INTERCELLULAR ADHESION AS A FUNCTION OF THE CELL CYCLE TRAVERSE

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ABSTRACT

Intercellular adhesion is assumed to play an important role in a multitude of biological phenomena governing cellular behavior. The rate of intercellular adhesion as a function of the cell cycle traverse has been investigated using, in the monolayer assay, synchronized Chinese Hamster Ovary-K1 cells. Results obtained demonstrate that cells in G1 adhere to G1 cells at twice the rate that S cells adhere to each other. G1 cells adhere to S cells at an intermediate rate. The additive adhesiveness seen in G1 is abolished by brief trypsinization, suggesting that in G1 a qualitative or quantitative change occurs with respect to the presence or exposure of components involved in intercellular adhesion.

MATERIALS AND METHODS

Materials

The following media were used unless stated otherwise:

Growth Medium (Medium A): The growth medium, Medium A, was prepared according to R. G. Ham (1965, Proc. Natl. Acad. Sci., 53:288). This medium (F12) was supplemented with 10% fetal calf serum (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) which had been heat-inactivated at 56°C until no glycosidase activity could be detected using p-nitro-phenyl-a//3-13-hexopyranosides (Sigma Chemical Co., St. Louis, Mo.) as substrates. The heat-inactivated serum was then dialyzed for 4 d against 4 × 6 liter Ca²⁺-, Mg²⁺-free phosphate-buffered saline (PBS). PBS contained (in

KEY WORDS intercellular adhesion • cell cycle • cell surface • glycoprotein

Intercellular adhesion is assumed to play an important role in such cellular functions as cell growth and motility (1, 2). In developmental biology, cell surface components are thought to be associated with such phenomena as induction, cell movement, morphogenesis, and growth regulation (3, 4). The molecular mechanism by which intercellular recognition and adhesion, most likely a multistep phenomenon (5, 6), occur, is however, still unknown, although many models have been presented that attempt to explain the phenomena (7, 8, 9).

Recent reports have demonstrated that cells have specific recognition sites. Homotypic and heterotypic adhesion by embryonic cells from different species occur at different rates (10) and specific anti-adhesion antibodies can be demonstrated (11). The main obstacle facing biochemical investigations of the mechanism of adhesion is the difficulty in obtaining sufficient material. In addition, specific adhesive components may be present only in one particular state of the cell cycle and thus totally escape detection if cells analyzed have reached a different point in the cell cycle traverse. We present data in this report to suggest that the rate of intercellular adhesion is a cell cycle-dependent phenomenon and that cell surface glycoproteins may play a role in the cell cycle-specific adhesion process.
grams per liter) NaCl, 8.00; KCl, 0.20; Na$_2$HPO$_4$, 1.15; KH$_2$PO$_4$, 0.20, and was adjusted to pH 7.2.

Dispersing Solution: Cell suspensions from confluent plates were obtained by washing the cell layers twice with PBS and incubating with a crude trypsin solution (GIBCO Solution A), containing 0.25% crude trypsin, salts, glucose, and phenol red indicator.

Incubation Medium (Medium B): Experimental incubations were performed in N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid-buffered (0.01 M) Hanks' balanced salts solution (pH 7.3) without phenol red but with 1 g/liter of D-glucose, 2% 50 x minimal essential medium essential amino acids, and 1% 100 x nonessential amino acids (GIBCO).

Arresting Medium (Medium A=): Medium A= had the identical composition of Medium A but with L-isoleucine and L-glutamine omitted.

Chemicals and Isotopes: All media were used prepared in this laboratory from the highest purity grade chemicals available (Sigma Chemical Co.: vitamins, amino acids, glucose, and lipoic acid; Fisher Scientific Co., Pittsburgh, Pa.: salts and trace metals). The following isotopes were purchased from New England Nuclear, Boston, Mass.: [methyl-$^3$H]leucine (20 Ci/mmole) and L-[3,4-$^3$H]leucine (30-50 Ci/mmole).

Tissue Culture Procedures: Proline-requiring Chinese Hamster Ovary Cells (CHO-K1 American Type Culture Collection) were grown in Medium A at 37°C in a water-saturated atmosphere of 95% air, 5% CO$_2$ (pH of media 7.0-7.2) on Falcon tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The cells reached a monolayer density of 275,000 cells/cm$^2$ with a generation time of 14 h.

For passage, confluent plates washed twice with PBS and incubated for 2-4 min with 1 ml of crude trypsin solution (see above), suspended in Medium A, and split 1:10. The cells were fed after 24 h and were split again after another 48 h. Experiments were carried out with cells between passages 2 and 10 and new cultures were initiated from stocks of sealed ampules stored in liquid nitrogen.

Methods

Synchronization of Suspension-Grown Cells: Cells from confluent plates were dispersed and suspended in Medium A=. After centrifugation for 4 min at 200 g, the pellet was resuspended in Medium A=. Falcon Petri dishes (No. 1023) were inoculated with 20 ml of Medium A= containing 6 x 10$^6$ cells. The cell concentration was established by counting in a hemocytometer. After 40-48 h of incubation, cells were released from G1 (12) by adding 10 ml of Medium A supplemented with additional isoleucine and glutamine to give a final composition equivalent to that of Medium A. Labeled cells were obtained by adding 40 μCi of [3H]leucine to the dishes at the time of release from the G1 arrest. The isotope was cleared 3 h before an assay.

Preparation of Synchronized Monolayers: Cells from confluent cultures were dispersed, centrifuged, and resuspended as described above in Medium A= to a concentration of 350,000 cells/ml. Each well in multiple-well dishes (FB 16-24 TC, Linbro Chemical Co., Hamden, Conn.) was inoculated with 1 ml of this suspension. After 40-48 h the cells were released from the G1 block by changing the medium to Medium A=.

Cells synchronized as described above were also prepared on Falcon tissue culture dishes by plating 175,000 cells/cm$^2$. [3H]Leucine was added at the times of release, and the isotope was chased 3 h before an assay.

Assay for Intercellular Adhesion: The method described by Walther et al. (13) was used to measure the rate by which labeled, synchronized single cells in suspension adhere to a synchronized monolayer of cells. Cells grown and labeled in suspension were washed once and resuspended in 20 ml of Medium B, to a final concentration of 100,000 cells/ml.

The monolayers to be used for adhesion studies, which were present in different stages of the cell cycle by having been exposed for different lengths of time to Medium A=, were washed twice with Medium B before adding 1 ml of the labeled cell suspension. The cells were incubated at 37°C on a reciprocal shaker at 60 strokes per min. The cell-cell interaction was interrupted by aspirating off the cell suspension and washing the monolayer twice with 1 ml of Medium B. After completion of an assay, 0.5 ml of 0.5 M NH$_4$OH was added to each of the wells. The plate was then sonicated for 10 min and the resulting solutions were transferred to scintillation vials with prewet Pasteur pipettes. This procedure was repeated twice more. For quantitation of the number of adherent cells, the radioactivity in 1 ml of the original cell suspension to which 0.5 ml of 1.5 M NH$_4$OH had been added was compared with that of the cell suspensions transferred from the different wells.

To determine whether any isotope leaked by the single cells was taken up by the monolayers, an aliquot of the labeled cell suspension was centrifuged after the experiment's completion, and the radioactivity in the supernate was determined. No more than 0.5% of the radioactivity of the cells was found in the medium after 30 min, and <1% of this radioactivity was taken up by monolayers over a 30-min time period.

Pulse Labeling Procedures: Multiple-well Linbro dishes with cells growing in synchrony after release from arrest in G1 were used. Cells were released at different times to provide duplicate cultures in different stages of the cell cycle. Medium A was changed to Medium A supplemented with 1 μCi/ml of [methyl-$^3$H]thymidine 1 h later the monolayers were washed twice with Medium B and incubated for 15 min with 0.5 ml of Medium B and 100 λ of 0.25% trypsin solution. 25 λ of serum was then added and the cell suspension was transferred to test tubes and the well was washed with 0.5 ml of Medium B. The cells were counted in a hemocytometer, disrupted by sonication for 30 s, and 1.1
ml of 10% cold TCA was added. The solution was left for 2 h on ice before filtering and washing. After drying, the filters were counted in 10 ml of scintillation fluid.

Synchronous cell suspension cultures (20 ml) at different stages of the cell cycle were pulsed with [methyl-3H]thymidine by adding 5 ml of Medium A supplemented with 30 μCi/ml. After 1 h, the cells were transferred to tubes, dispersed, and counted in a hemocytometer before pelleting at 200 g for 4 min. The cell pellets were disrupted by sonication in 1.5 ml of 0.5 M NH4OH and TCA-precipitated as above.

CONTINUOUS LABELING: Continuous labeling of synchronized cells was performed by adding radioactive precursors (1 μCi/ml for thymidine) to the medium when the cells were released from G1 arrest.

The monolayers were then treated as described for the pulse-labeling experiments to obtain incorporation data for thymidine.

RESULTS

Synchronization

Synchronous cells grown in suspension, after release from a G1 block induced by isoleucine and glutamine depletion for 48 h, have a generation time of ~22 h. The time span between release from the G1 arrest and completed cell division is 18 h (Table I). Some cells, however, divide within 16 h. The amount of thymidine incorporated during 1-h pulses suggests that a majority of the cell population is in the second G1 phase between 18 and 24 h after release. The loss of synchrony is apparent by 30 h, when 25% of the population has passed the second mitotic stage.

The degree of synchronization obtained by the starvation procedure in monolayer cultures was determined by releasing the cells in the presence of 1 μCi [3H]thymidine and establishing the accumulation of label after release from G1. Microscope observation demonstrated that after 12 h the cells have already divided. The majority of the cells traverse G1 in 12–18 h (Fig. 1). The increase and subsequent decrease in thymidine incorporation, seen both between 12 and 16 h and between 25 and 31 h after release, possibly suggest a turnover of DNA in G1, although to my knowledge such a turnover has not been reported. Cells grown in monolayers have a generation time of 14–16 h.

Adhesion to Synchronized Monolayers

SUSPENSION GROWN SINGLE CELLS. To establish the effect of stage of growth of monolayer cultures in the cell cycle traverse on the rate at which single cells would adhere to such monolayers, the following sets of experiments were performed: Monolayer cells arrested in G1 were released at 2-h intervals and used after 25 h for 1-h [3H]thymidine
incorporation experiments, and after 26 h for
adhesion studies. Fig. 2 shows that the monolayer
cells in G1 are more adhesive than cells in S phase.

The rate of adhesion of random single cells to
monolayers in G1, G1/S, and S phases was shown
to be linear over a 30-min period and, again, G1
monolayers are more adhesive than G1/S and S
phase cells (Fig. 3). The percent of cells adhered
may vary from experiment to experiment for a cell
in one particular phase of growth. The relative
rate of adhesion of single cells, however, is always
2:1.5:1 to the monolayers in G1, G1/S, and S
phase, respectively.

When both monolayers and adhering single
cells were synchronized and the adhesion of G1
and S single cells to both G1 and S monolayers
was measured in parallel cultures, the data shown
in Fig. 4 were obtained. The added adhesiveness
seen with the G1 monolayers is present also in the
synchronized single cell population and is additive.
That is, G1 single cells adhere to G1 monolayers
(G1-G1) at twice the rate that S single cells
adhere to S monolayers (S-S), and G1 singles
adhere to S monolayers (G1-S) at the same rate
that S singles adhere to G1 monolayers (S-G1).

TRYPSINIZED SINGLE CELLS: Trypsinization, a
standard procedure used to disperse monolayer
cultures, was employed to obtain single cell popu-
lations for adhesion studies using synchronized
monolayers. Monolayers in G1, G1/S, and S were
used. The adhesion data obtained demonstrate
(Fig. 5) that trypsinization affects neither the rel-
ative rate of adhesion seen with nontrypsinized
single cells nor the cell cycle specificity of the
phenomenon. The number of cells adhering, how-
ever, is dependent on the degree of enzyme diges-
tion. Cells trypsinized for 20 min will adhere at
the same relative rate to the monolayers in G1,
G1/S, and S, but only 2% of the single cell popu-
lation will adhere to the G1 monolayer after such
prolonged digestion (data not shown).

Adhesion experiments similar to those described
for suspension grown synchronized single cells
were conducted using synchronized trypsinized
single cells adhering to synchronized monolayers.
The results presented in Fig. 6 show that trypsin
Most models for intercellular adhesion suggest the interaction of complementary sites on the two interacting cells. The lock and key mechanism proposed by Tyler and Weiss (7, 8), as well as the glycosyltransferase acceptor-oligosaccharide model of Roseman (9), are some examples of such mechanisms. The total adhesion phenomenon, however, must contain additional reactions involving reorganization of membrane macromolecules with the end result being formation of tissue.

The in vivo sequence of events regarding cell division is reflected in the behavior of our experimental cells in vitro, in that the rate of adhesion of cells to monolayers is high in G1 and decreases as the cells proceed into the S phase. The increased adhesion observed in G1 is demonstrated by both the single cell and monolayer cell populations. Increased adhesiveness could be explained if cells in mitosis lost part of the glycosyloxyl (14, 15), and thereby exposed specific adhesive recognition sites as they enter G1. A quantitative increase in recognition sites or the appearance of one of two interacting sites in G1 would explain the observed elevated degree of adhesiveness. When trypsinized single cells adhered to monolayers, however, the latter were found to exhibit the same cell cycle specificity as observed with nontryptsinized cell suspensions. This observation may suggest that two complementary components are present on the cell surface in G1, but only one in S, and that one of them is readily removed by protease digestion, whereas the other is much less susceptible. The results may also suggest that the decreased adhesiveness is caused by disruption of other secondary phenomena involving macromolecular structures. We have not yet succeeded in deciding between these possibilities. On-going comparative adhesive studies in our laboratory with selected ricin-resistant clones have shown these not to vary in adhesiveness throughout the cell cycle traverse. Thus, comparing membrane glycoproteins in parental and lectin-resistant clones may aid in exploring the biochemistry of the demonstrated cell cycle variations in intercellular adhesion.

The skillful technical assistance of Ms. Brenda Snowman and Ms. Virginia Brown is gratefully acknowledged.

This work was supported by a grant CA-16865 from the National Institutes of Health, U. S. Public Health Service.

Received for publication 18 December 1978, and in revised form 30 March 1979.
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