PROLIFERATION AND AGGLUTINABILITY OF PRIMARY AND TRANSFORMED HUMAN EPITHELIAL CELLS IN CULTURE

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SUMMARY

Primary epithelial populations (HAM) were obtained by dissociation of the amniotic membrane stripped from human placentae. Agglutinability of cells from such normal populations and of cells from the transformed epithelial line WISH was then compared using concanavalin A as mediator. Extensive similar studies have previously been reported with cell strains isolated from other species. Freshly dissociated HAM cells from primary cultures agglutinated much less readily than did cells from WISH populations. Furthermore, the former exhibited a drastic decline in agglutinability as a function of time in suspension culture after trypsinization. Short-term exposure (60 h) of HAM cells in monolayer culture to 5-bromodeoxyuridine (BrdU) elicited heightened agglutinability detectable through 22 days in vitro.

Addition of the protease inhibitors L-tosyl-L-lysyl-chloromethyl ketone (TLCK) or L-tosyl-L-arginine-methyl ester (TAME) to the culture medium inhibited proliferation of the WISH line by 40–50% while effecting only a 10–15% inhibition of HAM cells. These results also confirm data with other cell species indicating that high proteolytic activity at the surface of transformed cells may be related to the rapid proliferation rate.

INTRODUCTION

The differences in membrane structure of normal cells and their transformed counterparts have been the subject of intensive investigation in recent years. An elevated agglutinability with plant lectins (Burger, 1973), an apparent increase in membrane fluidity (Rosenblith et al. 1973), and the presence of unique extracellular, cell-produced proteins (Hynes, 1973) are among the features which have characterized membranes of transformed cells. Interesting ancillary studies suggest that manipulation of the culture environment can lead to a modulation of the two extremes of cellular property. For example, Biquard (1974) reported that exposure of chick embryo fibroblasts to low doses of 5′-bromodeoxyuridine (BrdU), led to an increase in their agglutinability by concanavalin A (Con A) to a degree similar to that observed for virally transformed fibroblasts. In the converse sense, Collard & Smets (1974) and Schnebli & Burger (1972) reported a preferential decrease in the proliferative capacity of transformed mouse and hamster cells when exposed to protease inhibitors. One

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interpretation advanced to account for the latter observation implicates surface protein turnover (Borek, Grob & Burger, 1973).

None of the above work has been conducted with primary epithelial cells derived from human tissue. The human amniotic membrane provides a salient tool in this regard since it gives rise, following appropriate dissociation, to a morphologically pure epithelial population in culture. Hence, the purpose of this study was to apply the primary human amnion (HAM) cell system and the transformed epithelial line (WISH) (Hayflick, 1961) to experimental manipulations similar to those noted above. The intent was to determine whether normal and altered human epithelial cell strains exhibit differences like those observed between normal and transformed cells of lower vertebrates. Specifically, a comparison of the agglutinability by Con A was conducted using HAM and WISH cell populations. In a related experimental series, a range of concentrations of BrdU was introduced into the medium of HAM populations and agglutinability by Con A was measured over a subsequent 22-day period. In addition, the 2 population types were exposed to the protease inhibitors p-tosyl-l-arginine-methyl ester (TAME) or n-tosyl-l-lysyl-chloromethyl ketone (TLCK) and observed subsequently for alterations in proliferation rates.

MATERIALS AND METHODS

Cell cultures

**HAM.** Human placentae were obtained within 2 h of the time of delivery from the maternity ward of Kettering Memorial Hospital (Kettering, Ohio). The amniotic membrane was stripped from the placenta with sterile forceps and was washed 2 or 3 times with Hanks' Balanced Salt Solution (BSS) to remove blood and mucus. For predigestion, the tissue was placed in 200 ml of prewarmed 0·25 % trypsin solution for 10 min at 37 °C. The fluid was then replaced with fresh trypsin. The membrane was incubated at 37 °C for 30 min and agitated manually at intervals to enhance tissue dissociation. Following this period, the cell suspension was decanted and centrifuged at 510 $g$ for 10 min. These latter dissociation steps were repeated twice. After each centrifugation, the supernatant was discarded, and the cell pellet was resuspended in an appropriate volume of Ham's F12 medium supplemented with 15 % foetal calf serum, penicillin G (100 U/ml), streptomycin (50 µg/ml) and Fungizone (2 µg/ml) (growth medium). The resulting cells were seeded into 15-cm plates at a density of $4·7 \times 10^4$ cells per cm$^2$ of culture surface.

**WISH.** One ampoule of cells in solid carbon dioxide was obtained from the American Type Culture Collection (ATCC-CCL 25). Controversy concerning the tissue origin of human cell lines does not detract from this study since the central concern was to compare properties of normal and transformed human epithelia *in vitro*. After quick thawing the cells were seeded at a density of $4·2 \times 10^4$ cells per cm$^2$ of culture surface. This density ensured confluence within 7 days.

Cells, both WISH and HAM, were routinely grown in medium which was replenished on an alternating 3–4 day schedule. The cultures were incubated at 37 °C in water-saturated air with 5% carbon dioxide. Cells were assayed for pleuropneumonia-like organisms (Chanock, Hayflick & Barile, 1962) at intervals and were found to be free of contamination.

Agglutination assays

For both the WISH and the HAM cell populations, ethylene diaminetetra-acetic acid (EDTA) in a wide range of concentrations (20–100 mg/100 ml) proved ineffective in harvesting. Hence, the protease trypsin was employed. Since Burger (1969), Borek *et al.* (1973), and others have reported an increase in the agglutinability of cells following protease treatment, a recovery
period for resyntheses in suspension was afforded the cells after subcultivation. This time period resulted in a decreased agglutinability of the cells after 13 h incubation (see Results section below). Cells were harvested and counts were performed using a haemocytometer. After centrifugation at 246 g for 5 min the growth medium was removed and the cell pellet was resuspended in an aliquot of fresh medium to give densities of about 0.5–1 x 10⁶ cells/ml. The cell suspension was placed in screw cap flasks and equilibrated with 5% carbon dioxide in air. The flasks were then arranged in a shaking water bath at 37 °C and were maintained at 70 agitations per min for 13 h. After this incubation, the flasks were removed, and cell counts were performed using diluent containing erythrocin B at 1 mg/ml. Numbers of viable, non-viable and clumped cells were determined.

Con A, in concentrations dictated by the experiment, was dissolved in growth medium. Equal amounts of cell suspension and Con A solution (0.2 ml of each) were placed in depressions of a porcelain spot plate. Gentle rotation by hand for 5 min followed, after the method described by Noonan & Burger (1973). One drop of each suspension on a coverslip placed on a depression slide was then examined microscopically for agglutination. At least 3 fields of view were observed for each determination. The total number of cells per field was tallied as was the number of cells in clumps. Percent agglutination was computed for the 3 areas. Non-viable cells were not considered in the agglutination assay, as they did not participate in the aggregation. The percentage of clumped cells observed in the viable count was subtracted from the percent agglutination figures. This yielded a more valid figure for estimating Con A-mediated aggregation. The 3 assays were then averaged.

Exposure of HAM cultures to BrdU

Ten-day-old cultures of HAM cells were subcultivated and seeded into 6-cm plates. Twenty-four hours later, the growth medium was substituted with medium containing BrdU at 20, 40, or 100 μg/ml. After 60 h of incubation, fresh medium without inhibitor replaced the BrdU-containing medium. At intervals of 4, 8, 12, and 22 days following BrdU removal, agglutination assays were performed, using Con A at 500 μg/ml. Percent agglutination was then determined as described above.

Exposure of WISH cell populations to protease inhibitors

Cells were harvested, counts performed, and cell suspensions of 0.6 x 10⁶/ml cells were placed in screw cap flasks, as in the agglutination assays. In several of the flasks, the medium, in which the cells were suspended, was supplemented with either 30 or 75 μg/ml of a protease inhibitor (TLCK), which acts irreversibly on trypsin. Likewise, the medium in the remaining flasks was supplemented with either 300 or 750 μg/ml of TAME, which acts competitively with proteases and esterases. Control cultures were set up in parallel using standard growth medium. The concentrations of protease inhibitors chosen were similar to those used by Chou, Black & Roblin (1974) and Collard & Smets (1974).

Cell counts were performed on aliquots at intervals of 24, 48, and 96 h. After each test interval, the cells of each flask were spun at 246 g for 5 min. Fresh growth medium with the appropriate concentration of protease inhibitor was then used to resuspend the cell pellet. The suspensions were placed again in flasks, and maintained with constant agitation until the next testing period.

Comparative effects of protease inhibitors on HAM and WISH cells

Both cell types were subcultivated, and seeded into 6-cm plates. After 24 h, the growth medium was removed and medium containing either TLCK (50 μg/ml) or TAME (500 μg/ml) was added. Control plates were again carried in parallel using the standard growth medium. At periods of 24, 48, and 96 h of incubation, cells were harvested with trypsin, and counts were performed. The appropriate medium from each plate was replenished after 48 h of incubation.
RESULTS

Agglutination assays

After HAM and WISH cells were harvested with trypsin, they were allowed to repair membrane damage while being maintained in suspension culture. At 2-h intervals, agglutination assays were conducted using the standard system with Con A at 100 μg/ml. The HAM population exhibited a steady decrease in agglutinability until the 7th hour of incubation, whereupon a plateau was attained (Fig. 1). This plateau of comparatively minimal agglutinability was maintained throughout the remainder of the incubation period. In marked contrast, the transformed WISH cells exhibited high agglutinability throughout the same time interval. Furthermore, the decline of this property was less pronounced than that observed with normal HAM cell populations.

Exposure of HAM to BrdU

The primary cells were exposed to a range of concentrations of BrdU for 60 h. At subsequent intervals, after being returned to normal growth medium, agglutination assays were conducted using the standard system with Con A at 500 μg/ml. Percent agglutination is plotted as a function of incubation time in Fig. 2. Even at only 4 days...
after treatment, cells exposed to BrdU agglutinated much more readily than did the control populations. After 12 days in culture, a disparity in percent agglutination, that may be attributed to the concentration of BrdU, was observed. The cells exposed to BrdU at 20 and at 40 μg/ml exhibited an apparent increase in agglutination. Cells exposed to BrdU at 100 μg/ml, however, agglutinated less readily with time. These apparent trends were amplified after 22 days incubation.

![Graph showing effect of protease inhibitors on WISH cell proliferation](image_url)

**Fig. 3.** Effect of the protease inhibitors TLCK and TAME on proliferation of WISH cells in suspension culture. Vertical lines indicate total range of results. ○, control; △, □, cells exposed to 30 and 75 μg/ml, respectively of TLCK; ◆, ▲, cells exposed to 300 and 750 μg/ml respectively of TAME.

**Effect of protease inhibitors on WISH cell proliferation**

Proliferation of WISH cells in the presence or absence of the inhibitors, TLCK and TAME is presented as a function of incubation time in Fig. 3. A concentration-dependent inhibition of WISH by the protease inhibitors was apparent. Schnebli & Burger (1972) have explained this phenomenon by suggesting that an inhibitor-sensitive, protease-like activity is required for growth of transformed cells.

**Comparative effects of protease inhibitors on HAM and WISH cells**

HAM and WISH cells were seeded into 6-cm plates to give 4.7 x 10⁴ cells/cm², and 4.2 x 10⁴ cells/cm², respectively. After 24 h, they were exposed to TLCK (50 μg/ml) or TAME (500 μg/ml) for an additional 96 h. Fig. 4 depicts the growth curves obtained from each population over the incubation period. The transformed WISH...
line was more sensitive to the action of both inhibitors than was the HAM population. These findings are further emphasized in Fig. 5, in which the growth rate of inhibited cultures is presented as a percentage of that observed in the control cultures. Proliferation of HAM cells in the presence of these reagents was slightly reduced. However, the inhibitory effect on cells of the transformed WISH line was much more distinct. In this case, after 96 h of incubation with TLCK or TAME, the increase in cell number was only 50–60% of that found with uninhibited WISH cell populations.

**DISCUSSION**

The results from agglutination studies reported here parallel findings of many investigators which indicate that transformed cells have altered surface components. A higher level of agglutinability has been reported for neoplastic counterparts of the 3T3 fibroblast line (Burger, Bombik & Noonan, 1972; Turner & Burger, 1973), the baby hamster kidney cell line, chick embryo fibroblast strains (Burger, 1969), and a rat liver cell line (Borek et al. 1973). Likewise, cultures of epithelial transformed cells expressed a greater affinity for agglutinin than did the non-transformed parental
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cells (Borek, 1972). This study reveals that mild protease treatment of normal HAM

cells appears to cause membrane change such that these cells resemble more closely

transformed WISH cell populations. Rapid recovery was apparent for the normal

human cell type after trypsinization. In marked contrast, the transformed human

epithelial line showed only slight decrease in agglutinability during the identical

recovery period.

Conflicting theories have been advanced to explain this general phenomenon.

Burger (1969, 1973) suggested that, upon transformation, the increase in agglutinability

may be due to an increase in membrane receptor sites. In non-transformed cells,

most agglutinin sites are hypothesized to be masked, allowing only a small degree of an

agglutinin-cell reaction. After proteolysis, however, the receptors are exposed, thus

explaining the heightened aggregation. Additional support for this theory was provided

by Noonan & Burger (1973). A comparison of the amount of tritiated Con A bound
to virally transformed fibroblasts versus parental cells indicated that neoplastic
cells bound up to 4.5 times more lectin than did the normal. Alternative explanations

for heightened agglutinability in transformed cells include increased mobility of

lectin receptors (Singer & Nicholson, 1972; Rosenblith et al. 1973); increased

centration of exposed sites due to decrease in cell size (Ben-Bassat, Inbar & Sachs,

1971); reduced electronegative charge (Grimes, 1970) or increased flexibility of

membranes of neoplastic cells (Barnett, Furcht & Scott, 1974). Extensive reviews

of the subject have been provided recently by Rapin & Burger (1974) and by Nicolson

(1974).

The effect of BrdU addition to enhance agglutinability of cultured HAM cells is an

interesting ancillary finding. Biquard (1974) obtained similar results from studies with

chick embryo fibroblasts in culture. The analogue has been found to inhibit differen-
tiation in myoblasts (O'Neill & Stockdale, 1974) and chondrocytes (Daniel, Kosher,

Lash & Hertz, 1973) in vitro. Interestingly, addition of BrdU to 'virus-free' Balb/c-3T3 cell cultures has also been reported to lead to production of viral particles

(Aaronson, Todaro & Scolnick, 1971). One might speculate that the thymidine

analogue may be incorporated into DNA sequences as shown by Schwartz, Horio &

Kirsten (1974) with rat embryonic cells in culture. Such non-random incorporation

might unmask controlling gene sequences to interrupt differentiation or permit

expression of oncogenic information (Todaro & Huebner, 1972). This latter could

include increased agglutinability due to altered surface properties.

The preferentially inhibitory action of TLCK and TAME on proliferation of

transformed cells, shown previously with lines from other species, was demonstrated

here to apply also with human epithelia. Schnebli & Burger (1972) suggested that

protease inhibitors induce a reversion of transformed cells to a more normal pheno-
type. The action could occur by inhibiting active catabolism of surface proteins

(Borek et al. 1973). While such mode of action at the cellular level is not firmly

established (see for example Collard & Smets, 1974), the requisite of high surface

proteolytic activity for optimal proliferation of transformed cells is generally accepted

(Rapin & Burger, 1974; Collard & Smets, 1974).
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