

ALTERATION IN THE SURFACE MORPHOLOGY OF SYNCHRONIZED B-16 MELANOMA CELL DURING THE CELL CYCLE

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Abstract. B-16 melanoma cells were synchronized for G_1 -S periods by mitotic shake-off and S- G_2 periods by thymidine block. The surface morphology of the B-16 melanoma at low density partially confluent cultures revealed that, as in other cell systems investigated, the surface morphology could be correlated with four periods to cell cycle: G_1 , S, G_2 and M. G_1 cells were rounded and possessed microvilli, blebs and ruffles (late G_1). During S period, blebs and microvilli diminished and disappeared, but ruffles were more evident. Late G_2 cells acquired microvilli, surface blebs, and extended filopodia and began to thicken and round themselves for mitosis.

Key words: Melanoma; Cell synchrony; Scanning electron microscopy

Among the prerequisites for *in vitro* investigations of morphologic changes of cells during various cellular responses are (a) the establishment of a population of cells growing in synchrony, and (b) the cognizance of morphologic alterations occurring as the cells course through their life cycles. Recently, scanning electron microscopy (SEM) has provided an additional dimension for the observation of cellular events and has provided information on the dynamics of cell surfaces. Scanning electron microscopy has been especially helpful when subtle changes of surface morphology have been anticipated.

For a considerable period of time it has been known that progression of a cell through its life cycle is a continuous process. The two major events which characterize this continuum of activities are DNA replication and nuclear division. These events may, however, be further subdivided into four periods: G_1 , S, G_2 and M. The G_1 period has been associated with the preparatory events for DNA replication (S), while the G_2 period encompasses those events preparatory for mitosis (M).

In a variety of cell types examined by SEM, G_1

cells are characterized by a surface morphology showing ruffles, a large number of microvilli, and blebs. Except for ruffles, microvilli and surface blebs appear to diminish in prominence during S period and the cells attain a relatively smooth surface and spread over the supporting media. During G_2 , microvilli increase in numbers and the cells thicken in preparation for mitosis (13, 20).

Although previous investigations have been similarly directed with various cell populations (1, 4, 6, 8, 20, 21), no criteria exist for melanoma or any melanin pigment producing population of cells. Thus, the purpose of the present investigation was to attain a synchronous population of melanoma cells and define the surface characteristics of these cells as they course through their cell cycle. These data would provide information by which to compare the effects of various environmental conditions and agents on the cell surface morphology of B-16 melanoma cells grown *in vitro*.

MATERIALS AND METHODS

Observations were made on a line of B-16 melanoma derived from C57BL/6J host mice (Jackson Laboratories, Bar Harbor, Maine). The cells were cultured over numerous generations in Dulbecco's modified Eagle media supplemented with 10% fetal calf serum (GIBCO, Grand Island, New York). All cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 . Cells were grown in plastic vessels and subcultured weekly, using a solution of 0.5% trypsin to free cells from supporting surfaces. Pleuropneumonia-like organism contamination excluded the use of the cells for future study or subculture. Cultures observed were determined to be free of pleuropneumonia-like organisms.

Cells were synchronized by the mitotic shake-off method (27). The mitotic cells were planted on cover glasses and allowed to remain in an atmosphere of 95% air and 5% CO_2 at 37°C for periods of 15 minutes, 1, 2, 3, 4, and 5 hours. The samples were exposed to [3H]thymidine for 10 minutes

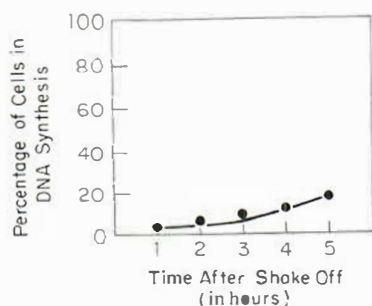


Fig. 1. Percentage of cells in DNA synthesis are plotted against time in hours after mitotic shake-off.

before fixing and autoradiography. The latter were used to assess whether cells had completed G_1 and entered S period.

Additional mitotic cells were plated on coverslips and a double thymidine block was employed to provide cells in the S period (3). The blocked cells were released by removal of the thymidine by a change of culture media.

Samples were fixed at 15 minutes, 1, 2, 3, and 4 hours after release of the thymidine block for cells in early, middle and late S periods. A sample was fixed at 6 hours for assessment of cells in G_2 period. Likewise, samples were exposed to [3H]thymidine for 10 minutes to determine the number of cells in S and G_2 periods.

Scanning electron microscopy

All cells were initially fixed in 0.1% glutaraldehyde in White's saline, pH 7.3, for 15 minutes. The cells were then fixed with 3% glutaraldehyde in the same buffer for 2 hours. After initial stages of fixation the cells were washed once in White's saline and then three times in distilled water. The washed cells were then post-fixed in buffered 1% osmic acid for 1½ hours and rinsed three times in distilled water. Cells were dehydrated in a gradient series of alcohol (30–100%) and then processed through a gradient series of freon (30–100%). All specimens were then critical point dried in a Bomar critical point dryer from 100% freon. Cover slips were mounted on aluminium stubs and coated with 50 Å of carbon-gold in a vacuum evaporator. All specimens were examined in a Cambridge Stereoscan scanning electron microscope at 20 kV.

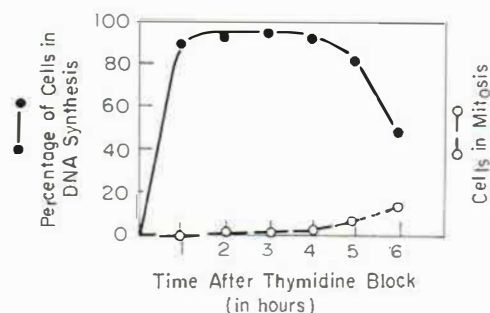


Fig. 2. The percentage of cells in DNA synthesis are plotted against time in hours after release from thymidine block.

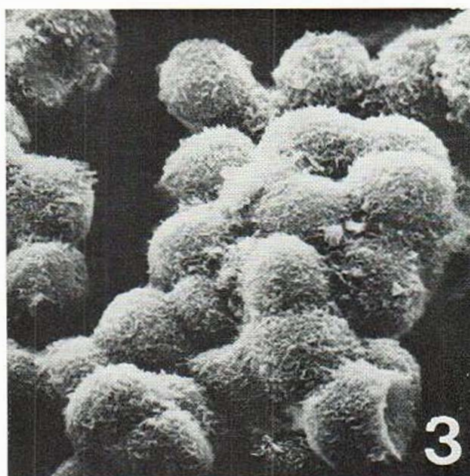


Fig. 3. Melanoma 15 minutes after shake-off. Early G_1 cells are attached by filopodia and is covered by numerous microvilli. ($\times 1000$)

RESULTS

Synchrony

The mitotic index of the cell populations used in these studies was from 60–65%. The loss of synchrony through G_1 was monitored as the percentage of cells that entered DNA replication and plotted against time (Fig. 1). At 1 hour, 94% of the cells were in G_1 ; however, this decreased to 90% at 3 hours and 85% at 5 hours.

Resynchronization of the cells with thymidine (Fig. 2) resulted in 90% of the cells in early S phase after removal of the block. Three hours after removal of the block, 95% of the cells were in middle to late



Fig. 4. Melanoma 15 minutes after shake-off. Early G_1 cells with numerous microvilli of varying length. ($\times 4260$)

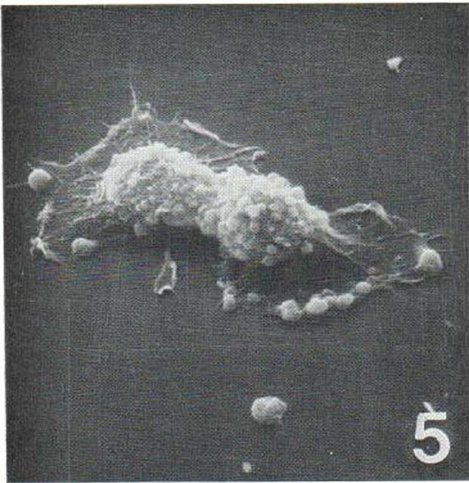


Fig. 5. Melanoma 1 hour after shake-off. G_1 cells show microvilli, surface blebs and connecting filopodia. ($\times 1\,200$)

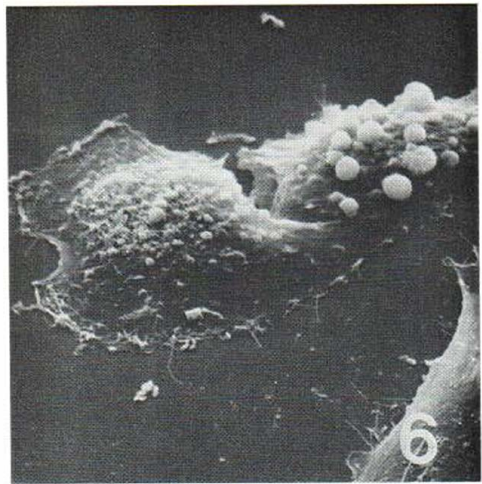


Fig. 6. Cell harvested 3 hours after shake-off contains surface blebs and microvilli; however, cells now begin to spread and become thinner and larger. ($\times 1\,200$)

S period. Fixation at 6 hours resulted in 50% late S period cells, 15% mitotic cells and 35% G_2 cells.

Morphology

Many of the cells fixed at 15 minutes were in late stages of cytokinesis or very early G_1 (Fig. 3). The cells were round and often times associated with many other cells. They contained numerous microvilli and extended filopodia to the supporting media. However, they showed little or no blebbing of the surfaces or ruffling of their peripheries. The microvilli were small structures, approximately $0.1\ \mu\text{m}$ in diameter, with variable lengths (Fig. 4). Most microvilli were approximately $1.7\ \mu\text{m}$ in length.

After 1 hour, most of the cells were still rounded; however, many began to show blebbing and extension of filopodia to the supporting media. Microvilli were still prominent. The surface blebs were usually concentrically distributed over the cell surface, especially thicker portions of the cell. The blebs varied in size from 0.15 to $1.7\ \mu\text{m}$ and occurred singly or in clusters.

The cells harvested at 3 hours had begun to lose their spherical shape and spread over the surface of the supporting media (Figs. 5, 6). The surfaces of the cells were covered with spherical blebs mixed with microvilli. Although there was some evidence of ruffling at the edges, this was not a consistent finding. The ruffles were usually manifest as thin folds ($5\ \mu\text{m}$) extending above the surface of the cell periphery and were not evident at sites of cell contact.

Late G_1 cells fixed at 5 hours after shake-off revealed a large variation in cell size and shape. Many of the cells were thinly spread and probably represented early S period cells, while others were thicker and less extended over the surface. Surface blebs were still persistent but reduced in numbers, and membrane ruffling had increased at the margins of the cells (Fig. 7).

The examination of S period cells 15 minutes after release from thymidine block revealed that they were now larger than G_1 cells. The cells had a greater variability in shape and were spread thinly over the

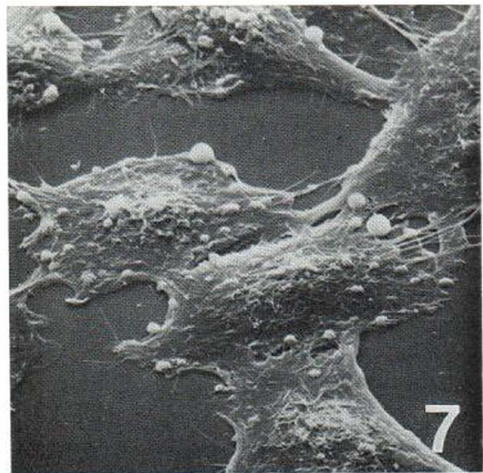


Fig. 7. Cells in late G_1 spread in bipolar directions; however, surface blebs and microvilli are still prominent. ($\times 960$)

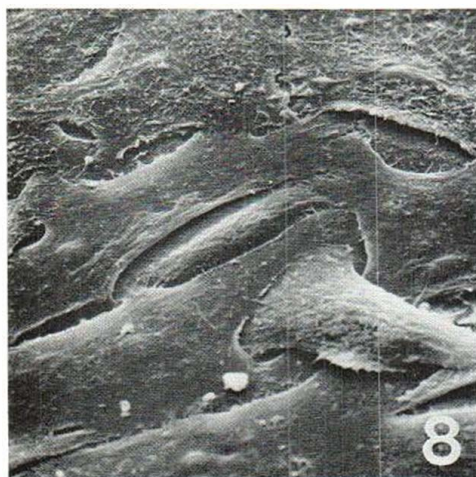


Fig. 8. Melanoma cells after thymidine block resynchronize (S), are greatly flattened, and show few microvilli. ($\times 700$)

surface of the supporting media (Fig. 8). Surface blebs were rare or absent, and microvilli were reduced in numbers. Ruffling was prominent, especially at the free edges of cells which were not in contact with other cells.

Cells at 1 and 3 hours (middle S) were similar, being thinly spread and containing few microvilli and blebs. Small colonies of cells could be noted, and occasionally confluence of cell contact could be noted. Where cell margins were free of contact, ruffling was still persistent, surface blebs were absent, and microvilli were greatly reduced. At 4 hours after thymidine release (late S), the cells appeared to

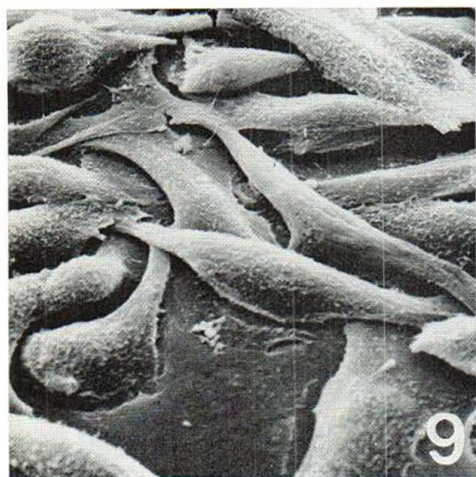


Fig. 9. Cells 4 hours after thymidine block (G_2) reveal increased thickness and reappearance of microvilli. ($\times 960$)

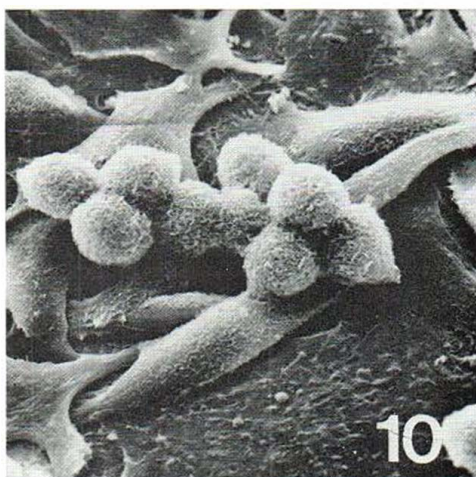


Fig. 10. Melanoma cell 6 hours after thymidine block (G_2 -early M) thickened and prepared for division. ($\times 960$)

thicken, with an increase in ruffling and microvilli, but absence of surface blebs.

Cells fixed at 6 hours (G_2 populations) after thymidine block was removed revealed a mixed population. Many of the G_2 cells were thickened and dendritic. Filopodia extending to the surface could be noted on some of the thicker cells, and microvilli began to increase in numbers (Fig. 9). Some of the cells began to round up in preparation for mitosis (Fig. 10).

DISCUSSION

The present study revealed that there are several distinct alterations in surface morphology as B-16 melanoma cells course through their life cycle. G_1 period ensued with the cells in a round form and the cell surface covered with microvilli. The cells were attached to the supporting media by filopodia. In the mid- G_1 period, the cells began to flatten and surface blebs began to form. These blebs at times dominated the surface morphology such that microvilli appeared to be replaced. However, high magnification SEM examination disclosed the persistence of microvilli in the presence of extensive blebbing. At the mid- G_1 period, B-16 melanoma appeared to spread in bipolar or tripolar directions.

During the period of transition between G_1 and S, there was a change in surface morphology. Blebs disappeared from the cell surface and peripheral ruffling occurred with greater frequency. After cells had entered S (15 minutes after thymidine block), cell surfaces still revealed microvilli; however, they

appeared to be less numerous than in G_1 . The cells had become completely flattened in mid-S (3 hours after thymidine block) and few or no microvilli were apparent on the surface.

At 6 hours after removal of the block, 45% of the cells were in G_2 . During this period, the cells began to acquire microvilli, thicken, and extend filopodia, a characteristic of cells approaching mitosis. Thus, in the present study, relatively low density partially confluent cultures revealed surface morphologies which were susceptible to contact influences over the cell cycle by presenting a spectrum of cell forms correlated to specific periods of the cell cycle.

Although the succession of changes in surface morphology of B-16 melanoma parallel the cell cycle, the exact relationship between these events is not completely known. Abercrombie, et al. (1) described many of the morphologies of the G_1 period in normal cells which included the appearance of microvilli, blebs and ruffles. Follett & Goldman (8) noted that as cells passed from G_1 to S, the microvilli faded and the cells became thinly spread. The approach of G_2 resulted in separation of cells from their neighbors and thickening in preparation for mitosis. Porter et al. (21) summarized these events over the cell cycle with transformed Chinese hamster ovary (CHO) cells. Rubin & Everhart (24) extended these investigations on CHO cells to include high and low density populations. If population density was low so that contact was inhibited, many of the cells possessed G_1 morphologies. However, as surface contacts were increased the spectrum of morphologic changes, previously outlined, became apparent. These data suggested that contact influences were a mediating factor in cell surface morphology during the cell cycle.

The understanding of the relationship between internal cell architecture and external morphologies is similarly incomplete. Taylor (26) and others (10, 11, 12, 28) have observed that colchicine and Colcemid disassembled microtubules and produced rounded cells similar to cells in preparation for mitosis. Similarly, experiments with neuroblasts have revealed that these cells failed to extend bipolar processes, a microtubular event essential to the formation of nerve fibers (7). Recent evidence that bipolarity in other cells is dependent on cyclic AMP concentrations during the cell cycle (9) have implicated cyclic AMP in certain changes of cellular form by inducing assembly and orientation of microtubules (16, 17, 19, 21, 25, 30).

The microvilli prominent in melanoma cells of G_1 period and G_2 period as cells prepare for division are similarly prominent on other transformed cells and many malignant cells (4, 18, 23). Boyde et al. (4, 5) also have noted that after transformation by oncogenic virus microvilli were present on cells otherwise free of microvilli. These villus structures have been noted to consist of an axial bundle of 40–60 Å filaments (8, 26). Although the specific function of microvilli remains obscure, they have been suggested to serve as regions of active transport for sugars (4, 15, 29). An alternative view is that microvilli represent a conservation device for the maintenance of surface membrane area while transition through other changes in cell form are occurring (8).

The presence of surface blebs usually occurred during G_1 , few persisted into S, and until late G_2 the cell surfaces are devoid of these structures. Price (22), examining epithelial cells, and Porter et al. (21), studying CHO cells with transmission electron microscopy, have observed large concentrations of ribosomes in bleb structures. Harris (14) has suggested that blebs developed initially at the cell margin and migrate centripetally back from the edge, thus accounting for the accumulation of large numbers of small blebs over the center of G_1 cells. These observations may account for the absence of blebs in S and G_2 in more densely populated cultures and their persistence in low density cultures (24).

Ruffling, although not present during mitosis, became evident in late G_1 and increased during S and G_2 periods. These structures originate at the advancing edge of cells and are absent in confluent cultures where there is extensive cell contact (1, 2, 17, 21, 24). Although the exact function is unknown, it has been suggested that ruffles are a device for the increase of membrane area during periods of metabolic need. Abercrombie et al. (1) have described the formation of ruffles (lamellipodia) as a process involving membrane assembly rather than flow and provided evidence that membrane flow was away from rather than toward the advancing edge of the cell. Transmission microscopy of ruffle sections has provided no specific evidence for their origin or function.

In conclusion, the present study has investigated the surface morphology of B-16 melanoma in low density partially confluent culture and has revealed that, as in other cell systems investigated, the surface morphology could be correlated with four periods of

the cell cycle: G_1 , S, G_2 and M. G_1 cells were rounded and possessed microvilli, blebs and ruffles (late G_1). During S period, blebs and microvilli diminished and disappeared, but ruffles were more evident. Late G_2 cells acquired microvilli, surface blebs, extended filopodia, and began to thicken and round themselves for mitosis.

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