CELL ADHESION AND CELL SURFACE TOPOGRAPHY IN AGGREGATES OF 3T3 AND SV40-VIRUS-TRANSFORMED 3T3 CELLS

Visualization of Interior Cells by Scanning Electron Microscopy

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ABSTRACT

A technique for exposing the interior of aggregates of cultured cells has been developed and is described in this report. Using this technique, we have examined for the first time, by scanning electron microscopy, cell morphology and cell contact ultrastructure in the interior of aggregates of BALB/c 3T3 and SV40-transformed 3T3 cells. The 3T3 cells make initial intercellular contact by means of microvillar processes. Over a period of 3–8 h, some of these microvillar contacts are replaced by broader projections. In contrast, the SV40-transformed cells make initial intercellular contact by means of blebs or blunt projections which are also broadened and extended over a period of 3–8 h. For both 3T3 and SV40-3T3 cells, the surfaces of the cells which form the outer layer of the aggregate resemble the surfaces of single cells fixed in suspension, regardless of how long the aggregates have been cultured. These cells are covered with many cellular processes and are roughly hemispherical in profile. The surfaces of the internal cells of the aggregates, however, lose many of their cellular processes, develop smooth patches, and many become irregular in shape. This smooth morphology was also observed on the interior surfaces of the peripheral cell layer. From these observations we conclude that: (*a*) the stabilization of adhesive contacts is a slow process which takes at least 3–8 h; (*b*) the outer surfaces of peripheral cells differ significantly from the surfaces of interior cells; and (*c*) clear differences in surface topography exist between nonmalignant 3T3 cells and their malignant SV40 transformants.

KEY WORDS cell contact · cell adhesion · scanning electron microscopy · cell aggregates · viral transformation · BALB/c 3T3

A considerable interest in recent years has been focused on the nature of cell adhesions at the ultrastructural level. The visualization of cell

shape and cell contact morphology has been greatly aided by the application of scanning electron microscope techniques and sample preparation procedures, such as critical point drying, which minimize cellular artifacts. With these techniques, the surface topography of cells attached to plastic or glass dishes, or free in suspension, has been studied by several laboratories (e.g., references 1, 2, 6, 13, 15, 16, and 18). These studies have provided valuable information primarily on cell-dish adhesions but also on intercellular adhesions. In this report we describe the morphology of intercellular adhesions in the interior of three-dimensional solid masses or aggregates. We believe that, because of the multiplicity of cell-cell contacts and the absence of solid artificial surfaces, studies of cellular aggregates can provide information not obtainable from either conventional suspension cultures or flat cultures. To expose the surfaces of internal cells for observation, we have opened the cell aggregates by a method of blunt dissection.

By correlating our current findings with our previous studies which employed transmission electron microscopy of aggregates and scanning electron microscopy of the exposed peripheral cells of aggregates (10), we have concluded that our dissection procedures do not distort the shapes or surface features of individual cells or alter their relative locations within the aggregate. We have, therefore, used this technique to compare the cell surface morphology of peripheral cells with that of internal cells, and also to examine the time-course of adhesion in aggregates. In addition, we have used this method to examine shape and contact morphology of nontransformed BALB/c 3T3 cells and of their SV40-virus transformants, SVT-2 cells.

MATERIALS AND METHODS

Chemicals and Biochemicals

All chemicals were purchased from commercial sources and were either biological grade, if available, or else the highest grade available. Serum and powdered culture medium were pruchased from Grand Island Biological Co. (Grand Island, N. Y.) and were stored at 4°C (powder) or −20°C (serum).

Cell Lines and Their Growth

The cells used in this study were mouse fibroblast BALB/c 3T3 cells, and SV40-transformed BALB/c 3T3 cells (designated SVT-2), which were originally derived from the A31 clone isolated by Aaronson and Todaro (1). Cells were maintained as frozen stocks and used between the 12th and 18th passages. No mycoplasma contamination was detected by an autoradiographic assay (5, 8). Cells were grown in Eagle's Minimum Essential Medium (MEM) 4× in Earle's salts plus 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The formation of cellular aggregates has previously been described in detail (8, 9). Briefly, cells were removed from subconfluent cultures with EGTA (0.5 mM in Ca^{++}-Mg^{++}-free phosphate-buffered saline, 7 min, 37°C), washed with complete culture medium several times, then centrifuged gently (790 *g*, 5 min) to make a pancake-shaped pellet. The pellet was incubated for varying times (usually 1.5–2 h, but as little as 10 min in experiments examining the time-course of adhesion) at 37°C, and then removed from the centrifuge tube. The pellet was cut up into small aggregate-sized pieces, and maintained in culture medium at 37°C on a gyratory shaker (7).

Preparation of Cells and Aggregates for Scanning Electron Microscopy

Aggregates were rinsed several times in prewarmed culture medium lacking serum and antibiotics and resuspended in the following fixative, which was modified from one originally developed by Karnovsky (12): 2% paraformaldehyde plus 2% glutaraldehyde in MEM 4× culture medium buffered to pH 7.4 and lacking serum (10). After 60 min at room temperature, the aggregates were rinsed three times in phosphate-buffered saline, dehydrated in graded water-ethanol mixtures, then in graded ethanol-Freon 113 mixtures, and then critical point dried with Freon 13 (3). During these transfers and procedures, aggregates were retained in cylindrical chambers consisting of two stainless steel washers, two stainless steel screens, a polyethylene washer, and a Pendaflex (Oxford Pendaflex Corp., Garden City, N. Y.) steel file folder clip (Fig. 1). One or two chambers could be clamped by each clip. After critical point drying, the aggregates were glued to the screens and the screens affixed to aluminum sample stubs as follows: a small dab of carbon conductive cement ("Tube Koat," G. C. Electronics Co., Rockford, Ill.) was placed on

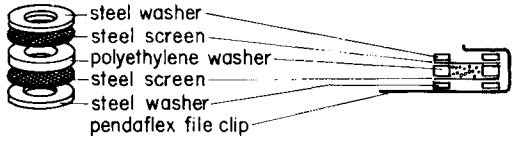

FIGURE 1 Schematic diagram of the chambers used to hold aggregates during processing for scanning electron microscopy. The circles of stainless steel screen and the polyethylene washers were cut from larger pieces by means of an arc punch. The steel washers and clips were purchased locally.

the upper screen. After a 1-s pause to allow the solvent to partially evaporate, the chamber was inverted and gently tapped. The dried aggregates fell on the still tacky cement and were immobilized on the screen. The procedure was then repeated with the opposite screen to immobilize any remaining aggregates. After allowing a few minutes for the solvent to evaporate completely, the chamber was disassembled and the steel screens were glued to SEM sample stubs with the same cement. It is essential in this procedure to minimize the amount of cement used in order to prevent the aggregates from being completely coated by capillary action. This artifact, when present, is granular in appearance and may be incorrectly interpreted as extracellular material of biological origin or even as specific cellular surface features. Therefore, some experience is necessary to allow identification of this potential artifact. In developing these procedures, mock runs were made with 10-μm-diameter polystyrene latex beads (Polysciences Inc., Ft. Washington, Pa.) as samples. These spheres are smooth-surfaced, and their coating with even small amounts of cement can be detected unambiguously. During the development of these procedures, a number of alternative methods was used to immobilize the fixed aggregates: (*a*) dried aggregates were allowed to adhere to double-sided transparent tape (Scotch Brand, 3M Company, St. Paul, Minn.), affixed to aluminum sample stubs, or (*b*) copper conducting tape (SPI Supplies, West Chester, Pa.) glued to sample stubs with the adhesive side facing up, or (*c*) sample stubs previously sprayed with an aerosol adhesive (Scotch Brand Photo Mount Spray Adhesive). (*d*) Aggregates suspended in fixative were also attached to polylysine-coated cover slips by the same procedure described below for single cells. The cover slips were then dehydrated and critical point dried, then mounted on sample stubs. None of these four alternative methods immobilized aggregates as successfully as the method utilizing carbon conductive cement, since they resulted in greater loss of aggregates during processing and charging in the electron beam. However, no differences were noted in the appearance of the peripheral cells of aggregates immobilized by any method used. We have, therefore, concluded that the carbon conductive cement routinely employed did not introduce any artifacts.

We also considered the possibility that apparent differences in cell contact morphology might be the result of fixation artifacts. That is, differential rates of penetration of fixative into the aggregate interior under various conditions might allow partial withdrawal or rearrangement of cellular processes. To test this possibility, we prepared a population of aggregates of 3T3 cells, and, after 24 h of culture, half of them were fixed as described above. The remaining aggregates were torn apart, without fixation, and the fixative was immediately (within 1 s) applied to the exposed surfaces. Both groups were then prepared for scanning microscopy as described. The intact group was, after coating, subjected to blunt dissection as described. As expected, opening the aggregates before fixation resulted in significant rearrangement of the cells and some breakage of cellular processes. However, a comparison of the two groups indicated that the number, type, and arrangement of cellular processes (microvilli) were the same in the interior of the two groups of 3T3 aggregates.

In addition, we have observed no differences with respect to surface morphology of internal cells, regardless of location within the aggregates, including cells just inside the periphery. Furthermore, the size of the aggregates used had no effect on the morphology of the internal cells. We have therefore concluded that differential penetration of fixative could not explain the differences observed between exterior and interior cells of aggregates.

Single cells fixed in suspension were rinsed twice with prewarmed culture medium lacking serum and antibiotics, then fixed for 60 min at room temperature in the fixative described. Drops of the fixed cell suspension were placed on glass cover slips coated with polylysine (14) (30 min, 1 mg/ml in water) and allowed to sit in humidified chambers overnight. The cover slips were rinsed in buffered saline and processed for scanning electron microscopy as described for aggregates, except that the cover slips were retained in specially constructed carriers instead of stainless steel screen chambers.

Immediately before examination, the samples were coated with gold-palladium in a Hummer sputter coater (Technics Corp., Alexandria, Va.) three to seven times at 1 min per coating at a current of 10 mA, with at least 3 min between coatings for the samples to cool. Samples were examined in a Cambridge Stereoscan S4-10 scanning electron microscope, operating at 20 kV. 20–50 aggregates were examined in each preparation, and at least three separate preparations were made for each type of treatment reported here.

The density of microvilli on 3T3 cells was quantitated from photographs. Prints at a final magnification of 2,000–10,000 were made and areas were randomly selected for measurement, except that only fields which lay in approximately the plane of the photograph were used. These areas of minimum curvature were overlaid with a 1-μm^2 transparent grid and the number of microvilli per square micron was determined. Data in the text are given as mean ± SD. Because processes on SVT-2 cells were very variable in size and shape, quantitation was not attempted.

Blunt Dissection of Aggregates

Dried aggregates were glued to screens, coated with gold-palladium, and examined in the scanning electron microscope before they were opened. Although dried, non-coated aggregates could also be broken open, they usually disintegrated into a number of fragments rather than two pieces. The metallic coating tended to protect the surface cells from mechanical damage and also

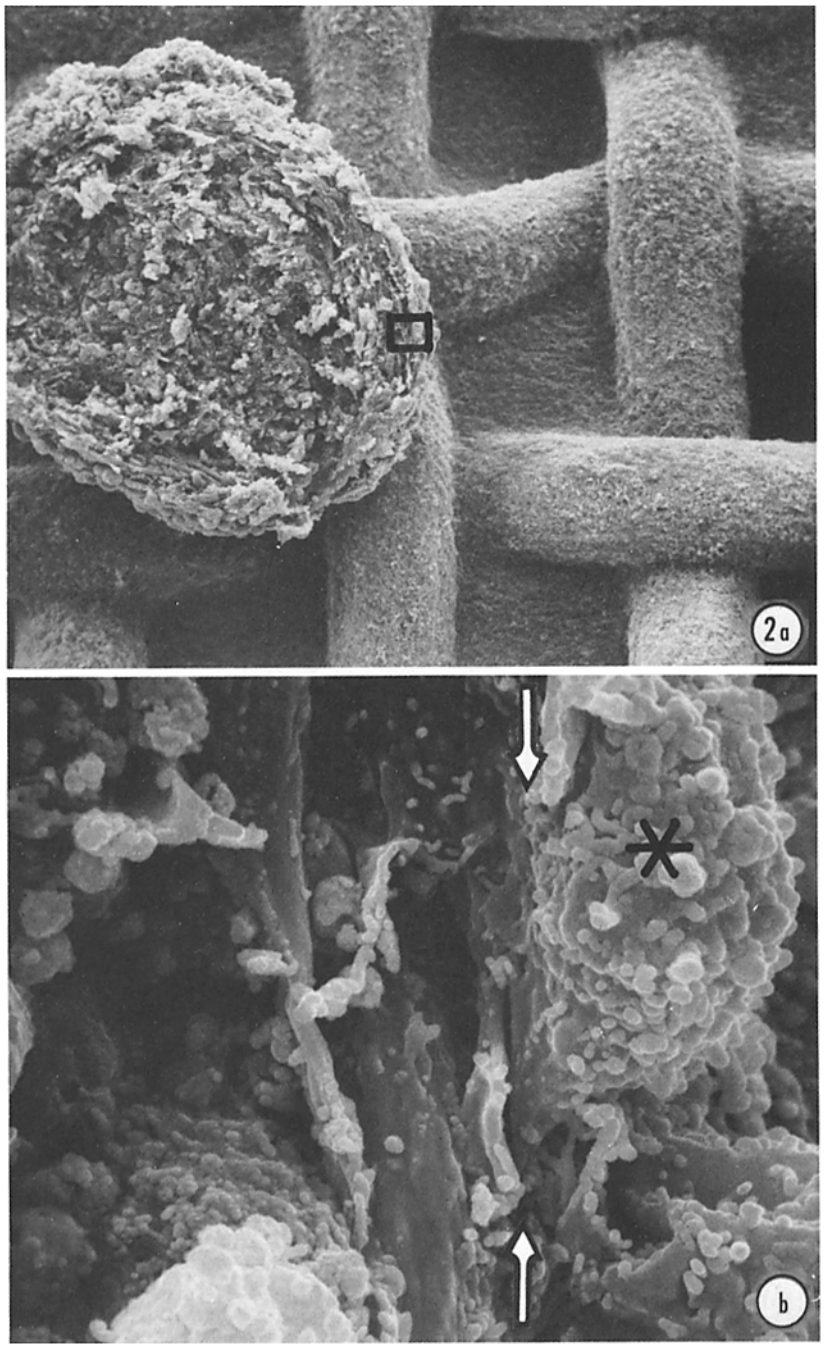

FIGURE 2 (*a*) Aggregate of SVT-2 cells, critical point dried then fractured by blunt dissection. The broken aggregate was cemented to a stainless steel screen as described in Materials and Methods. This photograph is an *en face* view of the exposed interior cells of the aggregate. The inscribed rectangle indicates the area shown in Fig. 2*b*. × 260. (*b*) A higher magnification view of the same aggregate shown in Fig. 2*a*. The fracture plane is indicated by arrows. The cells to the right of the arrows are on the surface, the cells to the left of the arrows are on the interior. Note the decrease in surface projections on the interior cells as compared to the surface cells. For one peripheral cell, indicated by an asterisk, both the surface exposed to the bulk medium and the surface exposed to the interior can be seen. Note the relative lack of surface processes on the interior surface of this cell relative to the exterior surface. × 6,500.

appeared to insure a clean fracture with minimum disturbance of the internal cells. The dissection, or fracturing, was done under a stereo dissecting microscope at × 10–50 magnification by means of microscalpels (Roboz Instruments, Washington, D. C.). Although two micromanipulators were used in the early stages of this work, they were discarded in favor of direct manual dissection. After the aggregates were fractured, the loose piece, generally a hemisphere, was reglued to the screen. If the complementary hemisphere had loosened, it was also reglued. The identification of the exposed surfaces was facilitated by the absence of deposited metal. Metal-coated surfaces were dark metallic grey, whereas newly exposed surfaces were nonreflective pale yellow. The samples were then recoated several times (described above) before examination in the scanning electron microscope.

A low magnification scanning electron micrograph of a single fractured SVT-2 aggregate on a stainless steel screen is shown in Fig. 2*a*. The same aggregate at higher magnification is shown in Fig. 2*b*. An equivalent fractured 3T3 aggregate is shown in Fig. 2*c*.

RESULTS

Comparison of Peripheral vs. Interior Cells

Observation of peripheral cells of aggregates of BALB/c 3T3, and SV40-transformed 3T3 (SVT-2) cells in aggregates cultured for 24 h, were consistent with previous preliminary observations (10) made with conventional techniques. The external surfaces of 3T3 cells were rounded and were covered with large numbers of projections 0.1 μm in diameter and 1.0–5.0 μm in length. We have chosen in this report to refer to these projections as microvilli, to be consistent with the terminology used in previous reports from other laboratories (e.g., references 4 and 16) as well as our own (10). It should be noted, however, that similar projections are also referred to as filopodia by other workers. Our criteria for the identification of a cellular process as a microvillus were a diameter of about 0.1 μm, a constant diameter over the entire length of the process, and an axial ratio of at least 2. Cells appeared to make contact with one another by means of these microvillar projections. Peripheral cells were often separated from one another by 0.5–1.0-μm gaps across which the microvilli extended. A view of several such peripheral 3T3 cells is shown in Fig. 3*a*. There were two important differences between interior 3T3 and peripheral 3T3 cells. First, the interior cells had considerably fewer processes than the peripheral cells. The density of microvilli

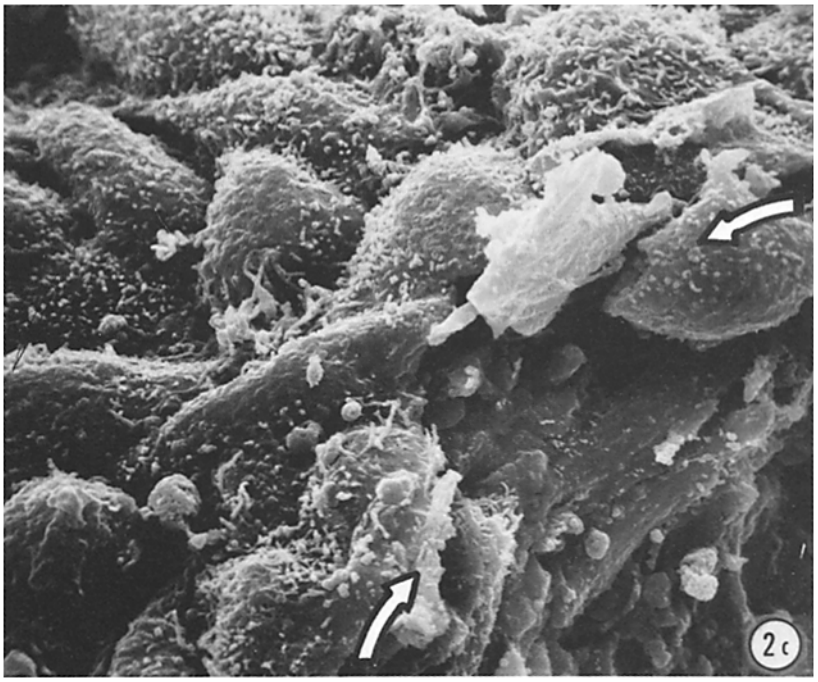

FIGURE 2*c* Portion of an aggregate of 3T3 cells fractured by blunt dissection. The fracture plane is curved and is indicated by two arrows. Note the large number of microvilli on the exposed surface of the exterior cells and their relative paucity on the surfaces of the internal cells. × 2,500.

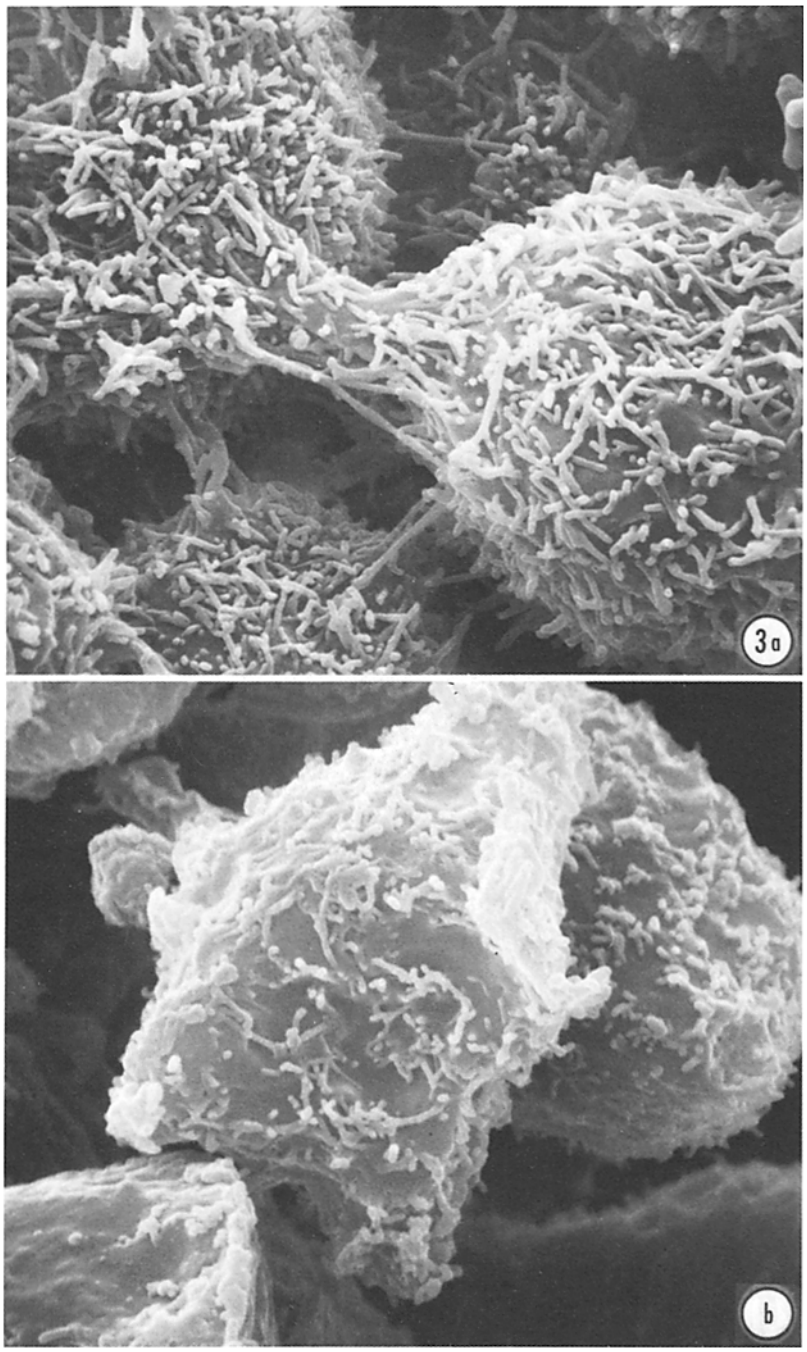

FIGURE 3 Aggregates of 3T3 cells formed by pelleting and then cultured for 24 h. (*a*) Several cells on the surface of an aggregate. Note that large numbers of microvilli are present and that cells are making contact by means of these microvilli, as well as by means of a few broader extensions. (*b*) Cells in the interior of an aggregate. Many of the cells are more irregular in shape than the exterior cells and have fewer surface projections. Both photographs × 6,800.

on peripheral 3T3 cells was 6.34 ± 1.40 per μm^2 (n = 49 fields), while on internal cells the density was 3.40 ± 1.10 per μm^2 (n = 25 fields). Although the density of microvilli was decreased, they were still present on interior cells and were a primary means of contact with neighboring 3T3 cells. A micrograph of 3T3 cells in the interior of an aggregate cultured for 24 h is shown in Fig. 3*b*. The second difference between peripheral and interior 3T3 cells was their overall shape. The portions of the peripheral cells exposed to the medium were hemispherical, while the cells in the interior were often more irregular in shape and tended to conform to the shape of neighboring cells.

Aggregates of SVT-2 cells which were cultured for 24 h also displayed differences between peripheral and interior cells. The peripheral cells were rounded, fairly tightly attached to their neighbors, and devoid of microvilli. In contrast to the peripheral cells of 3T3 aggregates, the peripheral SVT-2 cells were covered with short, blunt processes (blebs or pseudopodia) by which they appeared to make contact with one another. A micrograph of some cells on the periphery of an SVT-2 aggregate is shown in Fig. 4*a*. The interior cells of SVT-2 aggregates had fewer processes than did the peripheral cells, and displayed extensive regions that were quite smooth and free of cellular processes. These interior cells also tended to be more irregular in shape, and conformed more to the shapes of their neighbors. A view of some SVT-2 cells in the interior of an aggregate is shown in Fig. 4*b*. Both the decrease in number of cellular processes and the heteromorphic character of interior cells relative to peripheral cells were much more pronounced in SVT-2 aggregates as compared to 3T3 aggregates (compare Figs. 3*b* and 4*b*).

Time-Course of Intercellular Adhesion

In the course of this study, we noted that the cells on the periphery of aggregates resembled cells freshly removed from dishes and suspended in culture medium, and that they retained this resemblance for at least several days. In contrast, cells in the interior had modified cell morphologies, presumably as a result of cell-cell adhesions. We, therefore, examined the time-course of this modification in the interior of aggregates.

Aggregates of 3T3 and of SVT-2 cells were prepared as described and fixed 10 min, 3 h, 8 h, and 24 h after centrifugation. For all the timepoints except 10 min, the pellets were cut up with microscalpels 1 h after centrifugation and incubated in culture medium as described until they were fixed, then critical point dried and opened. In the case of the samples fixed after 10 min, the entire pellet was fixed and subsequently dehydrated, critical point dried, and opened. Micrographs of cells in the interior of 3T3 aggregates fixed at various times and of a 3T3 cell fixed in suspension before centrifugation are shown in Fig. 5*a–d*. 3T3 cells fixed 10 min after cell contacts were initiated by centrifugation (Fig. 5*b*) appeared very similar in appearance to 3T3 cells fixed in suspension (Fig. 5*a*). Both were spherical and covered with a forest of microvilli of similar density. The density of microvilli on cells fixed in suspension was 8.89 ± 1.74 per μm^2 (n = 18 fields). In cells fixed in pellets after 10 min, the density of microvilli was 6.89 ± 1.39 per μm^2 (n = 28 fields). Large gaps of up to 1 μm were present between cells, and numerous microvilli extended into these gaps and interconnected the cells. No broad, protruding contacts or extensive areas of cell-cell apposition were visible. By 3 h after aggregation (Fig. 5*c*), the density of microvilli on interior 3T3 cells had decreased (3.67 ± 1.20 per μm^2; n = 24 fields). Prominent gaps between cells were visible, and intercellular contact was still exclusively by means of microvilli. Most interior cells were still spherical, but a few cells were elongated and appeared to be conforming to one another in shape. Aggregates of 3T3 cells cultured for 8 h before fixation were still covered with microvilli, but the microvilli tended to be shorter and the cells also displayed some broader processes (Fig. 5*d*). The density of microvilli after 8 h was 3.80 ± 0.76 per μm^2 (n = 25 fields), approximately the same as at 3 and 24 h. Cell-cell contacts after 8 h were by means of microvilli, and also by these broader extensions. The gaps between cells were somewhat decreased. Some of the internal cells were still approximately spherical, although many irregular or elongated cells were also present (Fig. 5*d*). No further changes in cell contact morphology were noted between 8 and 24 h of culture in aggregates of 3T3 cells. Fig. 3*b* is an example of 3T3 cells in the interior of an aggregate cultured for 24 h.

The time-course of adhesion of SVT-2 cells in the interior of aggregates was also examined. Like

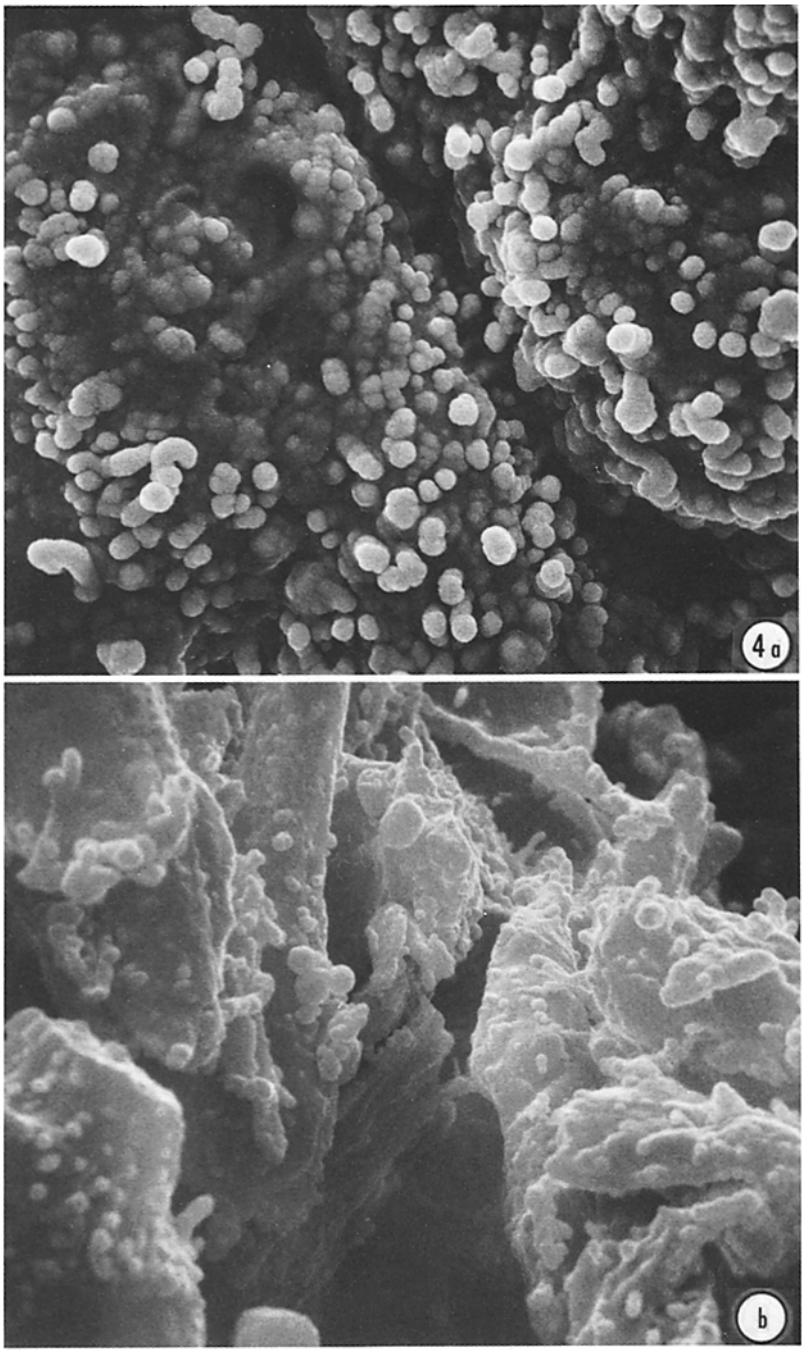

FIGURE 4 Aggregates of SVT-2 cells cultured for 24 h. (*a*) Two cells on the surface of an aggregate. The cells are covered with numerous short, broad projections. (*b*) Several cells in the interior of an aggregate. Note that the cells are more irregular in shape and have fewer projections than do the surface cells. Both photographs × 6,800.

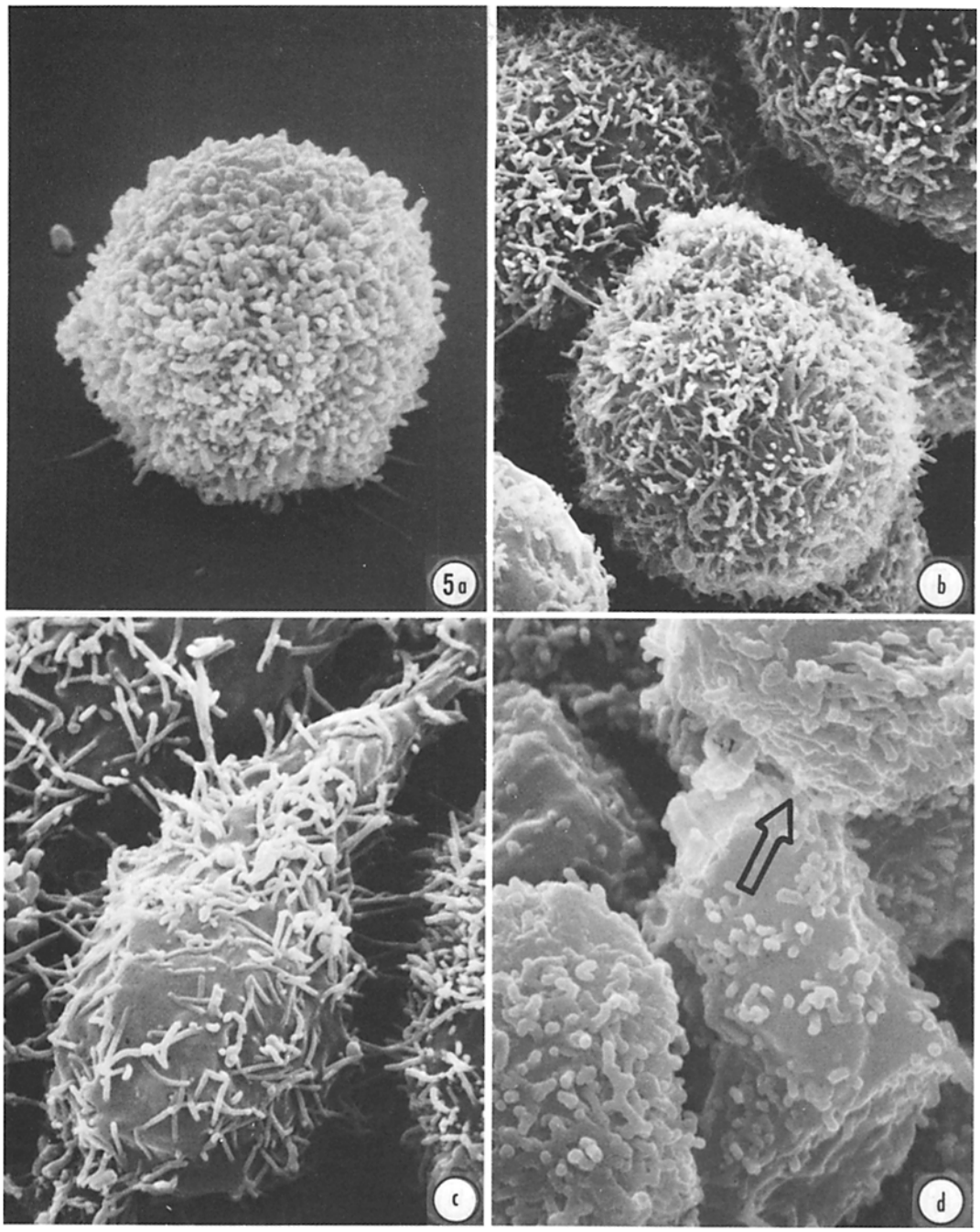

FIGURE 5 Time-course of intercellular adhesion in aggregates of 3T3 cells (*a*) A 3T3 cell fixed in suspension. The cell is spherical and covered with microvilli. The attachment of the cell to the substratum, polylysine-coated glass, with glutaraldehyde is nonspecific and occurred after fixation. (*b*) Several cells in the interior of an aggregate fixed 10 min after the cells were centrifuged into contact. Cells are spherical and covered with microvilli. Cells are in contact by means of these microvilli. (*c*) Cells in the aggregate interior 3 h after centrifugation. The cells are covered with microvilli but are not so spherical as those shown in Fig. 5*b*. Intercellular contact is still by means of microvilli. (*d*) Cells in the aggregate interior 8 h after centrifugation. Cells bear numerous microvilli, but also some broader cell processes. Intercellular contact is by means of microvilli, but some broader areas of contact are also present (arrow). All photographs × 6,200.

3T3 cells, SVT-2 cells in the interior of aggregates underwent a progressive series of morphological changes with time (Fig. 6*a–d*). Cells fixed in suspension, before centrifugation, were spherical and covered with short, broad extensions and blebs. Cells in the interior of SVT-2 aggregates fixed after 10 min were still roughly spherical. They made contact with one another by means of

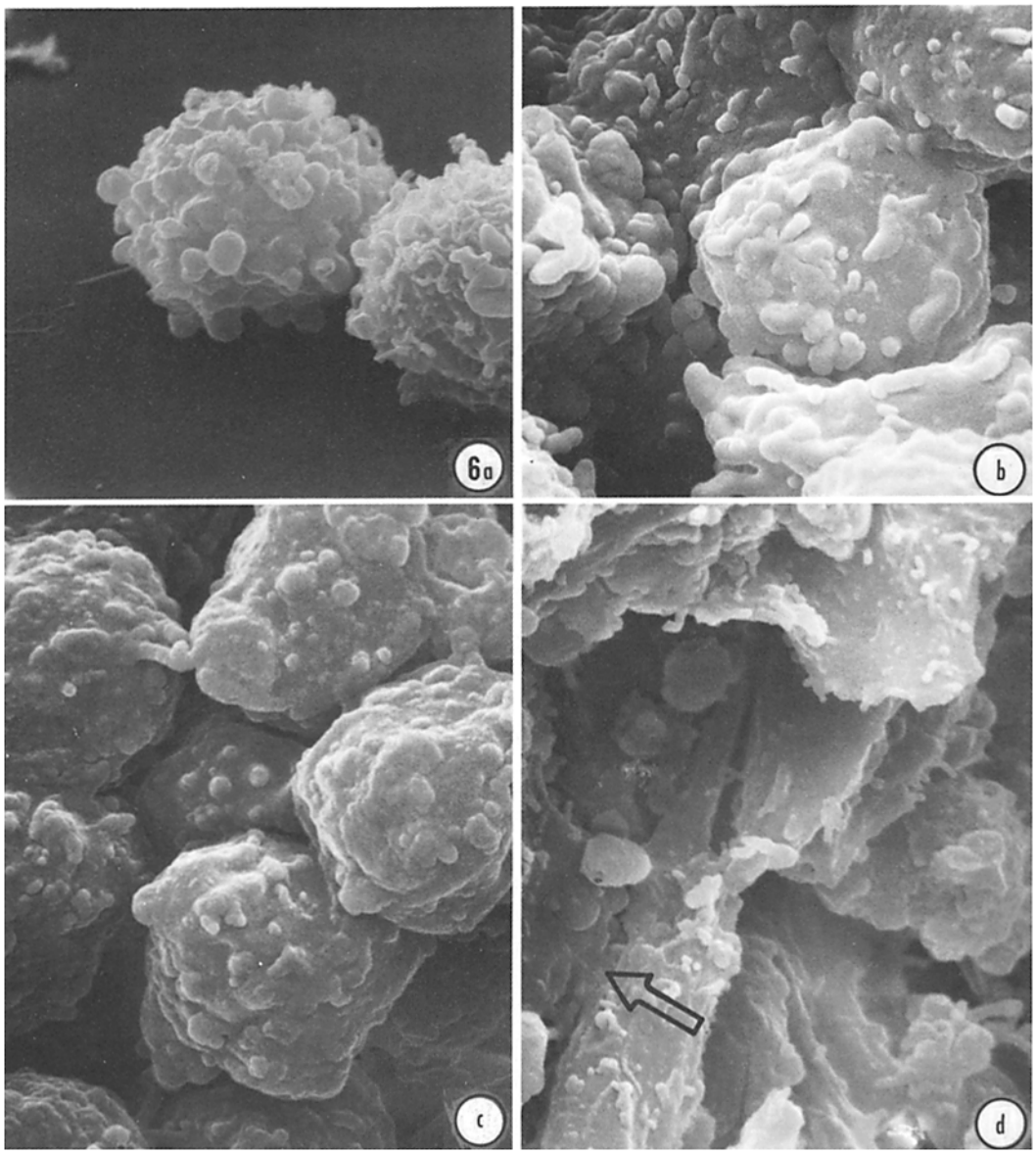

FIGURE 6 Time-course of intercellular adhesion in aggregates of SVT-2 cells. (*a*) An SVT-2 cell fixed in suspension. The cell is spherical and covered with blebs and short, broad protrusions. (*b*) Cells in the interior of an aggregate of SVT-2 cells fixed 10 min after being centrifuged into contact. Cells are spherical and covered with short, broad processes. Even at this time, intercellular contact is extensive. (*c*) Several cells in the interior of an aggregate fixed 3 h after centrifugation. Cells are still roughly spherical, and extensive areas of intercellular contact are present. (*d*) Cells in the interior of an aggregate fixed 8 h after centrifugation. Cells have fewer processes, and some have assumed very nonspherical shapes. Contact areas are still extensive (arrow). All photographs × 6,200.

broad extensions, often over large areas of their surface. Even at this early time, therefore, the area of contact between SVT-2 cells was extensive, as compared to the 3T3 cells. A micrograph of an SVT-2 cell fixed in suspension and a micrograph of a cell in the interior of an SVT-2 aggregate fixed 10 min after centrifugation are shown in Fig. 6*a* and *b*. By 3 h, the cells were still largely spherical and the cell contacts still extensive (Fig. 6*c*). Overall, little change in cell shape or cell surface features was seen during the first 3 h of contact. SVT-2 aggregates fixed after 8 h (Fig. 6*d*) showed somewhat more heteromorphism, with a significant number of elongated or flattened cells. Some short filopodial processes were present, but these were fairly uncommon and of a larger diameter (0.20–0.28 μm) than the 0.1-μm diameter processes referred to as microvilli here and elsewhere (6, 10, 13, 15, 16). By 8 h after the initiation of adhesion, there was a noticeable increase in the extent to which cells assumed irregular shapes (Fig. 6*d*). As with 3T3 aggregates, there were no significant changes in cell contact morphology between 8 and 24 h, although the gradual changes in cell shape did not stabilize until about 48 h after the initiation of cell adhesions.

DISCUSSION

Time-Course of Intercellular Adhesion

Despite considerable interest, the ultrastructure of intercellular adhesions has generally proven recalcitrant to study. Use of the techniques reported here has avoided several of the problems that hindered previous studies. Specifically, initiation of intercellular adhesions within the population has been synchronized to within 5 min. This was accomplished by centrifuging cells into contact with one another (8, 10, 20). While several previous studies have examined aggregating cells by scanning electron microscopy (2, 19), the initiation of intercellular adhesions in these studies was continuous over an extended period as aggregation continued, and it was impossible to distinguish between early and late adhesions. In addition, previous studies utilized either transmission electron microscopy or, alternatively, scanning electron microscopy, but were limited to examination of the surface cells of aggregates. While transmission electron microscopy has been very effective in the study of cell junctions, scanning electron microscopy has proven to be much more useful for examining overall cell shape and cell surface morphology. In this study, we have used scanning electron microscopy for the first time to examine the interior of cellular aggregates. The blunt dissection procedure, which has been previously used to advantage (21) in a study of *Drosophila* oocytes, complements a recent procedure reported by Vial and Porter (22) for examining the shapes of individual cells in isolation.

Using these blunt dissection techniques, we have made the following observations: first, the surfaces of cells exposed to the bulk medium (the peripheral cells of the aggregates) display many more surface projections than do the cell surfaces that face, or are in contact with, other cells in the interior of the aggregate. We have noted a striking similarity between the surfaces of cells fixed in suspension and the exposed surfaces of the peripheral layer of the same cells in aggregates. In contrast, the interior cells of aggregates were very different from both peripheral cells of aggregates and cells fixed in suspension. Although it is likely that this modification of the cell surface in aggregate interiors is mediated by cell contact, it is not possible from our observations to eliminate a short-range, chemically mediated effect. These observations indicate, however, that cells have the ability to polarize their morphology in response to an external stimulus. These data are, therefore, analogous to those of Revel and Wolken (17), which indicated that the surfaces of cells attached to plastic differed from their upper surfaces, which were exposed to the bulk medium.

Second, cells alter their overall shape and surface morphology only relatively slowly in response to intercellular adhesion. We have observed that changes in cell shape take at least 3–8 h to stabilize. In contrast, previous observations of adhesions between two cells colliding on dishes (11) indicate that formation of cell junctions may occur within seconds. Change of cell shape may, therefore, require synthetic processes or complex molecular rearrangements (e.g., polymerization of microtubules or microfilaments) not required to establish initial cell-cell adhesions. These changes in cell shape could result from an active internal process, such as rearrangement of a cytoskeletal system. Alternatively, they could be the secondary result of externally imposed forces, such as local changes in adhesive interactions, variations in cell density, or cell packing arrangements. Our observations do not permit us to distinguish among these possibilities.

Third, our observations indicate that, for the pair of normal/transformed cell lines examined, clear differences exist in cell contact ultrastructure. The nonmalignant 3T3 cells made initial contact by means of numerous microvilli. Over a period of 8–24 h, many of these microvillar contacts were converted to, or replaced by, broader areas of cell-cell apposition. In contrast, transformed SVT-2 cells displayed extensive intercellular contact within 10 min of the initiation of adhesions. These large contact areas were also increased over the first 8–24 h of aggregate culture. These data are consistent with observations of aggregates of BALB/c 3T3 and SVT-2 cells made by transmission electron microscopy (10).

Evaluation of the Fracture Technique

Two potentially serious artifacts were envisioned as the result of physical manipulation of cell aggregates. First, the fracture lines might run through the cytoplasm, exposing nuclei and other subcellular organelles and cytoplasmic debris. These internal components might, after coating, be mistaken for surface features. Second, the fracture plane might occur between cells, but the geographic relationships between the cells might be altered. For example, gaps between cells might be produced or enlarged artificially. The first artifact would introduce errors into the visualization of surface topography, while the second would result in errors in the visualization of cell contact ultrastructure.

Examination of several hundred fractured aggregates has provided no evidence that the fracture plane passes through the cell body rather than between cells. Although occasional debris was seen, it was nearly exclusively associated with aggregates that had been in culture for more than 3 days. Transmission electron microscopy of parallel aggregates (10) has indicated that this apparent debris is actually necrotic cells, or highly vacuolized cellular processes, and therefore not a result of the blunt dissection technique. In any case, the vast majority of fields did not show such debris. In addition, the surface features seen here by scanning electron microscopy of 3T3 and SVT-2 cells in aggregates are consistent with those previously observed by transmission electron microscopy of the same samples (10): numerous microvilli on 3T3 cells, and broad, short processes on SVT-2 cells. Thus, no evidence was found for distortion of surface features or of cell shape.

As an independent method of exposing internal cells, some aggregates from several different experiments were fixed as described, then placed in 70% ethanol overnight. The aggregates were then cut into hemispheres by means of microscalpels, and prepared for scanning electron microscopy as described. The cut edges of these hemispheres were compared to those of fractured aggregates. No differences in surface features of cells in these two kinds of preparations were seen, and both are consistent with previous transmission electron microscope data. For these reasons, we have concluded that the surface structures of internal aggregate cells as visualized by the technique described here are generally unaffected by the fracturing process.

The spatial arrangements of internal cells with respect to one another also appear to be unaffected by blunt dissection. The evidence for this is threefold. First, recovery of both hemispheres of an aggregate revealed a jigsaw puzzle-like fit of the two fragments, suggesting little or no cell displacement. Second, broken cellular processes that might indicate rearrangement of cells after fixation were not seen in these samples. Third, the size of the intercellular spaces in fractured aggregates and aggregates visualized by conventional transmission electron microscopy appeared to be the same: 0.5–1.0 μm for 3T3 cells, and 0.1–0.3 μm for SVT-2 cells in aggregates.

We have, therefore, concluded that the fracturing technique described does not appear to change cell shape, surface features such as microvilli, or the spatial arrangements of cells in aggregates. The conclusions that we have drawn here concerning the time-course and morphology of cell adhesions are specific for cells of the 3T3 and SVT-2 cell lines. However, we believe that similar studies by scanning electron microscopy of other cell types in aggregates, and perhaps of solid tissues, are feasible and would be potentially valuable additions to our understanding of cell adhesion.

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