Cell division and cellular morphology of the chick retinal pigmented epithelial cells in culture

A time-lapse analysis

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ABSTRACT

Objective: To investigate the patterns of cell division, movement and shape during early stages of development of the chick embryo retinal pigmented epithelial (RPE) cells and to evaluate the morphology of dissociated embryonic cells with regard to their proliferation capacity.

Methods: We conducted this study at the Department of Histology and Embryology, Celal Bayar University, Manisa, Turkey, between 2002 and 2003. We isolated the cells from chick embryos. We analyzed the images of the embryonic cells originated from neuroepithelia using a computer-based time-lapse acquisition system attached to a differential interference contrast microscope.

Results: Retinal pigmented epithelial cells, despite being dissociated, depict a colony-type growth. Cells in the periphery of the colony and those outside the colony showed a tendency to proliferate and migrate and retained contact with the neighboring cells during division. Characteristics of cytokinesis were separation from the neighboring cell while retaining an attachment point, became rounded, moved up and started to shake and ascend to disseminate to the substrate to complete the division. The round-up stage was non-significantly shorter when the cell was closer to the center of the colony. Cells that were in the periphery of, or outside the colony had a round-up time of over one hour while cytokinesis-to-adhesion time was around 5 minutes. However, when we found the cells in the center of the colony, the times were half-an-hour and 1.5 hours for the daughter cells, a 2-fold difference between daughter cells with regard to the duration of attachment.

Conclusion: Cell division, migration and proliferation are complex procedures influenced by growth factors, cell adhesion, matrix molecules underneath and the signal mechanisms and can be studied in detail using time-lapse microscopy, immunohistochemistry and confocal microscopy.

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We know about the development itself compared with the details of cell behavior during development. Embryonic retinal pigmented epithelial (RPE) cells first appear then differentiate through mechanisms involving adhesion, proliferation, migration and apoptosis, under the

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influence of cell-cell adhesion molecules, extracellular matrix proteins, growth factors and signal transduction. The ultimate goal of the cell is to become perfect to its function by being at the right place at the right time, with the most appropriate number of cells. Previous studies used a chick in research on cell behavior during the developmental process in a short time and low cost.[1,2] Time-lapse video microscopy as a histological tool is a simple technique which allows us to examine the cell behavior in culture without time limitation. Previous studies used this modern experimental tool for easy research on molecular or protein levels.[3,4] Information obtained using this tool provided important clues to elucidate cell behavior in vivo. In this study, we investigate the developing RPE cells in culture for their morphology during proliferation to explain the relationship between cell behavior and structural organization. Initiation of the proliferation depends upon multiple factors. Factors that affect the cell cycle include cyclin D1, age, energy of cell, colony formation capacity, conformational changes on the chromatin, contact between the cells as well as the cell-substrate relationship. We determined the adhesive cells that spread in the substrate that does not proliferate or produce blebs. While a strong contact, usually stimulates cell proliferation, capable to inhibit proliferation.[5-7] Similarly, Gibbins[8] shows the relationship of lamellipodia to the proliferation. Understanding of the relationship among migration, adhesion, proliferation and differentiation help us to understand the organization of the cells in the tissues, and where and when they should be present during development.[10,11] We investigated the proliferation capacity of the embryonic cells, cells capable of forming colonies and the relationship between the cell and the colony using the time-lapse differential interference contrast (DIC) microscopy.

Methods. The study was conducted at the Department of Histology and Embryology, Celal Bayar University, Manisa, Turkey, between 2002 and 2003. Fertilized eggs from a chick were incubated for 3 days in a humidified, forced-draft air incubator at 38°C.[12] The isolation and culture of chick's RPE cells and their characterization is a routine procedure in many laboratories and has been well documented. Eyes from chick embryos were enucleated, and then a scleral incision was made posterior to the limbus using sterile technique. The incision was extended circumferentially with fine scissors to remove the anterior portion of the eye. The eye remnants were placed in serum-free medium, and further dissection was carried out under a Zeiss dissecting microscope. The vitreous and the neural retina were gently teased with fine forceps from the posterior of the eye and discarded. Eyecups with exposed RPE cells were incubated for 30 minutes at 37°C in a sterile 0.1 % (w/v) solution of dispase (GibcoBRL, Life Technologies, UK). Pieces of RPE cells sheets were carefully dissected from their underlying basement membrane, washed in phosphate buffered saline (PBS) and dissociated into a single cell suspension by a short (2-3 minutes) treatment with 1 ml 0.25% (w/v) trypsin/0.53 mM tetra-sodium ethylenediaminetetraacetic acid (EDTA, GibcoBRL, Life Technologies, UK) in PBS, accompanied by gentle agitation using a pasteur pipette. Examination under the inverted phase microscope showed that the dissociation procedure produced mostly isolated cells but aggregates of <10 cells were found. These aggregates were identified as connected small circle cells instead of small explants after dissociation. The trypsinization was then inhibited by adding 100 µl undiluted foetal calf serum (FCS), (GibcoBRL, Life Technologies, UK) and the cells were pelleted and resuspended in fresh medium. Cells were plated in 2 ml of medium in 35-mm tissue culture plastic dishes (Falcon, UK). Culture medium consisted of a-minimal essential medium (a-MEM, GibcoBRL, Life Technologies, UK) containing 10% (v/v) FCS (GibcoBRL, Life Technologies, UK), 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Fungizone, and supplemented with fresh 2 mM L-glutamine (all GibcoBRL, Life Technologies, UK). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.[12-15] Time-lapse filming has enormous advantages for following the behavior of single RPE cell. For time-lapse video microscopy, primary cultures of chick RPE cells were seeded (100,000 cells/per flask) into tissue culture flasks (Sterilin, UK) and allowed to attach for 4 hours at 4°C. Non-attached cells were removed by pipetting off the old medium from flasks, which were then completely filled with growth medium buffered with 20 mM 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES). The flasks were placed in the warm stage of a Nikon inverted microscope with phase-contrast optics (Nikon, Japan). The behaviors of the attached cells were filmed with a video camera (Hitachi KP40 Solid State, Hitachi, Japan) attached to a time-lapse video cassette recorder (Panasonic SVHS, Matsui Industrial Co. Ltd., Japan) at a speed which expanded at 3-hours to 480 hours. Cells in a single field were observed on a monochrome monitor (Hitachi, Japan) for a period of ≤10 days and the videotape (SVHS Professional, Fuji, 180E) was replayed at 160 times at normal speed so that the real time which is 24 hours could be condensed into 9 minutes. The behaviors of the cells were divided into stages and photographic stills of each stage were taken directly from the monitor screen and transferred to the computer.[3,4,11,16]

Results. Colonies in HEPES buffered medium were formed after 3 days. It was observed that the

cells had a tendency to produce colonies and one of these colonies in the microscope field was observed. Cells in the periphery of the colony were more migrative and proliferative compared to the cells to the center of the colony. Morphology of the cells in periphery, midline and the center of the colonies were also different which undifferentiated, well-spread peripheral cells as well as in the differentiated, polygonally packed central cells of the colony. Two neighboring cells undergoing division in the periphery of the colony were observed. Before the beginning of the proliferation, cells were in the resting phase and were completely surrounded by the neighboring cells. There were cells that were spreading or migrating as well as dead cells around (**Figure 1**). Proliferation of the cells started with disconnection from the other cells by retracting themselves, but they retain an attachment with another cell on one side and became rounded. Cells moved up and started to shake, but it still kept its connection to the other cell (**Figure 2**). In the meantime, the cells started to ascend to the substrate and were rather dynamic. At the beginning of the cytokinesis stage in which the cells started to divide, the cell next to the neighbor cell also started to proliferate (**Figure 3**). Cells divided into 2, but the sizes of the daughter cells were not same. The adjacent cell became elongated. While one of the daughter cells returned to the substrate, the other one retained its position. The second dividing cell became smaller and was shaking strongly (**Figure 4**). Daughter cells of the first divided cell adhered and spread to their original position on the substrate. The second divided cell almost completed the cytokinesis. At the end, daughter of both divided cells went back to the substrate, adhered, spread and completely filled the area where they regained their connections with neighbor cells (**Figure 5**). Observation from time-lapse microscopy showed that 4 hours after plating, primary culture of cells adhered to the substrate and started to proliferate. Division of the cells was completely different. Examining cells under the microscope showed 51 minute division time for the first dividing cells and 25 minutes on the second dividing cell. The second divided cell with shorter division time was more centrally placed compared to the first divided cell. Important observation was that, there was always blebbing and shaking on the surface of the cell before the beginning of the mitosis. These actions were very important for the initiating mitosis. It was also difficult to establish the time and the ended division too. Cytokinetic analyses performed during colony-forming stage prior to cell division have examined the duration of 3 phases: round-up time between the break-up of the cell-cell or cell-substrate adhesions and the beginning of cytokinesis; completion of division time between the end of cytokinesis and the re-establishment of the

Figure 1 - The image of resting cells at the periphery of the colony (× 40).

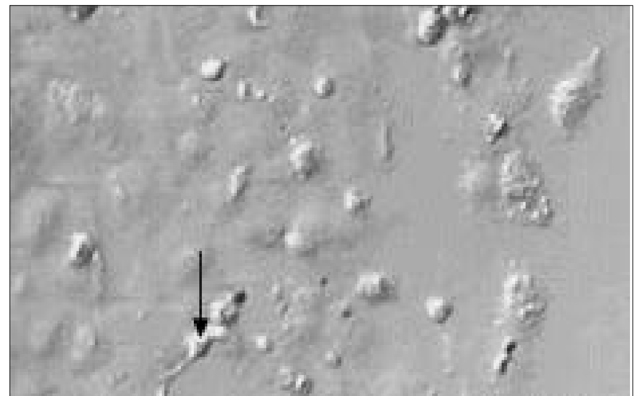

Figure 2 - Cell preparing to divide suddenly retracted and became elongated but still kept their connections with the neighboring cell by one side (× 40).

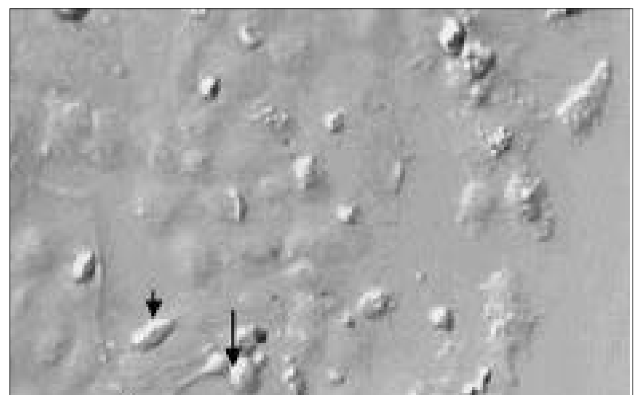

Figure 3 - Cell became clearly rounded, with the side connections tight and completely touching the neighboring cell (× 40).

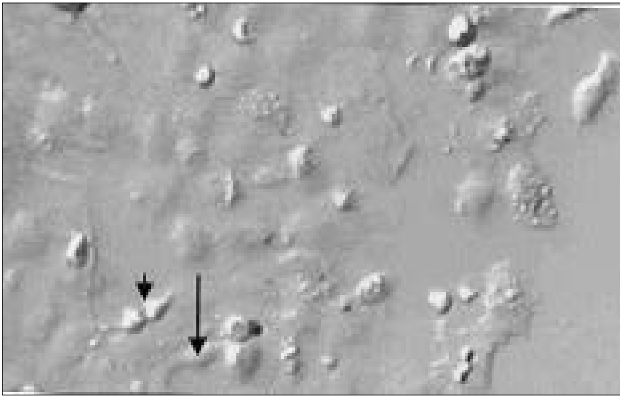

Figure 4 - One daughter of the first divided cell kept connected to the neighboring cell while the other one disconnected itself from its adhesion to the neighboring cell (× 40).

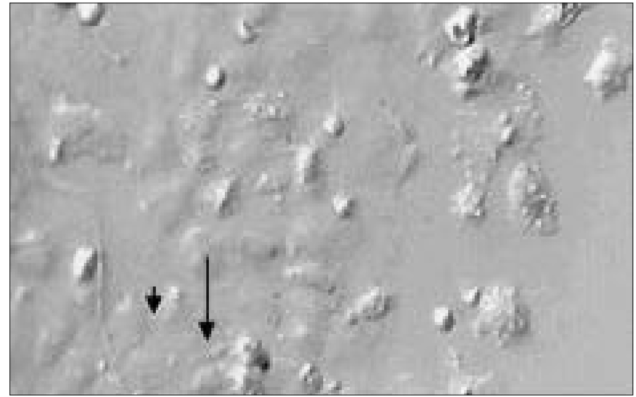

Figure 5 - Daughter cells of the first divided cells completed adhesion and became almost equal in size (× 40).

adhesion and the total time. These times were measured on cells that were outside the colony, 58 cells were on the periphery of the colony and 48 cells were within the colony. Mean round up time for cells outside the colony was 00:19:01 ± 00:09:04, time between cytokinesis and adhesion was 00:08:12 ± 00:04:29 and total time was 00:26:55 ± 00:10:53. In 33 of them, adhesion after cytokinesis did not occur simultaneously. Therefore, cytokinesis to adhesion time (00:14:53 ± 00:09:00) and the total time (00:32:04 ± 00:15:48) was prolonged in these cells. Among cells which were in the periphery of the colony all the times were non-significantly shorter. Round-up time was 00:12:06 ± 00:07:11, cytokinesis to adhesion time was 00:07:32 ± 00:04:14 and the total duration was 00:19:38 ± 00:08:49. In 16 of them, adhesion after cytokinesis did not occur simultaneously. Cytokinesis-to-adhesion time was 00:13:30 ± 00:07:03, with a total duration of 00:27:29 ± 00:10:13. For those cells located towards the center of the colony, the times were non-significantly shorter: round-up time was 00:08:53 ± 00:05:49, cytokinesis-to-adhesion time was 00:05:34 ± 00:03:43 and the total duration was 00:14:27 ± 00:07:46. In 20 of them, adhesion after cytokinesis did not occur simultaneously. Cytokinesis-to-adhesion time was 00:12:27 ± 00:08:57 and the total duration was 00:24:10 ± 00:15:25. Some of the cells far exceeded the mean values. Round-up times of 2 cells that lay outside the colony were 03:36:43 and 01:14:44. Cytokinesis-to-adhesion times were 00:07:38 and 00:04:25, with a total duration of 03:44:21 and 01:19:09. One cell that lay in the periphery of the colony had a round-up time of 01:09:35, cytokinesis-to-adhesion time of 00:04:44 and a total duration of 01:14:19. Among the cells that lay to the center of the colony, 2 had an unusual long time. Round-up times of these cells were 01:01:36 and 01:18:48. Cytokinesis-to-adhesion times were 00:25:34 and 00:05:10, with total durations of 01:27:10 and 01:23:58. One of the daughter cells of the first cell was very late to go down and adhere. It took 01:35:55 to adhere, causing the cycle to complete in 02:35:31.

Discussion. Cell division and proliferation are coordinated functions that depend upon cell-cell adhesion, cell-substrate relationship, growth factors and genetic code.[2,15,18] We endowed the 2 daughter cells resulting from cytokinetics with a complete set of chromosomes and cytoplasmic organelles. We mediated the conceptually simple event by a complex and dynamic interplay between the microtubules of the mitotic spindle, the actomyosin cytoskeleton, and membrane fusion events. The study of cytokinesis based on morphological studies, molecular and genetic approaches have lead to great insights into the cellular structures that orchestrate cell division, and the initial steps in the development of a molecular understanding of this fundamental event in the life of a cell.[5,17,19,20] In this study, we examined the behaviors of the primary culture of RPE cells for their proliferation capacity. Proliferation behavior to form epithelium evolves as a result of interactions between cell cycle and adhesions. It is not possible to think that division and proliferation are not coordinated. The basic components of this coordination are cell-cell adhesion, cell-substrate relationship, growth factors and genetic code.[10,13,17,21] Cytokinesis creates 2 daughter cells endowed with a complete set of chromosomes and cytoplasmic organelles. A complex and dynamic interplay mediated this conceptually simple event between the microtubules of the mitotic spindle, the actomyosin cytoskeleton, and membrane fusion events. For many decades, the study of cytokinesis was driven by morphological studies on specimens amenable to physical manipulation. The studies led to great insights into the cellular structures that orchestrate cell division,

but the underlying molecular machinery was largely unknown. Molecular and genetic approaches have now allowed the initial steps in the development of a molecular understanding of this fundamental event in the life of a cell.[19,22] Likewise, we noted the changes in morphology and cell characteristics during mitosis in any cell. However, it was noteworthy that cells did not undergo contact inhibition or rather, contacts with the walls of other cells seem to prompt cell division. We observed these steps of mitosis in our cells and most of the behavior was similar with the previous studies. It is noteworthy that cells do not undergo contact inhibition although they contact each other. Being alone does not induce a stimulus for mitosis. Instead, contacts with the walls of other cells seem to prompt this stimulus.[18,23] It is well established that proliferation limits the space. The cells can migrate and thus increases cell-cell adhesion.[24,25] When the attachment of the cells is not tight enough, cells produce filopodia and lamellipodia. Blebs formed on the surface of the cell by this way directly affects cell behavior and enables proliferation and migration. Therefore, we deter-mined the coordination of cell behavior for proliferation by adhesion of the cells. For that reason, some of the cells undergo mitosis while under the same condition, others do not. The effect of adhesion is unlimited to the proliferation. After a cell divides into daughter cells, adhesion maintains the contact of the daughter cells with the adjacent cell.[26,28] The duration of the division and the time between the breaking down of the nuclear envelope and the completion of cytokinesis is 51 and 25 minutes. Only by itself is sufficient to reflect the importance of factors affecting the cells. The duration of the division increases when the cell is the center of the colony. As reported previously, cells start to get spherical from their apical side during proliferation and daughter cells adhere to the basement through their basal side after cytokinesis. At this stage, it is still not clear which mechanisms determine the orientation of the daughter cells or what adhesion they will exhibit once they are attached to the basement. Nevertheless, adhesions with adjacent cells play an important part in signal transduction. Cells that did not adhere after proliferation must obtain it by losing the contact with the neighboring cells. Elongation of the cell before the division may affect adhesion during or after the mitosis.[27,29] We allocated the observations into 3 groups; namely, center of the colony, periphery of the colony and outside the colony. We assessed the cell division in 2 stages: 1) round-up stage, which is the detachment and moving up and 2) the second stage which starts with cytokinesis and end with adhesion. We found that the round-up stage was non-significantly shorter if the cell was closer to the center of the colony where adhesion and contact inhibition are expected to be stronger. We did not expect this finding, suggesting an interesting interaction between cytokinesis and cell-cell adhesion and cell-matrix adhesion of cells within the colony. After the cytokinesis, cell adhesion lasts for almost half of the previous times and, interestingly, is constant. The duration of attachment of the daughter cells that attached later than the other after cytokinesis was variable but the means of these times among late attaching cells were comparable. There was a 2-fold difference between daughter cells with regard to the duration of attachment. Another interesting observation was the presence of a very slowly dividing cells. Among these, cells that were outside or in the periphery of the colony had round-up time of over one hour while cytokinesis-to-adhesion time was around 5 minutes. However, when we located the cells to the center of the colony, the times were half-an-hour and 1.5 hours for the first and late attaching daughter cell. Despite the variability of these times, prolongation in round-up and cytokinesis-to-adhesion times is difficult to explain, especially in the cells that adhesion did not take place. The effect of adhesion molecules on the elongation of the cell before mitosis is another question. Especially, dis-connection of the adhesion from neighboring cells and beginning of retraction during proliferation need explanations. Time-lapse video microscopy with immunohistochemistry of adhesion molecules may help for these mechanisms. Demonstrating changes in matrix proteins and receptors as well as cell-cell adhesion molecules will especially be useful. Moreover, the observation of more cells in different culture conditions will help to understand the behavior of the cells during mitosis. These mechanisms occur during development and pathologic conditions where cells proliferate. Adhesion molecules such as cadherin, laminin, collagen, fibronectin and their receptors are organization factors during migration, proliferation and death of the cell. Either their original effect or their effects to the cytoskeleton affect cell behavior.[30,31,35] We observed the loss and gain of the cell-cell contact from the cell surface with complete separation of cells from each other under time lapse video micros-copy during proliferation. The findings strongly suggested that cell-cell adhesion molecules may be involved in regulation of the proliferative activity of the cells and may be very important for the differentiation and maintenance of these cells during development. Cells in contact display enhanced spreading, in contrast to poor spreading of the dissociated cells, a phenomenon first described by Middleton and Pegrum.[31] The higher density cultures altered the rate of attachment. However, colony formation of the cells was increased by the higher density cultures. This observation showed the effect of local production of contacting cells in islands on adhesion, which was not observed in

dissociated cells. The factors that were produced by the contacting cells in islands increased the stable adhesion of the cells and increased the colony formation of the cells in other islands, which was an indication of the production of soluble factor(s) by these cells. Other studies suggested that the dramatic changes in the behavior of contacting cells compared to dissociated cells on tissue culture plastic were, most likely, due to the production of extracellular matrix proteins by the cells themselves.[7,28,33] The reason for the different behavior of the cells in contact can be explained by the effect of cell-cell adhesion on the cell-substratum adhesion properties of the cells in vitro. This effect may also be important for the behavior of these cells in vivo during development by changing the composition of the matrix proteins in the basement membrane. We shows the effect of cell contact in E5 chick retinal RPE cells which survived, proliferated and differentiated into neurons only in the presence of cell to cell contact on a laminin substrate.[31,33,34,37] MacDonald et al,[36] described the effect of conditioned medium from RPE cells on island formation of neurons and suggested the factor(s) produced by contacting cells and their effect on other cells in islands. Morphologic and molecular observations made by different techniques agree that adhesion of the cells determines the structure and differentiation. Proliferation is a very important component of embryonic development it is under the effect of migration and adhesion. The different time requirement for dividing cell needs exact phase analyses with specific fluorescent dyes, cell cycle inhibitors and flow cytometry in each cell.[34,35] We still in the process of evaluating these complex mechanism works. The explanation of these mechanisms will not just help us to understand the developmental process of an embryo, it will also helps in treating pathologic conditions. Time-lapse microscopy in conjunction with immuno-histochemistry of adhesion molecules may help understand the mechanism for the behavior of the dividing cell where they play a crucial role for pathologic conditions.

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