Trypsin Effect on Intercellular Adhesion of Chick Embryo Fibroblasts

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Abstract. The paper shows that addition of trypsin (1—100 μg/ml) to a suspension of chick embryo fibroblasts dispersed from monolayer cultures at confluent state with EDTA gives rise to a pronounced cell aggregation. The aggregation begins just after the cells are brought in contact with trypsin and takes a few minutes. The trypsin-induced aggregation of chick embryo fibroblasts has two stages. At the first stage the amount of aggregated cells increases linearly with time and at the second stage, which occurs after about 10% of single cells have been aggregated, the rate of further aggregation increases sharply and nonlinearly. The total amount of the aggregated cells depends on the concentration of trypsin brought into the suspension and amounts to 40 to 80%. Data are presented, which support the suggestion that the second stage is induced by structural rearrangement of the plasma membranes, which involves cleavage of small but a critical number of peptide bonds on the cell surface.

Key words: Trypsin — Cell aggregation — Plasma membranes

Introduction

Adhesiveness is one of the most important properties of the cell surface which is of the utmost significance for some processes such as reproduction, morphogenesis, metastasing and invasive growth of malignant tumors. In spite of considerable advances made in the investigations of intercellular adhesion, the mechanisms which underly the mutual adhesion of the cells are still obscure.

Information on the intercellular adhesion mechanisms is mostly obtained from studies of trypsinised cells (Steinberg *et al.* 1973; Deman *et al.* 1974; Edwards *et al.* 1975; Mc Guire 1976; Wisseman and Hammond 1978). However beginning with Moscona's works (Moscona and Moscona 1952; Moscona 1956), trypsin has usually been used for nondissociated cells, i.e. monolayers, tissue fragments. Such a method involving quite durable subsequent operations for preparation of cell suspensions makes it impossible to follow the changes of adhesive properties of the cell surface at early stages immediately after the proteolytic action of the enzyme begins.

The present work contains results of the experiments in which trypsin was applied to tissue culture cells not in a monolayer but after their dissociation and suspending with EDTA. The data obtained testify to the fact that trypsin brought into chick embryo fibroblast suspension at a low concentration (1 to 100 μg/ml) induces a pronounced cell aggregation. Aggregation occurs at two stages and is accomplished within a few minutes. The effects observed are probably related to the structural rearrangement of plasmic membranes caused by proteolytic cleavage of a small but critical number of peptide bonds at the cell surface.

Some of the results obtained in our laboratory and concerning intercellular adhesion are included into the monograph (Konev and Mazhul 1977).

Material and Methods

Cells

In the experiments secondary cultures of normal 10-day old chick embryo fibroblasts were used. The cells were grown at 37 °C in 1500 ml glass dishes on 5% hemohydrolysate for tissue cultures (produced by the Belorussian Research Institute of Epidemiology, Microbiology and Hygiene, Ministry of Public Health, BSSR) with 10% calf serum. The medium was supplemented with penicillin at a final concentration of 100 units/ml. The initial seeding density was 5.3×10^5 cells/ml. The medium volume in a dish was 200 ml.

Preparation of cell suspensions

Single cell suspensions were prepared with 4 day-old confluent cultures. The cellular monolayers were washed three times with warm Hanks' solution and incubated for 10 min at 37 °C with 250 ml of 0.02% EDTA in Dulbecco's phosphate buffered Ca^{2+} and Mg^{2+}— free solution (pH 7.4). The cells were then washed three times with warm Hanks' solution, incubated for 3 min at 37 °C with 250 ml of deoxyribonuclease (5 μg/ml) and washed again with warm Hanks' solution. The cells were suspended by gentle shaking and pipetting in Hanks' solution. In order to remove nondissociated cells the suspension was filtered through a filter with 10 μm pore nylon. In experiments with Ca^{2+}— and Mg^{2+}— free media the cells were suspended in Hanks' solution without Ca^{2+} and Mg^{2+} or in 0.015 mol/l HEPES buffer prepared on 0.14 mol/l NaCl solution (pH 7.4). The test cell concentration of the suspension was 5×10^6 cells/ml. Such concentration provided optimal conditions for cell aggregation.

Formaldehyde and glutaraldehyde treatment

To the cell suspension in Hanks' solution formaldehyde or glutaraldehyde (Serva, FRG) was added to give the final concentrations of 2% and 0.1% for formaldehyde and glutaraldehyde, respectively. The cell suspension was then incubated for 2 min at 20 °C, centrifuged (500 g, 5 min, 4 °C) and washed three times with warm Hanks' solution. For preparation of the test suspensions the fibroblasts treated with formaldehyde or glutaraldehyde were suspended in Hanks' solution at a concentration of $5 . 10^6$ cells/ml. Cell viability was checked by the standard trypan blue exclusion test (Paul 1970).

Aggregation measurement

The experiments were carried out during the first two hours after the cell suspension had been prepared. The amount of aggregated fibroblasts was estimated by measuring the number of single cells lost from the suspension. Kinetics of the trypsin-induced aggregation was measured with the nephelometric method following the two procedures (A and B).

Procedure A

The aggregation was measured by continuous recording the light transmission at 540 nm in a rotating suspension on a spectrophotometer VSU2-P with a KSP-4 automatic recorder. The test cell suspension of 1 ml was placed in the thermostatted spectrophotometer cuvette, 2 ml in volume. The suspension was rotated with a magnetic stirrer at a rotation speed of 120 rpm. Linearity of the device was controlled with a hemocytometer. In all the experiments crystallized trypsin (Spofa, ČSSR) with a specific activity of 3750 TU/g (by casein test) was used and 0.1 ml of trypsin solution prepared on Hanks' solution was added to the suspension. Corrections were made for the respective dilution of the suspension.
The enzymatic activity of trypsin was inhibited by contrical ("Germed", DDR), which is a competitive protease inhibitor isolated from cattle lungs. In the present experiments trypsin was inhibited with a two-fold contrical amount which was brought into the cell suspension as a solution (0.1 ml in Hanks' solution).

Procedure B

The measurements in a millisecond range were performed on a stopped-flow instrument, the "Durrum 100" type (USA) where equal volumes (0.2 ml) of fibroblast suspension and trypsin solution were mixed almost instantly.

All the experiments which followed procedures A and B were performed at 20 °C.

Results

In the present experiments chick embryo fibroblasts dispersed from monolayer culture with EDTA and suspended in Hanks' solution exhibited a relatively low rate of adhesion since for the first 30 min only about 10% of the cells aggregated. After trypsin at a concentration of 1 to 100 μg/ml (0.001 to 0.01%) had been brought into the rotating fibroblast suspension, the adhesiveness of cells increased sharply which was manifested by their coalescence and formation of large aggregates (Fig. 1).

The trypsin-induced aggregation has two characteristics: its rapid development (it is accomplished within a few minutes) and occurrence of two stages of the process. At stage I the number of the aggregated cells increased linearly with time. After the critical level corresponding to a loss of 10% of the single cells from the suspension was reached, a sharp and nonlinear increase of fibroblast incorporation into aggregates was observed, i.e. stage II of the trypsin-induced aggregation occured. The total number of the cells incorporated by aggregates, after stage II had been completed, was 40 to 80% (Fig. 2).

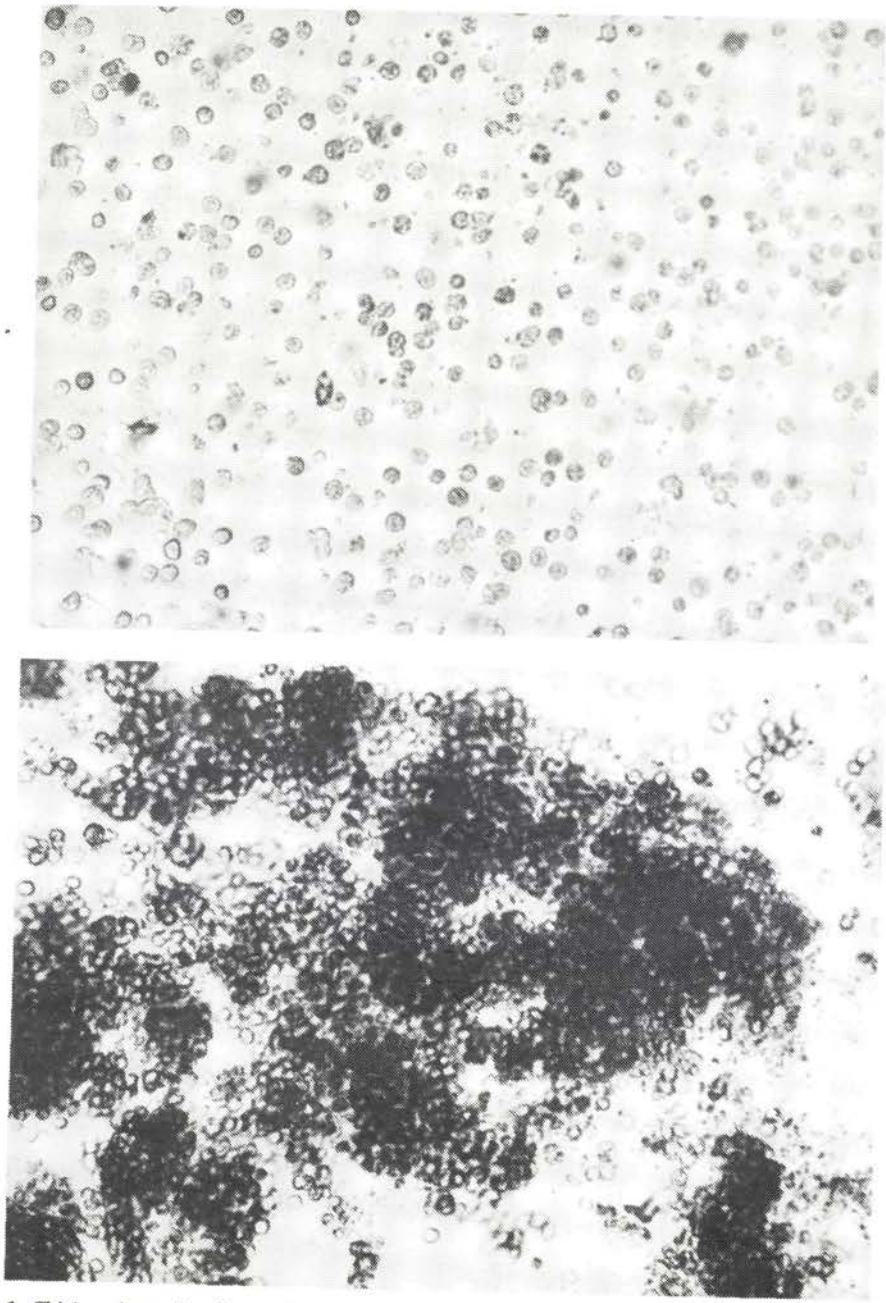

Fig. 1. Chick embryo fibroblasts dispersed from the monolayer culture with EDTA and suspended in Hanks' solution (*above*) before trypsin treatment; (*below*) 2 min after trypsin at a final concentration of 10 μg/ml was brought into the the cell suspension.

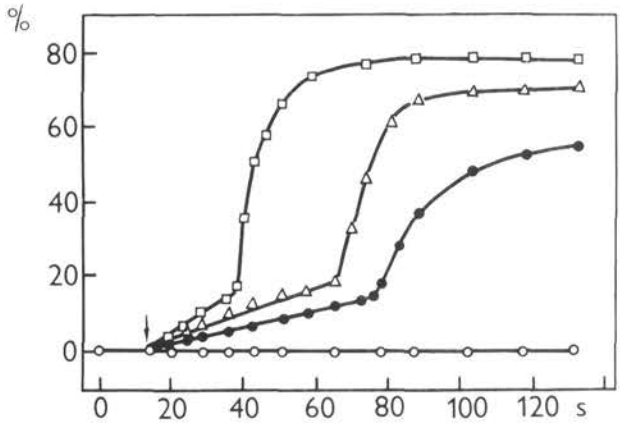

Fig. 2. Kinetics of trypsin-induced aggregation of chick embryo fibroblasts shown as percent of aggregated cells versus time. The tissue culture cells were dissociated with EDTA and suspended in Hanks' solution. Trypsin solution was brought into the prepared suspension. The arrow shows the moment of trypsin addition. The final trypsin concentration in the suspension: 5 μg/ml (● — ●); 10 μg/ml (△ — △); 50 μg/ml (□ — □). Trypsin-free suspension was used as control (○ — ○).

The duration (t) of stage I depended on the added enzyme amount: it decreased as the trypsin concentration increased, the most pronounced changes in the $t = f(C_{tr})$ curve being found at $C_{tr} = 1$ to 10 μg/ml (Fig. 3a). The fraction of the cells incorporated by the aggregates after completion of stages I and II varied with the trypsin content in a similar way: over the concentration range $C_{tr} = 1$ to 10 μg/ml incorporation of the fibroblasts by the aggregates increased sharply, then $C_{cell} = f(C_{tr})$ curve became virtually a plateau (Fig. 3b).

Similar relationships of the trypsin-induced aggregation were observed also in the case when measurements were made not in a rotating suspension but at the stopped-flow instrument where the cell suspension and trypsin solution were quickly mixed. The aggregation also appeared to be a two-stage process. During stage I a monotonous, rather slow decrease of single cell concentration is observed (due to cell incorporation into aggregates) and at stage II the rate of this process increases abruptly. In a millisecond range the rise of the cell adhesiveness was shown to proceed immediately after trypsin had been added.

An additional trypsin portion brought into the suspension at stage I accelleretes transition of the cells to a highly-adhesive state. Trypsin addition at stage II has however no effect on the trypsin-induced aggregation kinetics (Fig. 4a).

The experiments using contrical revealed that suppression of the trypsin enzymatic activity by the protease inhibitor at stage I blocks further development of the process. If the protease inhibitor is added into the suspension a few seconds after completion of stage I or during stage II of the trypsin-induced aggregation, i.e.

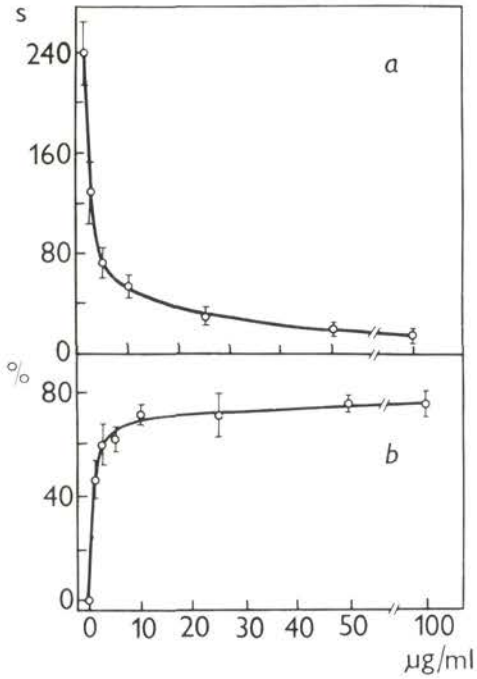

Fig. 3. (a) A plot of the time of stage I of the trypsin-induced aggregation of chick embryo fibroblasts versus the trypsin concentration added to the suspension. (b) A plot of the number of cells which were incorporated into the aggregates after stages I and II of trypsin-induced aggregation of chick embryo fibroblasts had been completed versus the trypsin concentrations added to the suspension.

after the critical level of membrane protein proteolysis is reached, the avalanche —like growth of the cell number in the aggregates smoothly continued (Fig. 4b, curves 2—5). As demonstrated by the present experiments, the trypsin-induced aggregation is independent of calcium and magnesium cations which may be present in the medium. The aggregation kinetics in completely balanced Hanks' saline, in Ca^{2+}— and Mg^{2+}—free Hanks' solution and in HEPES buffer were essentially the same upon introduction of equal trypsin portions. The aggregation factor molecules released into the medium also do not participate in the development of the intercellular adhesion contacts, since the supernatants obtained from trypsin-treated suspensions (after contrical had inactivated the enzyme) do not induce aggregation of the trypsin-untreated fibroblasts which were suspended in them for the second time.

When ribonuclease or protamine sulphate (proteins with polycationic properties) was added into the suspension instead of trypsin no cell aggregation was found

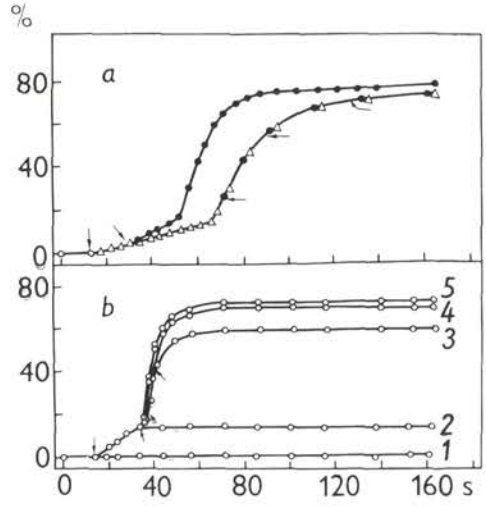

Fig. 4. (a) The trypsin re-addition effect on the kinetics of stages I and II of trypsin-induced aggregation of chick embryo fibroblasts. The tissue culture cells were predissociated with EDTA and suspended in Hanks' solution. The trypsin solution was brought into the prepared suspension at stage I (upper curve) and at different substage II (lower curve). The vertical arrow common for both curves shows the time when the first trypsin portion was added; the sloped arrow shows the second trypsin addition (into the same suspension) at stage I; the horizontal arrows indicate the second trypsin addition (each time into new suspension) at different substages of stage II. The final trypsin content in the cell suspension: 0 μg/ml (○ — ○); 10 μg/ml (△ — △); the control curve 20 μg/ml (● — ●). (b) The effect of contrical (the protease inhibitor) on the trypsin-induced aggregation development in chick embryo fibroblast suspension. The tissue culture cells were dissociated with EDTA and suspended in Hanks' solution. The vertical arrow, common for curves 1 to 5 marks the moment when trypsin at a final concentration of 50 μg/ml was brought into the suspension. The sloped arrows at curves 1 to 4 show the moment when contrical was added to the suspension. Contrical added (1) before trypsin; (2) at the end of stage I; (3) at the beginning of stage II; (4) in middle of stage II; (5) control (without contrical).

then. There was no aggregation also in the case when trypsin was brought into the cell suspension after protease inhibitor had been added (Fig. 4b, curve 1).

The cells pretreated with 0.1% glutaraldehyde or 2% formaldehyde completely lost their ability of trypsin-induced aggregation. As follows from the trypan blue exclusion tests, in those conditions the cells preserve their viability.

Discussion

As is known, trypsin does not penetrate into the cell (Turner and Burger 1973; Carney and Cunningham 1977) and therefore all the primary events take place at the cell surface.

The trypsin-induced effects are not a mere formation of complexes with the

negatively charged cell surface, since protamine-sulphate and ribonuclease which, just like trypsin, possess polycationic properties, did not induce fibroblast aggregation. On the other hand, aggregation was not observed also in those cases when the fibroblast suspension had been treated with inactivated trypsin. Thus, the sharp increase of the rate of cell adhesion may be attributed to the proteolytic activity of trypsin.

That trypsin-induced aggregation also develops in the absence of calcium and magnesium cations as well as of the aggregation factor implies that adhesion of the cells occurs because of interactions of the cell surfaces, no molecular links being involved.

Structural lability of the membranes and ability of protein components to lateral mobility is of great importance in the formation of adhesion contacts and in the development of trypsin-induced aggregation. The aggregation is completely arrested when the mobility of the membrane proteins is limited by glutaraldehyde or formaldehyde.

It may be thought that the cells are brought into the state of a "high adhesive activity", which is characteristic of stage II, because of structural rearrangement of cytoplasmic membranes induced by cleavage of the critical number of the peptide bonds in the membrane proteins. This is indicated by a sharp deviation of the aggregation kinetic curve from linearity after stage I was completed and by the effects of trypsin and contrical addition on the kinetics of stages I and II. As can be seen from Fig. 4a, after another trypsin portion was added to the cell suspension at stage I, transition of the cells to a high adhesive state became accelerated. However, when stage I had been completed, at a high level of proteolysis which lead to structural rearrangement of cytoplasmic membranes, the cells became trypsin "insensitive" as at stage II trypsin had no effect on the aggregation kinetics.

Similar results have been obtained in the experiments with contrical (Fig. 4b, curves 2—5). Suppression of the trypsin enzymatic activity at stage I inhibits the process development, however contrical introduced after stage I was completed or during stage II has a slight effect on the trypsin-induced aggregation kinetics. This is evidence for spontaneous aggregation at stage II, i.e. this stage does not require additional cleavage of the peptide bonds for its development.

The number of peptide bonds which must be hydrolyzed in order to bring the cytoplasmic membranes to a new structural state is probably small as is shown by a high aggregation rate and a low effective concentration of the enzyme of 1 to 10 μg/ml (0.0001 to 0.001%).

It is important that the cell aggregation is induced by the same trypsin concentrations (1 to 10 μg/ml) which cause a number of properties of the transformed cells to appear in normal fibroblasts: the block of contact inhibition of reproduction is eliminated, the lateral mobility of the receptors of concanavalin A and other lectins and agglutinability due to lectins increases, the arrangement of

the cytoskeleton system units is disturbed (Turner and Burger 1973). It may be noted that just as in the present experiments the time of trypsin treatment necessary for the above effects to manifest themselves amounts to a few minutes. Some data have been recently reported showing that the transformation is accompanied by an increase of adhesiveness when the contacts are formed de novo (Cassiman and Bernfield 1975; Wright et al. 1977). Treatment of normal fibroblasts by trypsin at low concentrations makes them similar to the transformed cells in yet another parameter, i.e. in the ability of forming intercellular adhesion contacts.

Thus, the authors have obtained data which support the idea that trypsin-induced cleavage of a small but a critical number of peptide bonds of the proteins on the cell surface of chick embryo fibroblasts results in a transition of cytoplasmic membranes into a new structural state characterized by a great ability of intercellular adhesion.

Further information which proves the existence of structural rearrangement of the chick embryo fibroblast membranes induced by low trypsin concentrations gained with help of electron microscopy (freeze-fracture), by registration of room-temperature tryptophane phosphorescence and from measured electrophoretic mobility of cells will be presented in the following paper.

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