INTERACTIONS BETWEEN EPITHELIAL AND FIBROBLAST-LIKE CELLS IN CULTURES DERIVED FROM MONKEY PERIODONTAL LIGAMENT

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SUMMARY

Explants of adult monkey periodontal ligament produced outgrowths containing both fibroblast-like (F) and epithelial-like (E) cells. A number of multilayers of either E or F cells, or both E and F cells were seen. Electron-microscopic examination of mixed multilayers showed that E cells could form either the superficial or deep sheet of cells. Attachments by means of desmosomes between dorsal and ventral surfaces of multilayered E cells were demonstrated. Although the membranes of E and F cells were sometimes closely apposed, no special junctional structures could be detected between them. The E cell and F cell layers were also observed in a relationship, unusual for cell culture, in which the E cell layer was sandwiched between two F cell layers. This unusual relationship may be the result of cells mimicking in vitro their relationship in vivo, since the E cells are derived from the epithelial rests of Malassez which, in vivo, are surrounded by periodontal ligament F cells. When F cells were seeded on to the surface of E cells a significant fraction of them were found to attach, but the spreading of F cells on E cells was a rare event. These observations could be interpreted as being the result of heterogeneity in the F cell populations. A further indication of the heterogeneity of F cell populations was provided by time-lapse studies of colonies of F cells obtained by culturing cell suspensions of porcine periodontal ligament. In this system, it was found that statistically significant differences existed between the different F colonies with respect to their average cellular mobility. These findings suggest that light-microscopical morphological criteria do not adequately define cell types in terms of behavioural or functional characteristics.

INTRODUCTION

There have been many studies on the relationship of fibroblasts to epithelial cells in cell culture (Abercrombie & Middleton, 1960; Di Pasquale & Bell, 1974; Elsdale & Bard, 1974). The general rule that has emerged from such studies is that formulated by Elsdale & Bard (1974), namely that neither mesenchyme nor epithelium can attach or move upon the free surface of an attached epithelium. Pickett, Pitelka, Hamamoto & Misfeldt (1975) have pointed out that a defect in some of these studies involves the combined culture of cells from different sources. This is because it is possible that cultures derived from cells that are close neighbours in vivo may be more tolerant of

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one another than those derived from widely separated tissues. There is another problem in producing rules on cell behaviour. Abercrombie & Middleton (1968) have stated that generalizations can conceal 'what may be considerable variation between different kinds of cells within each of the two broad classes and a considerable variation correlated with developmental state or conditions of observation'. These considerations suggest that observations additional to those that have been reported are required on the relationship between epithelial and connective tissue cells in culture, and particularly on those combinations of cells that are closely apposed in vivo. In this report, we present electron-microscopic and time-lapse cinemicrographic observations made in vitro on periodontal ligament fibroblast-like (F) and epithelial-like (E) cells that demonstrate some unusual features.

MATERIALS AND METHODS

Periodontal ligament was obtained from permanent, fully erupted central and lateral incisors of 3-4 kg macaca Fascicularis monkeys by the method of Marmary, Brunette & Heersche (1976). Strips (roughly 1 mm wide) of attached gingiva were excised from the same animal from the area between the lateral incisor and the 1st molar using a no. 15 blade mounted in a Bard Parker scalpel handle. Care was taken to avoid any areas of clinically-detectable inflammation. Cell suspensions of porcine periodontal ligament, and explants of both periodontal ligament and gingiva were cultured as described by Brunette, Melcher & Moe (1976). Monkey periodontal ligament explants produced outgrowths that were primarily composed of fibroblast-like (F) cells. Trypsinization of these cultures removed the F cells and revealed, in about 10% of the cultures, underlying clusters of epithelial-like (E) cells (Marmary et al. 1976). The terms fibroblasts, fibroblast-like, and epithelial-like are employed as suggested by Fedoroff (1975). Outgrowths of both epithelial and connective tissue cells were also produced by the gingival explants.

Culture conditions

All cultures were grown in Falcon 3002 culture dishes using α-Minimal Essential Medium (α-MEM) (Stanners, Eliceiri & Green, 1971) plus 15% foetal bovine serum (FBS) and antibiotics (penicillin G (Sigma) 100 μg/ml, gentamycin (Sigma) 50 μg/ml and amphotericin B (Calbiochem) 3 μg/ml). Cultures were incubated at 37 °C in a humidified atmosphere of 95% air + 5% CO₂.

Time-lapse cinemicrography

Time-lapse sequences were filmed at the rate of 1 frame every 2 min on Kodak Type 7276 film by means of a 16-mm Bolex camera coupled to an intervalometer (U.N. Scientific, Toronto) and an automatic exposure control unit (Nikon AFM). The cells were cultured in Falcon 3002 culture dish in a plexiglass cabinet that was flushed continuously with 5% CO₂ and located in a warm room (37 °C). Phase-contrast optics were used to observe the cells. Films were analysed with a data analyser projector (L-W 224A, L-W Photo Inc., Van Nuys, California). A simple method of quantitating the motion of individual cells was devised. Film was projected at a magnification of 1000 times on a grid composed of 1.25-in squares. The number of moves per cell per frame of film in a colony was then determined for the time period over which a cell's nucleus was clearly discernible. A move was defined as the complete egress of a nucleus from the square it initially occupied. Only cells at the periphery of the colony were considered. The choice of a particular location in a colony is required because cells at the edge of a colony have less chance of being involved in contact reactions which inhibit mean square displacement per unit time (see Abercrombie, 1976) than those cells that are in the centre of a
colony. The other criterion of selection required that only cells that were in the field for at least 100 frames were included when computing average mobilities.

**Seeding experiments**

F cells were obtained by exposing subcultures of either monkey gingiva or periodontal ligament to a solution containing 0.25% trypsin (Difco, 1:250) in citrate saline (pH 7.8) + 0.1% glucose. The cells were removed from the trypsin solution by centrifugation at 186 g for 10 min and resuspended in α-MEM + 15% FBS. One million of the F cells were added to 60-mm culture dishes that contained E cell outgrowths of not less than 100 cells. These cells were obtained from explants of periodontal ligament or gingiva. The volume of medium in the cultures was 5.0 ml. The combinations of cells used are described in the Results section. Exposure of film was started immediately after the cells were combined.

**Electron microscopy**

At the end of the culture period, areas of interest were identified using phase-contrast optics and marked on the bottom of the culture dish. The medium was then decanted, and the cells processed for electron microscopy in situ. The cells were rinsed with 2.5% glutaraldehyde (TAAB Laboratories, Reading, England) in 0.1 M cacodylate buffer, pH 7.3, at room temperature, and were then fixed for 1 h in the same solution, still at room temperature. Following 3 10-min washes in fresh changes of 0.1 M cacodylate buffer at 4 °C, they were postfixed in 2% osmium tetroxide (B.D.H. Chemicals, Poole, England) in 0.1 M cacodylate buffer, pH 7.3, at 4 °C for 1 h. After a rapid rinse in 0.1 M cacodylate buffer at room temperature, the cells were dehydrated in an ascending series of ethanol to absolute ethanol. Thereafter, equal volumes of absolute ethanol:Araldite/Epon (Voelz & Dworkin, 1962) were added to the dishes and allowed to stand at room temperature for 3 h. This solution was replaced with absolute ethanol:Araldite/Epon (1:2) overnight at room temperature, and then with Araldite/Epon. The dishes containing Araldite/Epon were retained at room temperature for 2 h and then incubated at 60 °C for 3 days. The marks on the dishes designating the areas to be examined were then transferred to the surfaces of the Araldite/Epon and the embedded cells separated from the culture dish by trimming the edge of plastic from the periphery of the culture dish and then manually prizing the dish away from the embedded cells. The marked areas were excised with scissors and then re-embedded in Epon/Araldite so that sections could be cut at right angles to the surface of the cells. Grey-silver sections were cut on an LKB Ultratome using a diamond knife, stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965), mounted on 300-mesh copper grids, and viewed in a Philips EM 300 electron microscope.

**RESULTS**

**Time-lapse studies**

**Cell attachment.** In the seeding experiments, attachment was defined as the ability of a cell to remain behind after 2 cycles of removal of medium by suction followed by addition of fresh medium. Cells meeting this criterion of attachment were also found to move with the underlying cells when the culture dish was tapped gently. The results are shown in Table 1. When the F cells from periodontal ligament were added to E cells from periodontal ligament, it was found that 28% (12 out of the 43 that were filmed) attached to the E cells, while only 4% of periodontal ligament F cells (6 out of 165 that were filmed) attached to the E cells cultured from gingiva. When F cells derived from gingiva were added to E cells from periodontal ligament, 10% (5 out of 50) attached: 28% of gingival F cells (23 out of 82) attached to gingival E cells. We believe the total number of cells used is too low to demonstrate reliably any genuine
differences between the various combinations of cells, but the results do indicate the probability that under the conditions described here, F cells have a greater capacity to attach to E cells when the two are derived from the same tissue.

Table 1. **Attachments of trypsinized F-cells to epithelium**

<table>
<thead>
<tr>
<th>Epithelium source</th>
<th>Fibroblast source</th>
<th>Periodontal ligament</th>
<th>Gingiva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontal ligament</td>
<td>28%</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Gingiva</td>
<td>4%</td>
<td>28%</td>
<td></td>
</tr>
</tbody>
</table>

**Interaction between E and F cells in periodontal ligament explants.** Groups of periodontal ligament F cells were found to move over the periodontal ligament E cells, but the mechanism whereby this was achieved was not clear. In the majority of cases, it appeared that the F cells were attached to each other on their lateral aspects but, in contrast to the behaviour of these cells on a plastic substratum, little ruffling activity at the leading edge of the F cells could be detected. The only exceptions to this generalization were two F cells which were seen to spread on the E cells. These 2 cells were observed to move over the E cells in a manner similar to the movement displayed by F cells on a plastic substratum. One of these cells was a rounded post-mitotic cell (Fig. 1). The E cell clusters were often found at some distance from the explant and were not connected to it by a cord or sheet of epithelium. Hence, although the mechanism whereby these islands of epithelium are formed is not clear, it is unlikely that the spatial relationship of the E and F cells in the explant was maintained during the migratory process.

**Heterogeneity of F cells.** The above observations suggested that the F cells derived from periodontal ligament may not be a homogeneous population because a fraction of the cells attached to epithelium while the majority did not. Further evidence for this hypothesis was obtained by filming colonies of F cells derived from cell suspensions of porcine periodontal ligament, and analysing the rates of motion of individual cells. Table 2 lists the results obtained by studying 6 F colonies. It was found that the average mobilities of the cells at the periphery of different colonies varied. The significance of this variation was assessed via a one-way analysis of variance which is presented in Table 3. The F ratio was found to be significant at the 95% confidence level. No significant difference was found when the average mobility of cells of the same colony was measured on different days. This implies that the variation of cell mobilities is not due to random sampling fluctuations and that there are real differences between the different F colonies with respect to this behavioural characteristic.

**Ultrastructure of cell cultures from monkey periodontal ligament**

**Cell identification.** The ultrastructural criteria used to identify epithelial cells were the presence of bundles of tonofilaments and particularly of desmosomes. F cells were identified as elongated or spindle-shaped cells which lacked desmosomes and
Fig. 1. Sequence from a time-lapse movie showing a rounded post-mitotic F cell spreading on underlying E cells. The elapsed time starting with the first micrograph at $t = 0$ is given in min. $\times 270$. 
Table 2. Average mobilities of cells from different colonies

<table>
<thead>
<tr>
<th>Colony</th>
<th>Average mobility index, moves/frame × 10⁴</th>
<th>S.D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105·1</td>
<td>64·4</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>132·2</td>
<td>43·5</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>105·0</td>
<td>45·3</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>59·9</td>
<td>43·3</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>67·2</td>
<td>55·4</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>49·8</td>
<td>44·0</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3. Analysis of variance of cell mobilities

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between colonies</td>
<td>5</td>
<td>38032</td>
<td>7606</td>
<td>2·899*</td>
</tr>
<tr>
<td>Error</td>
<td>41</td>
<td>107593</td>
<td>2624</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0·05.

Fig. 2. Phase-contrast photomicrograph taken in situ of cells in a primary culture after 35 days. A sheet of epithelial-like (e) cells is covered by a ribbon of cells (c) that appeared in the microscope to be fibroblast-like. x 270.
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tonofilaments but generally contained organelles usually associated with fibroblasts such as Golgi membranes and rough endoplasmic reticulum. Numerous sections through the same cell were examined before identification was made.

Ultrastructure of multilayers. A number of examples of multilayers of either E or F cells, or both E and F cells were seen. In mixed multilayers, E cells were seen to form either the superficial or deep sheet of cells, or to be sandwiched between two layers of F cells. Fig. 2 illustrates a phase-contrast photomicrograph of the area in situ and Fig. 3 is composed of 4 representative montages selected from the 9 that were prepared for examination of the area marked c in Fig. 2. Additional sections of the area were examined in the electron microscope but not photographed. The cells in the figures are numbered so that they can be followed from section to section. In this example, 10 E cells (e1-e10) are covered superficially by a sheet of 3 F cells (c1-c3) and, in turn, lie on a sheet comprising at least 7 F cells (c4-c10). Although a part of an E cell was occasionally seen to project through the F cell sheet to attach to the base of the culture dish (cell e5, Fig. 4), careful examination of these sections has not revealed any processes from the superficial F cells to protrude through the multilayer to the base of the dish. Examples of tonofilaments and desmosomes characterizing the epithelial cells shown in Fig. 3A-D are shown at higher magnification in Figs. 5 and 6. Attachment by means of desmosomes between dorsal and ventral surfaces of multilayered E cells was seen for example between cells, e8 and e9 and e8 and e7 (Figs. 3B and 7).

The membranes of E and F cells were sometimes closely apposed (Fig. 8), being separated by distances that varied between 15 and 22 nm, but no special junctional structures could be detected between them. Some of the spaces between the cells, when narrow, did not contain demonstrable extracellular substance, except for material that appeared to be continuous with the cell coat (Fig. 8). In other spaces, some of them large, extracellular substance containing fibrillar material could be seen (Fig. 9). On occasions, the fibrils exhibited a periodicity of about 16–20 nm (Fig. 9), but this could not be measured accurately as the periods were poorly defined. These fibrils, which probably are collagen, were generally found in the deeper segments of the cell multilayer. In addition, material having an appearance reminiscent of basal lamina was seen occasionally (Fig. 9). Fibrillar extracellular substance and occasional banded fibrils, probably collagen, were also seen between cells of multilayers containing F cells only.

DISCUSSION

The unusual feature seen in cultures of monkey periodontal ligament cells is the formation of complex multilayers of cells containing both E and F cell layers and layers of E cells ‘sandwiched’ between layers of F cells. Multilayering has been observed in E cultures by Pickett et al. (1975) and Flaxman, Lutzner & Van Scott (1967) as well as others but, to the best of our knowledge, the formation of both underlying and superficial F cell layers in relation to a stratum of E cells is unique. A possible explanation of this unusual interrelationship between the F cells and the
Fig. 3A–D. Sections of the cell multilayer in the region marked c in Fig. 2 comprising fibroblast-like cells (c₁–c₉) and epithelial cells (c₁₀–c₃). The sections were cut at right angles to the floor of the dish. The aspect of the culture that was in contact with the dish is represented by the electron-dense line at the bottom of the multilayer (arrow). The cells are numbered so that they can be followed from one electron micrograph to the next. Note the epithelial cells sandwiched between the fibroblast-like cells, and the process (e₈p) of one epithelial cell (e₈) protruding to the floor of the dish. × 4500.
Fig. 4. Higher magnification of the epithelial process (a,b) from cell a, illustrated in Fig. 3a, but taken from a different section to avoid the grid bar. The base of the culture is indicated by an electron-dense line (large arrow) and desmosomes are ringed. Note the microvilli (small arrow). x 12800.
E cells may be the result of the origin of the E cells. Previous work has shown that the E cells cultured from adult periodontal ligament are derived from epithelial rests of Malassez (Brunette et al. 1976). The rests, which are present in adult periodontal ligament but which have no known function, are derived from embryonic dental epithelium and are unique in that they persist in the adult surrounded by connective tissue (Ten Cate, 1972). Hence, our observation that E cells derived from periodontal ligament are surrounded by connective tissue-like cells may be explained by the hypothesis that these E cells exhibited properties in vitro similar to the properties of rest cells in vivo. However, it should also be mentioned that the use of EM sections cut perpendicular to the culture plane (such as were employed in this study) is rare (Pickett et al. 1975) and multilayering of the type reported here may be more common than is generally realized.

Other features of interest in the ultrastructural studies were the presence of extracellular banded (probably collagenous) material and of a structure reminiscent of a lamina densa. The existence of these features suggests that some aspects of tissue differentiation are occurring in these cultures but more detailed observations remain to be made. The finding of fibrils having ultrastructural characteristics suggestive of collagen is not unexpected, as these cells have been shown biochemically to be capable of synthesizing collagen (Limeback, Brunette & Sodek, 1977).

The time-lapse cinemicrographic observations reported here suggest that cells of F morphology cultured from adult periodontal ligament are heterogeneous with respect to cell mobility and apparent capacity to attach to epithelium. That such heterogeneity exists may not be surprising for it is conceivable that one is growing together cells in vitro whose ancestors had different roles in vivo. Moreover, these findings may be interpreted to mean that morphological characteristics at the light-microscope level do not provide reliable criteria for unambiguous cell identification. This interpretation is not new. Willmer (1965) wrote: 'Cells which are morphologically indistinguishable may yet differ in many biochemical and physiological properties just as men in black coats and white collars may be performing widely different functions in the human community.' Hence, it is likely that cells from adult tissues exhibiting morphology consistent with that of F cells may be extremely diverse in other characteristics, such as their capacity to traverse various surfaces. We believe that much of the present controversy (Prop, 1975; Di Pasquale & Bell, 1975; Elsdale & Bard, 1975) on the ability of F cells to attach to and move upon the surface of an attached epithelium may, in part, be caused by the oversimplistic grouping together of apparently similar cells that in fact can exhibit diverse behavioural characteristics.

In so far as our observations bear on the controversy mentioned above, we found that a small proportion of F cells could attach to the underlying E cells, unlike the observations of Elsdale & Bard (1974) and Di Pasquale & Bell (1974). The variation between these observations could have resulted from the modification of the E cell surfaces in our experiments by the secretory products of F cells (such as collagen) or E cells. Alternatively, the difference may have been caused by the different cell types that have been used in the various investigations. As mentioned previously, the cells used by the aforementioned authors were taken from different sources and it is
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possible that cultures derived from cells that are close neighbours in vivo may be more tolerant of one another. Our data are consistent with this proposal. In addition, the cells used in the studies reported by the aforementioned authors included cells from embryonic tissues as well as cell lines such as KB and 3T3. Those model systems may comprise cells that are more homogeneous than the cell populations derived from mature tissues such as those used here. In this regard, Elsdale & Bard (1975) have conceded that Prop (1975) (based on the work of Visser, de Haas, Kox & Prop, 1972.

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Fig. 5. Bundles of tonofilaments (t) from cell e3 shown in Fig. 3B. × 21 000.

Fig. 6. A desmosome between 2 of the epithelial cells in Fig. 3B. × 50 800.

Fig. 7. Higher magnification of an area in Fig. 3B to illustrate desmosomes (arrowed) attaching the ventral surface of e6 to the dorsal surface of e6, and the dorsal surface of e6 to the ventral surface of e5. × 21 000.

Fig. 8A–C. Apposition between membranes of epithelial (e3 or e5) and fibroblast-like cells (c1) marked in Fig. 3B. Note 'fuzz' between plasma membranes. A, B, C, × 64 800, 50 800, and 64 800, respectively.
with cultures derived from mouse mammary glands) may have discovered an exception in a hormone-dependent tissue to the general rule that an attached epithelium is non-adhesive. The cells of the periodontal ligament may be yet another exception to this rule.

REFERENCES


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