Cell Surface Changes in HeLa Cells as an Indication of Cell Cycle Events

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SUMMARY

A HeLa cell line synchronized by double thymidine block and mitotic shake off was shown to have a characteristic surface morphology for each of the different cell cycle stages. Inhibitors of cell multiplication were used to arrest cells in specific cell cycle phases, and these cells had a surface morphology similar to that of synchronized cells in the same phase. The results indicated a close association between the cell surface topography and the cycles of DNA synthesis in the cell nucleus of this HeLa line.

INTRODUCTION

Many reports have appeared in recent years on the cyclic changes of membrane structure and function that occur when cells proceed through the different cell cycle phases. The carbohydrate content has been shown to fluctuate during the cell cycle, as well as the expression of blood group and transplantation antigens (7, 10). Concanavalin A and hormone receptors also display cyclic changes during cell cycle stages (9, 17).

Considering these fluctuations of plasma membrane components, it is not surprising to find a close association between different plasma membrane movement patterns and cell cycle phases (11). This has also been demonstrated by a morphological approach to CHO cells by SEM (14).

We studied synchronized HeLa cells by SEM and found variations in surface morphology when the cells progress through the cell cycle. Through the use of various inhibitors of cell multiplication, cells were arrested in specific cell cycle phases. These cells had the characteristic surface morphology of synchronized cells in that stage, which suggested that the structure of the cell surface was associated with different phases of DNA processing.

MATERIALS AND METHODS

Cell Culture. The HeLa cells were cultured in 90-mm Nunclon plastic dishes (Nunc, Roskilde, Denmark) in Eagle’s minimum essential medium supplemented with 10% calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland), 100 units penicillin per ml, and 50 μg streptomycin per ml. For the experiments the cells were transferred to round coverglasses placed in 30-mm Nunclon plastic dishes. Further culture conditions have been given earlier (8).

Synchronization was performed either by a double thymidine block, according to the principles given by Galavazi et al. (5), or by the mitotic shake-off technique (19). The thymidine concentration was 5 \times 10^{-3} M, and the 2 incubation periods lasted 16 hr each, with an interval of 8 hr. For the mitotic shake-off procedure, cells were grown in 75-sq cm plastic flasks (Falcon Plastics, Los Angeles, Calif.) until confluence was nearly reached, and the medium was manually passed gently over the monolayer 20 times. After centrifugation, the cells were resuspended in a small amount of medium and transferred to plastic dishes.

Autoradiography was used to characterize synchronization. After a 20-min pulse with 1 μCi [methyl-3H]thymidine per ml (Radiochemical Centre, Amersham, England), the cells were fixed in alcohol:acetic acid (3:1), washed in 70% ethanol, and air dried. The bottoms of the dishes were cut out, dipped in Ilford G5 emulsion (Ilford Ltd., Ilford, England), and exposed for 2 or 3 weeks. After being developed and stained with May-Grunewald-Giemsa, cells with labeled nuclei were counted by means of a light microscope and immersion objective.

Mitotic index was measured by light microscopy, and the total cell number was determined by an electronic cell counter (Linson Instrument AB, Linson Counter 411, Stockholm, Sweden).

Inhibitors of cell multiplication were used in the doses shown in Table 1. The inhibitors were dissolved in the medium or in a stock solution of ethanol (hydrocortisone), the final concentration of which was less than 0.01%. This concentration could be shown not to affect surface morphology. The cells were treated for 48 hr, and a trypan blue dye exclusion test determined that cell viability was not affected in the used concentrations. Vinblastine treatment could not be performed for more than 24 hr because of serious cell detachment.

SEM. At various times after the 2nd thymidine block was released or after mitotic shake off, the cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 1 hr. The cells incubated with the inhibitors were fixed immediately at the end of the incubation period. After fixation, the cells were washed once in buffer and post-fixed in 1% OsO4 in the same buffer for 1 hr. They were then dehydrated in increasing concentrations of ethyl alcohol up to 99% and thereafter in a graded series of amyl acetate in alcohol up to 100% amyl acetate. The preparations were transferred to a critical-point drying apparatus (Polaron E
Various inhibitors of cell multiplication used in the experiments and their modes of action

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mode of action</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Hydrocortisone, $10^{-4}$ M</td>
<td>Prolongation of G1</td>
<td>6, 21</td>
</tr>
<tr>
<td>$1-\beta$-D-Arabino-furanosyl-cytosine, 5 $\mu$g/ml</td>
<td>Arrest at G1-S</td>
<td>23</td>
</tr>
<tr>
<td>Hydroxyurea, $10^{-4}$ M</td>
<td>Arrest at G1-S</td>
<td>23</td>
</tr>
<tr>
<td>Mitomycin C, 1 $\mu$g/ml</td>
<td>Not specific</td>
<td>1</td>
</tr>
<tr>
<td>Bleomycin, 10 $\mu$g/ml</td>
<td>Prolongation of S</td>
<td>22</td>
</tr>
<tr>
<td>Vinblastine, 0.01 $\mu$g/ml</td>
<td>Arrest at M</td>
<td>20</td>
</tr>
</tbody>
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RESULTS

Synchronization and Surface Morphology. It can be seen from Chart 1 that the degree of synchronization was rather high up to 10 hr after release of the thymidine block. Nearly 75% of the cells were in the S phase after 5 hr, and a mitotic index of 20% was achieved after 9 hr. Apparently, the degree of synchronization was low after 15 hr, when the next S phase started. The mitotic shake-off procedure yielded about 80% mitotic cells (Chart 2). From 2.5 to 10 hr, no mitotic cells could be seen. Therefore, thymidine block was chosen for a study of the surface morphology of S, G2, and M cells, and mitotic shake off was used to study that of G1 cells.

The different surface features were best expressed when the cells were in contact with each other, a finding that is in agreement with studies on CHO cells (15). When the cultures were overcrowded, all cells appeared rounded and were covered with microvilli (Fig. 1).

Characteristics of the Cell Cycle Phases. Cells that were fixed immediately after the 2nd thymidine block was released showed no signs of toxic effects, compared with control cells. The thymidine block arrested the cells at the G1-S boundary; at this stage, the cells were rather flat and attached to the coverglass. Cell surfaces were sparsely covered with short microvilli and some cytoplasmic blebs (Fig. 2).

In the beginning of the S phase, the cells looked like G2 cells, except for the microvilli, which seemed to accumulate over the central parts of the cells. This phenomenon was more accentuated in mid-S when the microvilli were taller and more numerous (Fig. 3). The cells were still rather flat, and few blebs were seen. Toward the end of S, the microvilli increased in number and covered a greater proportion of the cell surface.

Before mitosis, the cells began to appear rounder, the entire surface was covered with microvilli, and blebs appeared only occasionally (Fig. 4). It seems reasonable to assume that these surface characteristics represented cells in G2, but the possibility exists that they were cells in very late S phase or even early G1, since, obviously, the degree of
synchronization had deteriorated in this stage (Chart 1). The mitotic cells were spherical and covered with microvilli of varying lengths (Fig. 5). Many cells were attached to the glass by long filopodia that often bifurcated at the ends. In late mitosis, just before separation of the 2 daughter cells, a general phenomenon was the appearance of many cytoplasmic blebs (Fig. 6).

In early G1, the cells were somewhat rounded and had begun to spread out. Microvilli of varying lengths appeared all over the surface. Blebs were often but not always seen (Fig. 7). During the G1 phase, the cells successively flattened, microvilli became shorter, and blebs were still present. Cells fixed at the G1-S boundary appeared similar to those fixed immediately after the thymidine block was released (Figs. 2 and 8).

The described surface features represented the main characteristics of each cell cycle phase. Chart 3 summarizes the percentage of cells displaying a certain surface character after the 2 methods of synchronization. The cells were counted in the scanning micrographs. Obviously, not all cells have the described typical appearance, since the degree of synchronization is never 100%. Moreover, the morphology changed gradually during the cell cycle, and it did not seem possible to assign all cells in an unsynchronized culture to a specific stage according to surface morphology.

### The Effect of Inhibitors of Cell Multiplication

To check the validity of these results, we tested some inhibitors of cell multiplication with well-characterized modes of action on the cell cycle. According to Table 1, some inhibited the cells in specific stages of the cell cycle, while others arrested the cells more unspecifically.

Hydrocortisone is known to prolong the G1 phase (6, 21), and the principal part of the cells incubated with this drug appeared as G1 cells, a similarity based on the general shape as well as the distribution and size of microvilli (Fig. 9).

Incubation with 1-β-D-arabinofuranosylcytosine and hydroxyurea has been shown to arrest cells at the G1-S boundary (23). Treatment with these drugs induced surface characteristics similar to those described occurring immediately after the 2nd thymidine block was released (Fig. 10; cf. Fig. 2), but the association between surface morphology and cell cycle stage seemed lower, as many cells with characteristics of other cell cycle phases could be seen.

Most of the cell population appeared as mitotic cells after incubation with vinblastine (Fig. 11). As a rule, microvilli were normal, compared with mitotic cells in the thymidine-synchronized population.

Cells with the appearance of all cell cycle phases were present after incubation with mitomycin C and bleomycin, and no specific cell cycle stage seemed to be predominant (Fig. 12). Some of the cells showed shrinkage of the cell surface and loss of microvilli, which could be a toxic effect, although trypan blue exclusion was not affected.

**Ruffles.** The phenomenon of ruffling of the cell membrane seen in CHO cells by SEM has been described (14). In our HeLa cells, similar structures appeared at the free margins of some cells, but not to the same extent as in the CHO cells. The ruffles did not seem to be more numerous in any specific cell cycle phase. After incubation with the inhibitors, ruffling was still visible. Fig. 13 shows ruffling in a cell treated with mitomycin C.

### DISCUSSION

Our observations indicate that it is possible to recognize different cell cycle phases by the surface morphology of the HeLa line. Microvilli increased in number during the S phase and were most abundant at G2. M. Porter et al. (14) found that microvilli were most numerous during G2, M, and G1, in CHO cells, and similar findings have been made in a melanoma cell line (16). This discrepancy may be due to different culture conditions or to the fact that the cell lines were derived from different species.

During the S phase, microvilli accumulated over the central parts of the cell, a phenomenon reported in unsynchronized rat sarcoma cells (13). Studies of unsynchronized HeLa S3 cells by SEM or transmission electron microscopy have not shown any unevenness in the distribution of microvilli (3, 12). Our HeLa cell line, continuously cultured separately from other HeLa cell lines since 1956, is obviously different. Accordingly, it was possible to ascribe certain surface details to different cell cycle phases of 1
specific cell line, but obviously this was not a general phenomenon.

The fact that the changing distribution of microvilli is expressed when cells grow in a monolayer and not in overcrowded cultures (3, 12) makes it difficult to explain in functional terms the nature of the changes in size and distribution of microvilli. The transitional nature of microvilli has been shown by trypsinization of HeLa cells (3). Cytoplasmic blebs were predominantly seen during late mitosis and G phase, which agrees with observations made on HeLa cells by transmission electron microscopy (2). We are inclined to consider these morphological changes as possible markers of nuclear activity, while their functional meaning must await other investigational approaches.

Cells arrested at a specific cell cycle stage by incubation with synchronizing inhibitors appeared much like synchronized cells in that particular phase. Some of the inhibitors, i.e., 1-β-D-arabinofuranosylcytosine, hydroxyurea, mitomycin C, and bleomycin, are known to inhibit DNA synthesis, but not RNA and protein synthesis (18, 22, 23). This means that, although cytoplasmic and cell membrane growth continued, the surface morphology did not change, suggesting that the surface morphology was directed by the cycles of DNA synthesis.

It is known that thymidine also has effects other than those on DNA synthesis, which may be the reason for the observed changes in surface morphology. However, mitotic shake off, without use of any inhibitors, is a gentle method of synchronization, and no principal differences could be seen between cells synchronized with the 2 methods in comparable cell cycle phases, although the degree of synchronization could be variable. Thus, the changes seemed to be due to the cell cycle phase and not the method of synchronization.

Our observation that ruffling of the cell membrane occurred throughout the cell cycle in the synchronized cells as well as after treatment with inhibitors of DNA synthesis is in agreement with the results of Gail et al. (4). They found locomotion in 3T3 cells that were cultured in medium lacking growth factor. Apparently, there was a dissociation between the rough molecular structure, indicated by surface morphology, and certain cell membrane movements like ruffling. The membrane components directing surface morphology seemed to be governed by nuclear activity, while ruffling was independent of it, which may indicate that in this HeLa cell line the attaching sites for the forces causing membrane movements were more constant than the cell membrane structures that maintained surface morphology.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Rounded cells covered with microvilli in an overcrowded culture. × 3000.

Fig. 2. Cells near the G1-S boundary fixed immediately after the release of the 2nd thymidine block. Note short microvilli evenly distributed over flattened cells. × 1100.

Fig. 3. Cells with accumulation of microvilli over the central parts of the cells. Fixation was performed 4 hr after thymidine release, i.e., during S phase. × 1100.

Fig. 4. Somewhat rounded cells with the surface covered with microvilli, probably representing G0 cells. Fixed 9 hr 40 min after thymidine release. × 1100.
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Fig. 5. A cluster of round, mitotic cells fixed 9 hr 40 min after thymidine release. × 1100.

Fig. 6. Late mitosis just before cytoplasmic separation. Note many cytoplasmic blebs. Fixed 1 hr after mitotic shake off. × 1100.

Fig. 7. Early G, cells spread out on the surface. Fixed 3 hr after mitotic shake off. × 1100.

Fig. 8. Cells fixed at late G, 7 hr after mitotic shake off. × 1200.
Fig. 9. Cells fixed after incubation with $10^{-8}$ M hydrocortisone for 48 hr. $\times$ 2400.
Fig. 10. Cells fixed after incubation with $10^{-2}$ M hydroxyurea for 48 hr. $\times$ 1200.
Fig. 11. Mitotic cells after treatment with 0.01 $\mu$g vinblastine per ml for 24 hr. $\times$ 2400.
Fig. 12. Cells with the appearance of cells in all cell cycle phases can be seen after incubation with 1 $\mu$g mitomycin C per ml for 48 hr. $\times$ 1200.
Fig. 13. Ruffling of the cell membrane (arrows) in a cell treated with mitomycin C for 48 hr. × 3250.
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