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Rapid aggregation and tight junction formation in single cell suspensions of tumor cells after very low dose trypsin treatment

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The possible participation of tight junction formation in intercellular adhesion of tumor cells was studied in single cell suspensions of HT29 adenocarcinoma cells. Very low dose trypsin treatment (0.15 μg/ml, 30 min, 37°C) induced rapid intercellular adhesion of suspended HT29 colon carcinoma cells. Intercellular adhesion was independent of the presence of divalent cations. Electron microscopy of freeze-fractured membrane fragments of trypsin-treated HT29 cells revealed a dramatic increase of typical tight junction structures during aggregation.

Aggregation; Tight junction; Colon carcinoma cell; Trypsin

1. INTRODUCTION

The tight junction (zonula occludens), a belt-like region of contact on the apical pole of epithelial cells, serves as a barrier controlling the paracellular pathway of large molecules. On freeze-fracture replicas of fixed and glycerinated membranes tight junctions appear as strands on the protoplasmic face (PF) and as corresponding grooves on the exoplasmic face (EF) [1]. A variety of experimental conditions have been reported to induce tight junctions in cultured cells or tissues, e.g. treatment with proteases [2-4], ammonium salts [5] or Triton X-100 [6]. The human adenocarcinoma cell HT29 in culture virtually grows without tight junctions. After short trypsin treatment (1-2.5 mg/ml, 15 min, 37°C) of adherent confluent cells grown on Petri dishes for 4-5 days the formation of tight junctions on 50-60% of plasma membranes counted on freeze-fracture replicas has been found [3]. Possibly trypsin treatment is an initial signal for the assembly of preformed structural elements of the junction, because tight junction formation is not diminished by incubation with protein synthesis inhibitors [7,8].

In order to further study the relationship between tight junction formation and intercellular adhesion of tumor cells we have examined the trypsin-mediated processes in suspensions of single tumor cells after their detachment from culture dishes. In such a system both aggregation and tight junction formation can be followed. Rapid tight junction formation and aggregation of suspended tumor cells was observed after very low dose trypsin treatment (0.15 μg/ml).

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2. MATERIALS AND METHODS

2.1. *Cell culture*

HT29 colon carcinoma cells obtained from ATCC (HTB 38) were maintained in cell culture as described [9]. Cells were harvested at their 6th or 7th day of culture by short treatment with 0.25% EDTA, rescued with their own medium and washed with phosphate-buffered saline (PBS).

2.2 *Trypsin treatment and aggregation assay.*

Cells (1.5×10^6) were suspended in 1 ml PBS containing up to 0.25 μg trypsin (Boehringer, Mannheim, Germany; 110 U/mg) and incubated within a pasteur pipette. Aggregation of cells was measured by sequential passaging of cells through several gauze nets with a mesh width of 100 μm, 40 μm and 10 μm, respectively. Due to their elasticity single cells were found only in the filtrate. The number of cells retained by the nets or collected from the filtrate was determined by measuring the protein content (Biorad, München, Germany). An aggregation factor was calculated, which is helpful to condense the result into a single figure:

$$\text{Aggregation factor (AF)} = (100 \times \text{protein}_{100\,\mu m} + 50 \times \text{protein}_{40\,\mu m} + 20 \times \text{protein}_{10\,\mu m} + 10 \times \text{protein}_{\text{filtrate}})/\text{total protein}$$

Possible AF-values range from AF = 10, equivalent to 100% single cells, up to AF = 100, which would mean that 100% of the cells were retained by the 100 μm net [9]. Viability of cells remained constant during the incubation period as shown by determination of lactate-dehydrogenase (LDH) activity in the supernatant. For inhibition of tryptic activity phenylmethylsulfonylfluoride (PMSF) was used (Sigma, München, Germany).

2.3. *Freeze-facturing*

Cells were pelleted by centrifugation and fixed with 2.5% glutaraldehyde for at least 3 h. The fixed material was infiltrated with a graded series of glycerol in PBS (final concentration 30% v/v), mounted on specimen disks and frozen in liquid propane. Specimens were processed in a Balzers BAF 300 freeze-etch unit. Fracturing was carried out at −100°C followed by etching for 30 s prior to platinum/carbon shadowing. Replicas were cleaned with chromic acid and examined with a Philips 301 electron microscope operated at 80 kV. Tight junctions were quantified as described [7]. Briefly, plasma membranes with and without tight junctions were counted in

freeze-fracture replicas. Every membrane fragment which could be unequivocally identified was taken as one unit irrespective of size. A minimum number of 125 membranes was evaluated. The occurence of tight junctions on membranes is given in % of totally counted membranes.

3. RESULTS

3.1. *Very low dose trypsin treatment induced rapid aggregation of suspended HT29 cells*

Cells were harvested as described and treated with 0.01–0.25 μg trypsin for 30 min at 37°C. As depicted in Fig. 1 even at a concentration of 0.1 μg/ml, which corresponds to about 4 nM, trypsin induced homotypic aggregation of suspended HT29 cells (Fig. 2a,b). Viability of cells was not impaired as determined by Trypan blue exclusion (data not reported) and by measuring LDH-activity in the medium (Table I). Aggregation started rapidly after addition of trypsin. Already after 5 min incubation with 0.15 μg trypsin/ml cells showed aggregation (number of experiments: n = 4; aggregation factor: AF = 16.6 ± 2.4) compared to untreated cells (n = 4, AF = 11.5 ± 0.3). Trypsin-induced aggregation was inhibited by adding 1 mM PMSF (n = 3, AF = 12.1 ± 0.3).

3.2. *Very low dose trypsin treatment induced tight junction formation of suspended HT29 cells*

Cells were treated as above and incubated with 0.15 μg trypsin in 1 ml PBS for 30 min at 37°C. Counting the number of formed tight junctions on freeze-fractured membrane segments showed that 6.5% of the native cells, but 88.5% of trypsin-treated cells carried the typical components of tight junctions (Fig. 3).

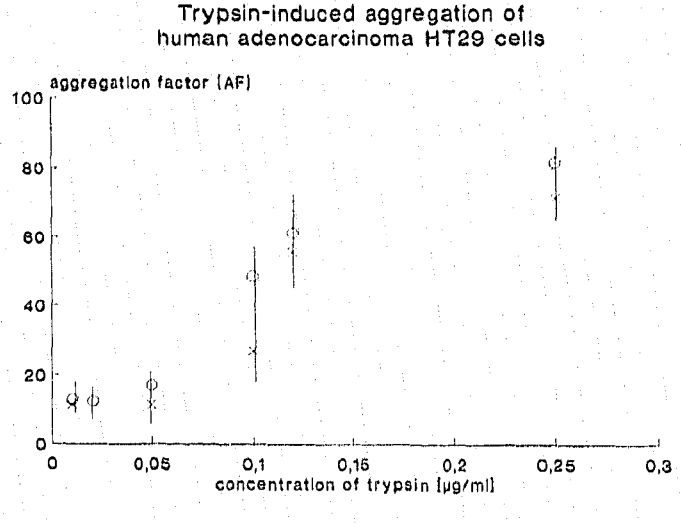

Fig. 1. Aggregation of human adenocarcinoma HT29 cells after treatment with several trypsin concentrations for 30 min at 37°C (○) or for 1 h at room temperature (×) (each point represents means of 5 experiments; vertical bars show SD).

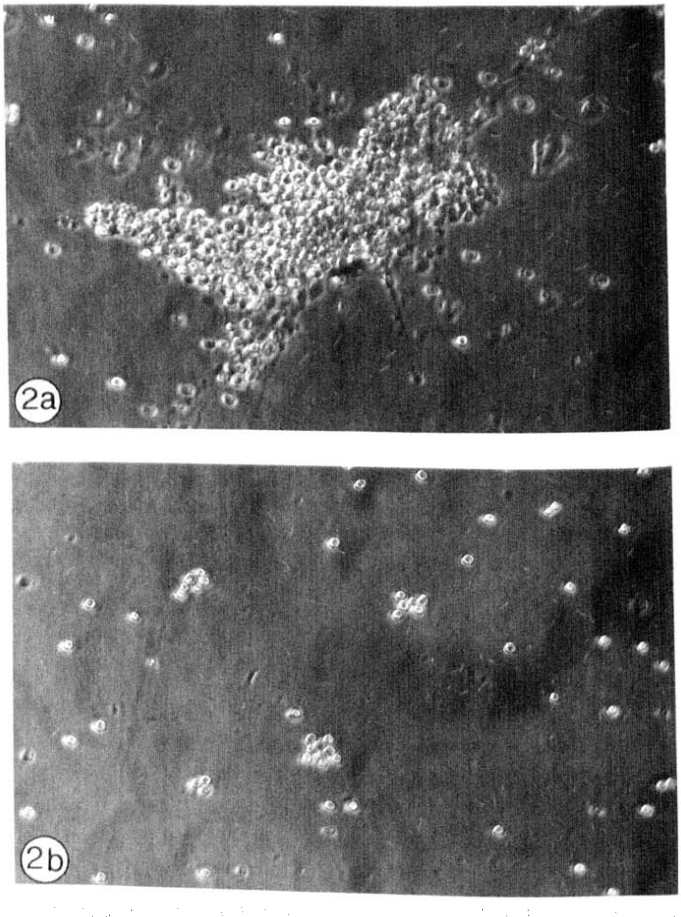

Fig. 2. (a) Light microscopy of aggregates of HT29 cells after treatment with 0.15 μg trypsin/ml for 30 min at 37°C. (b) Control without enzyme treatment.

3.3. *In the presence of Ca^{2+} ions trypsin-induced homotypic aggregation of HT29 cells is reduced*

Harvested cells were treated with 0.15 μg trypsin for 30 min at 37°C. Co-incubation of HT29 cells with $CaCl_2$ in concentrations above 1 mM reduced trypsin-induced aggregation (Table II). As expected, similar

Table 1

Determination of cell viability by measuring lactate dehydrogenase (LDH) activity.

	mU LDH/10^6 cells	(%)
Cells after 15 s sonification	1233 ± 60	100
Untreated cells	110 ± 15	8.3
Trypsin-treated cells	118 ± 22	9.0
Trypsin-treated cells, 1 mM $CaCl_2$	252 ± 18	19.1
Trypsin-treated cells, 2 mM $CaCl_2$	373 ± 33	33.6
1 U LDH in 1 ml PBS containing 0.15 μg protein	950 ± 101/ml	

All values are means of 3 experiments.
Cells (1.5×10^6) were treated with 0.15 μg trypsin for 30 min at 37°C. Aggregated cells were pelleted by centrifugation and LDH-activity was determined in the supernatant according to standard procedures [19]. Low LDH-activity in the supernatant is taken as a measure for high viability.

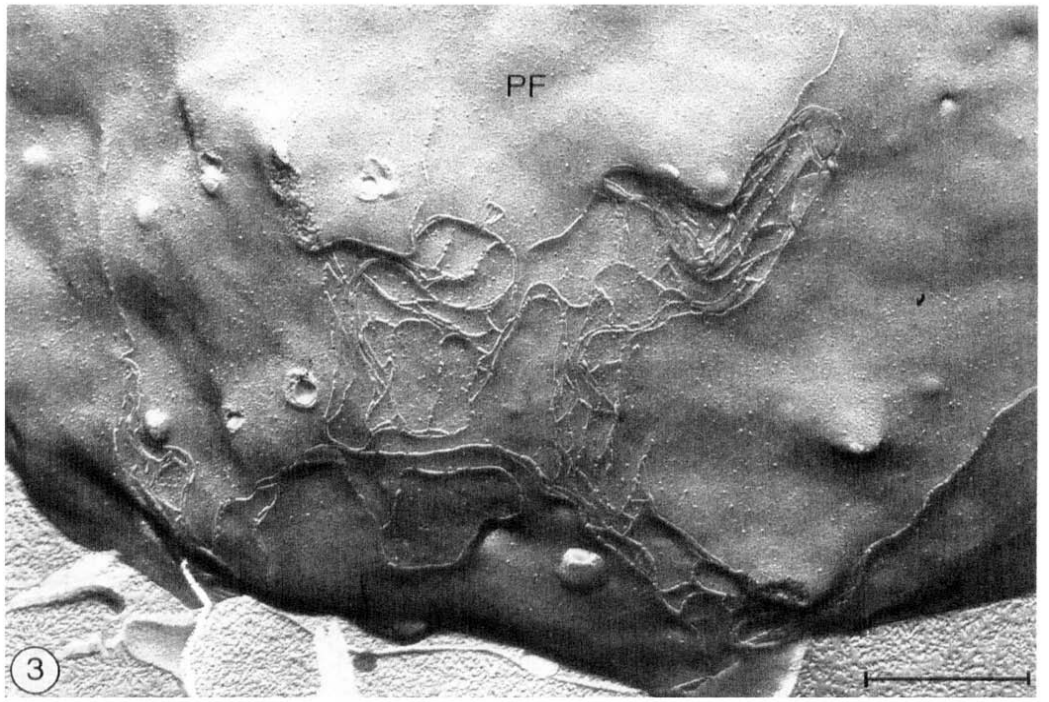

Fig. 3. Electron micrograph of a freeze-fractured membrane-fragment of HT29 cells treated with 0.15 μg trypsin/ml for 30 min at 37°C, showing the typical strand formation of tight junctions. PF, protoplasmic face. Bar = 500 nm.

concentrations of $CaCl_2$ are without any effect on the caseinolytic activity of trypsin (data not shown). Cell viability was measured by determination of LDH-activity in the supernatant of treated cells (Table I). Assuming that all cells are destroyed by sonication for 15 s, LDH-activity measured in the supernatant of sonicated cells was set equivalent to 100% dead cells. Trypsin-treatment decreased viability only slightly (91% viable cells; Table I, second column) compared to untreated cells (91.7%) but viability was reduced to 72% after incubation with 2 mM $CaCl_2$.

4. DISCUSSION

The presented data show that a brief and very low dose treatment of HT29 colon carcinoma cells in suspension induced strong aggregation and the formation of tight junctions. In general, tight junctions are thought to act primarily as an occluding barrier in epithelial cell layers established after adhesion between cells has taken place [10]. In MDCK-cells tight junction formation was found to be a secondary event which follows Ca^{2+}-dependent adhesion mediated by the cadherin uvomorulin [11,12]. From work on adherent, confluent HT29 monolayers it is known that already 5 min after trypsin treatment (1 mg/ml) tight junction formation has started [2] and that this process is not Ca^{2+}-dependent [4]. Our presented data on HT29 cells in suspension show that a 5 min incubation with 0.15 μg trypsin is sufficient to induce aggregation. This process depends on the catalytic activity of trypsin, because it was inhibited by addition of 1 mM PMSF. Calcium was not necessary. Moreover, incubation with 2 mM $CaCl_2$ led to reduced aggregation, but also to reduced viability of the cells. It has been found that Ca^{2+} is able to protect cadherins from proteolytic degradation [13]. Whether Ca^{2+} protects any proteins with adhesive potential from proteolytic activation (by trypsin or by trypsin-activated surface-bound proteases) or whether the reduced viability of the cells interferes with aggregation remains to be elucidated.

Table II

Incubation with $CaCl_2$ reduces aggregation of HT29 cells after treatment with 0.15 μg trypsin/ml for 30 min, 37°C

	Aggregation factor
control	79.8 ± 7
$CaCl_2$ 0.5 mM	74.4 ± 14
1 mM	69.0 ± 13
2 mM	44.1 ± 11

Means of 7 experiments.

In summary, the data show that Ca^{2+}-dependent adhesive proteins like the cadherins are not involved in trypsin-induced tight junction formation as in the case of MDCK-cells and that aggregation and tight junction formation are processes which occur simultaneously. The results obtained so far do not exclude the possibility that trypsin acts by inducing aggregation of suspended HT29 cells which then would give rise to tight junction formation. In this case, mild aggregation but no tight junction formation would be expected during the

very first minutes, followed by a massive increase in tight junction formation and tight junction provoked aggregation. However, the early start of tight junction formation suggests that aggregation is not a prerequisite for tight junction formation but rather a consequence of this process.

Very low dose trypsin-induced aggregation could be of physiological relevance, e.g. with regard to the formation of tumor metastases. An important step in metastasis of tumor cells is their dissemination in lymphatic and blood vessels. It has been speculated that intercellular adhesion of tumor cells facilitates their attachment to target organs [14,15]. Normally, serum contains a number of protease inhibitors which suppress the action of endogenous serine proteases like trypsin. However, it has been found [16] that in cancer patients with distant metastases 70% of the serum-α-proteinase inhibitor is inactivated, which is responsible for 90% of the serum trypsin-inhibitory capacity.

The molecular structure of tight junctions is still under investigation, although two of its elements have been characterized - the ZO-1 protein [17] and cingulin [18]. The results presented may establish the aggregation assay as a rapid tool to search for new drugs or antibodies interacting with tight junction formation. This may help in elucidating structure and function of the zonula occludens.

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