists.
I gratefully acknowledge the technical assistance of Mrs. H. Erber and Ms. M. McDonald. I also thank Dr. M. Hollenberg of the University of Calgary Medical School for his encouragement and for the use of the laboratory facilities in which a portion of this work was performed.

REFERENCES


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