Some Aspects of the Mode of Action of the Aggregation Promoting Factor Contained in the Conditioned Medium

KEI TAKAHASHI*

Department of Biophysics, Faculty of Science
Kyoto University, Sakyo-ku, Kyoto, 606 Japan

Abstract The presence of the factor which promotes cell aggregation in the conditioned medium (CM) prepared with a large number of cultured chicken embryonic cells was revealed by measuring the rate of aggregation based on a kinetics of the decrease of the number of total particles from the system. Surface negativity of fibroblasts of cultured chicken embryonic lung cells was increased by the treatment with CM. This was shown by comparing the binding capacity of cell surfaces with positively charged colloids, protamine sulfate, between cell pretreated with the factor from CM and non-treated cells. The pretreated cells with increased surface negativity were aggregated with faster rate than other cells only in the presence of Ca^{2+}. Fixed cells by glutaraldehyde attained higher surface negativity, after the treatment with CM. A possible role of Ca^{2+} on cell aggregation was discussed, particularly in relation with a role of the aggregation promoting factor contained in CM.

Introduction

Several factors have been known to influence cell-to-cell or cell-to-substrate adhesion of singly dissociated cells (Fisher et al. 1958; Orr and Roseman, 1969a, b; Curtis, 1969). However, chemical properties of active substances as well as their mode of action upon cell adhesion have not well been elucidated. Takahashi and Okada (1971) have shown that conditioned medium (CM) harvested from primary cultures of a large number of chicken embryonic cells contains at least two effective factors acting differently. The one is to promote cell-to-cell adhesion (aggregation) as well as cell-to-substrate adhesion, whereas the other enhances a growth rate of sparesely inoculated cells in cell culture. These factors were separated through Sephadex G–25 gel into two separate fractions, α and β. Substances with similar activities are included also in the serum and other sources (Fisher et al. 1958; Orr et al. 1969a, b; Curtis and Greaves, 1965; Rubin, 1969; Perterson and Rubin, 1969; Oellerman and Miller, 1969; Virolainen and Deffendi, 1967; George et al., 1971). This paper deals with an investigation of surface negativity of cells treated by the aggregation promoting factor(s) contained in α-fraction of CM. The increased surface negativity was revealed by employing colloid titration technique (Terayama, 1952). The action of divalent cations for realizling the function of this factor was also studied.

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*Present address: Department of Physiology, Shimane Medical University, Izumo, Shimane, 693, Japan

Materials and Methods

1. *Materials*

Muscles and lungs of chicken embryos at 11-days of incubation were used.

2. *Preparation of conditioned medium (CM) and the culture for assaying the effect of CM*

The procedures to obtain CM as well as cells to be used for assaying the effect of CM were the same as given in the previous papers (Takahashi & Okada, 1970; 1971). In brief, CM was harvested from primary cultures of a large number of 11-day-old chicken embryonic muscle cells. CM^{3-4} and CM^{5-6} were the medium which had been conditioned with cells between 3 to 4 and 5 to 6 days of culturing, respectively. The cells used for assaying CM were harvested from the monolayer sheet of fibroblasts in primary cultures of 11-day-old chicken embryonic lungs.

3. *Preparation of glutaraldehyde (GLA)-fixed cells*

In some experiments, fixed cells were used for assaying the action of CM. After harvesting cells with trypsin, they were washed with cold Hanks' balanced salt solution (HBSS, pH 7.3) two times by centrifugation. The cell suspension was mixed with the same amount of cold glutaraldehyde (GLA) in HBSS (5%, pH 7.3) and incubated without shaking for 10 minutes in ice cold HBSS. They were dispersed again in cold M/4 saccharose-0.01 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) solution, pH of which was adjusted to be at 7.3 by 0.1 M NaOH. Thus prepared fixed cells were stored 4°C and they were washed with cold HBSS to remove saccharose immediately before use.

4. *Preparation of Sephadex G-25 gel excluded (α) and retarded fractions (β)*

By filtrating CM^{5-6} through Sephadex G–25 column, two main fractions associated with the effect for promoting the plating efficiency (α fraction) and the growth rate of cells inoculated *in vitro* with low density (β fraction) were separated, according to the procedure given in the previous paper (Takahashi & Okada, 1971). Each of these two fractions were used for the experiments described below.

5. *Cell aggregating medium*

Cells were allowed to aggregate in HBSS, to which, CM^{3-4}, CM^{5-6}, α-fraction, β-fraction or the mixture of α and β was added. By adding an appropriate amount of 10% NaHCO3 solution, pH of these media was adjusted at 7.3. For the test examining the effect of divalent cations upon aggregation, various amounts of calcium or magnesium chloride were dissolved in Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution (CMF–HBSS), the pH of which was adjusted to be at 7.3 with 0.01 M HEPES and 0.1 M NaOH.

6. *Measurement of cell aggregation*

Aggregation was expressed by the rate of disappearance of single cells in a given system, as described in the previous paper (Takahashi & Okada, 1971). For closer

analysis of the initial stage of cell aggregation kinetics within about 30 minutes after the start of cell aggregation, the measurement was done by treating the aggregation as a bimolecular rate process (cf. Wilkins et al., 1962b). Therefore, in the present study, a following mathematical simplication of the process of aggregation was introduced for a kinetics of a decay of the total particle concentration,

$$1/N_{\infty(t)} = 1/N_{\infty(0)} + kt$$

where $N_{\infty(t)}$ = the number of total particles including both cell aggregations and single cells found at time t, whereas $N_{\infty(0)}$ is the total number of single cells at time 0. When $1/N_{\infty(t)}$ was plotted against time t, the slope k may indicate the rate of cell aggregation. The steeper the slope is, the higher the cell adhesiveness is thought to be, and *vise versa* (Fig. 1). Therefore, in the experiments to follow a kinetics of cell aggregation, $1/N_{\infty(t)}$ was plotted directly against time t.
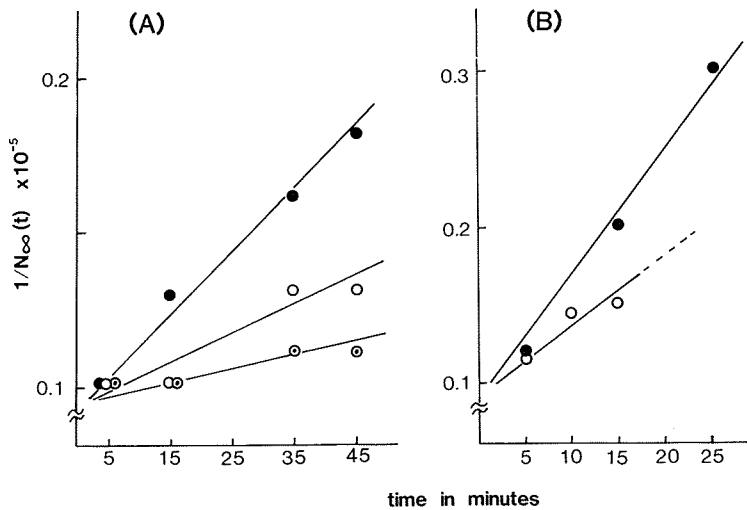

Fig. 1. A typical example of cell aggregation kinetics. 3.3×10^6 cells were inoculated in an Ehrlenmyer flask containing 3.0 ml of the aggregating medium to be agitated at 90 rpm at 37°C on a Gyratory shaker, and the number of total particles were counted every 5 minutes.

A. Ordinate : Rate of cellular adhesiveness ($1/N_{\infty(t)}$, see in the text). Abscissa : time in minutes. —●—●— , cell aggregation in the presence of α fraction; —○—○— , cell aggregation in HBSS; —⊙—⊙— , cell aggregation in the mixture of α and β fractions.

B. —●—●— , cell aggregation in the presence of Ca^{2+} ($1.8 \times 10^{-3}M$); —○—○— , cell aggregation in CMF–HBSS. Cells were pretreated with CM^{5-6} (α).

In the experiments to examine the effect of divalent cations on cell aggregation, $1/N_{\infty(t)}$ at 30 minutes after the start of cell aggregation, instead of plotting $1/N_{\infty(t)}$ to time course, was plotted against various concentrations of added cations. In all aggregation experiments, 3.3×10^6 cells were inoculated in each Ehrlenmeyer flask containing 3 ml of the aggregating medium and the flasks were agitated on a Gyratory shaker at 90 rpm at 37°C. The number of total particles at a given time was counted by hemocytometer.

7.   Adsorption of polycations on cell surfaces

Surface negativity was measured by the method of colloid titration by means of volumetric titration of polymer ions (Terayama, 1952). Protamine sulfate (PS) was used as positive colloid, whereas pottasinm salt of polyvinyl sulfate (PVSK), as negative colloid in a solvent. They were dissolved each in M/4 saccharose solution and pH was adjusted at 7.3 by adding 0.01 M of HEPES and 0.1 M of NaOH (saccharose-HEPES–NaOH buffer). In the control test, a definite amount of PS solution (0.018%, 5.0 ml) was mixed with cells at 0–4°C in an ice cold bath to avoid a possibility of cell's taking up PS by cell actions (Becker & Green, 1960), and the amount of non-adsorbed PS was titrated with a standard PVSK solution (0.01%, i.e. 4.9×10^{-4}N) at a room temperature after cells were removed by centrifugation. In the experimental test, cells were pre-incubated with CM for 40 minutes without shaking at 0–4°C in an ice cold bath, then they were washed once with an excess amount of cold HBSS by centrifugation. After removing the supernatant fluid carefully, PS solution was added to a final amount of 5.0 ml with mixing the cells. After leaving cells with PS in an ice cold bath for 10 minutes, they were removed by centrifugation and the supernatant fluid was immediately titrated by PVSK at room temperature. The end point of these colloid titrations was determined by colour change of an indicator substance, toluidine blue (0.01%). For the blank titration, 5.0 ml of PS solution was used in place of the cell suspension. When the binding capacity under various pH values was examined, pH was controlled by adding an adequate amount of HCl or NaOH (0.01 to 0.1 N, 0.1 to 1.0 ml) during the course of titration. Pilot tests have shown that the binding capacity of PS with PVSK is pH dependent (Fig. 2). Therefore, adsorption tests of PS on cell surfaces were done at pH 7.3 throughout the experiments.
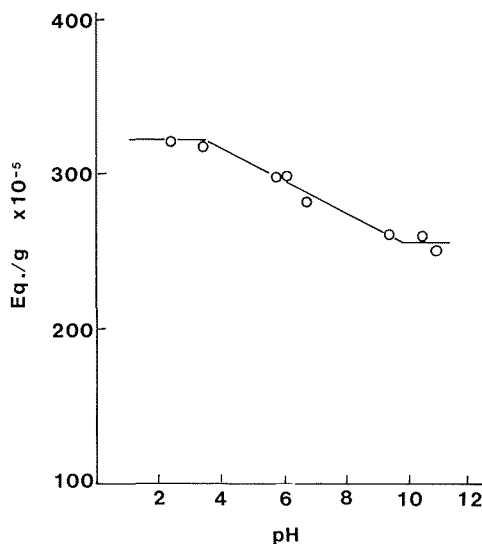

Fig. 2.   Binding capacity of negative polymer ion (PVSK, 0.01%) per unit weight of protamine sulfate (PS) under various pHs. Ordinate: gram equivalent PVSK per gram PS which was calculated from the following equation ; gram equivalents/gram $= 0.00048 \times \Delta \times 10^{-3}/0.018 \times 5 \times 10^{-2}$, where Δ is the difference between the titers of experiment and blank tests at the same pH. Abscissa: pH.

When binding of PS on cell surfaces was examined under various concentrations of PS, PS left unadsorbed with cell surfaces was tirated with 0.002% PVSK (0.98×10⁻⁴) (Figs. 3 and 4). Under conditions depicted in Fig. 4, the binidng capacity of unadsorbed PS with PVSK was constantly maintained at the level of 300×10^{-5} gram equivalent per gram of PS. This indicates that PS–PVSK binding occurs stoichiometrically. Therefore, as shown in Fig. 3, blank titrations for various concentrations of PS were performed by a standard PVSK solution $(0.98 \times 10^{-4} N)$.
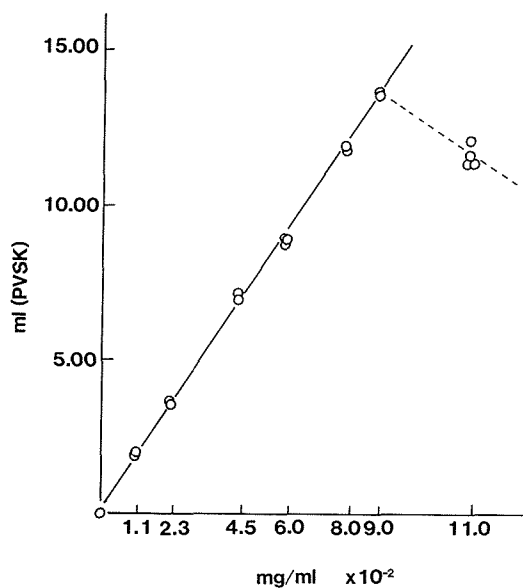

Fig. 3. Various concentrations of PS are each titrated with a definite amount of PVSK, $0.98 \times 10^{-4} N$. Ordinate: consumed volume (ml) of PVSK. Abscissa: concentrations of PS (mg/ml). At the concentrations over 9.0 mg per ml of PS, PVSK $(0.98 \times 10^{-4} N)$ connot bind with PS stoichiometrically.
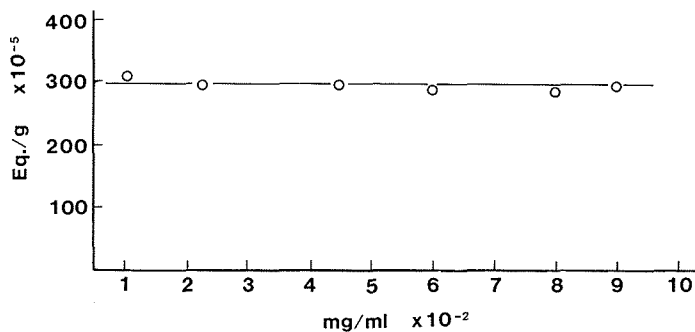

Fig. 4. Binding capacity of $0.98 \times 10^{-4} N$ PVSK with various amounts of PS at pH 7.3. Ordinate: binding capacity was calculated by gram equivalents/gram (see also Fig. 2). Abscissa: concentrations of PS (mg/ml).

Results

1. *Cell aggregation as a rate process*

Fig. 1A shows a typical example of a kinetics of cell aggregation. It clearly demonstrates that in the presence of α, the slope was steeper than in the control mediun (HBSS). When cells were aggregated in the presence of the mixture of α and β, the slope was much less steeper than in the control medium. These results suggest that α promotes cell aggregation, as revealed in the previous data obtained by counting single cell elimination (Takahashi & Okada, 1971), whereas the mixture of α and β inhibited cell aggregation at least in its initial stage up to 30 minutes after the start of inoculation.

The present treatment of a kinetics of initial stages of cell aggregation as a bimolecular rate process was also adopted to analyze the effect of divalent cations on cellular aggregation in later experiments.

2. *Changes of negativity of cell surface*

Generally, surfaces of cells are negatively charged (cf. a review: Curits, 1967). Several authors (Steinberg, 1958; Curtis, 1960; Wilkins et al., 1962a, b) suggested that aggregation is affected by surface negativity of cells. In considering the action of α on aggregation, it was thought that firstly α might be adsorbed on cell surfaces and secondly, properties of such surfaces with α might be electrically changed.

Concerning the first point, some indirect evidence has been given in the previous paper (Takahashi & Okada, 1970). In order to verify the possibility of changes of surface negativity of cells adsorbed with α, binding capacity of PS with cell surfaces was titrated with a standard PVSK solution.

Since some preliminary tests indicated that the effect of crude CM^{5-6} for changing the binding capacity of cell surfaces with PS was the same as that of separated α-fraction, the experiments below were performed by using CM^{5-6}, which will be designated as CM^{5-6} (α) in the following description. CM^{3-4}, which contains only β-fraction as shown the previous work (Takahashi & Okada, 1971), was also tested, though no test was made with the separated β-fraction.

Fig. 5 shows the effect of CM^{5-6} (α) on the surface electricity of cells. It was clearly observed that CM^{5-6} (α)-treated cells bound PS more effectively than untreated cells at all concentrations of PS tested. On the other hand, the binding capacity of CM^{3-4}-treated cells with PS was the same as untreated cells. Since PS is polycationic colloid and binds to negative charge on cell surfaces, it was thought that the cells treated by CM^{5-6} (α), but not by CM^{3-4}, increased their surface nagativity. In both CM^{5-6} (α)-treated and control cells, the amount of PS adsorbed on cell surfaces increased linearly depending to the amount of PS added.

A question arises, however, why cells with increased surface negativity by the treatment with CM^{5-6} (α) can aggregate much faster than untreated cell, notwithstanding that cells with increased negative charge should be expected in theory to be more repulsive each other. It was experienced that CM^{5-6} (α)-treated cells were aggregated at much slower rate, when aggregation was performed in Ca^{2+} and Mg^{2+} free HBSS (CMF–HBSS) (see Fig. 1B). Several authors (Steinberg, 1958; Curtis,
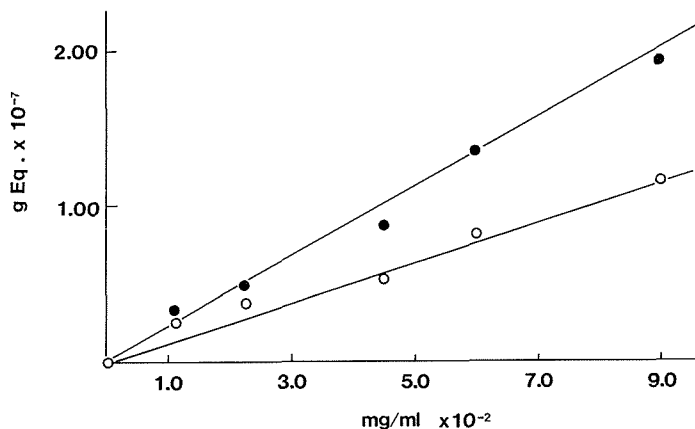
Fig. 5. Kinetics of PS adsorption of cell surfaces of cultured lung cells under various concentrations of PS. Ordinate: gram equivalents PVSK per 9.0×10^6 cells. Abscissa : concentration of PS (mg/ml). —●—●— , cells pre-treated with CM^{5-6} (α); —○—○— , untreated control cells.

1967; Wilkins et al., 1962b) suggested that divalent cations greatly affect cell aggregation. Therefore, it was presumed that the presence of divalent cations such as Ca^{2+} and Mg^{2+} is relevant to the enhanced aggregation of cells, whose surface negative charges are increased by adsorption of CM^{5-6} (α).

3. *Effect of divalent cations on cell aggregation*

Cells pretreated by CM^{5-6} (α) or CM^{3-4} for 40 minutes at 0–4°C or nontreated cells were resuspended in cold CMF–HBSS (pH 7.3 with HEPES–NaOH) with an addition of an appropriate amount of $CaCl_2$ or $MgCl_2$.

Fig. 6 shows that aggregation of both pretreated and control cells became faster depending to the concentration of Ca^{2+} in some range. As expected, cells pretreated with CM^{5-6} (α) were aggregated always at higher rate than control cells.

Aggregation of cells pretreated with unconditioned fresh medium (FM), which consisted of Eagle's minimum essential medium supplemented with glutamine and 10% calf serum, was not affected with Ca^{2+} concentration. On the other hand, aggregation of cells pretreated by CM^{3-4} was somewhat inhibited with the increase of Ca^{2+} added.

The effects of Mg^{2+} on cell aggregation were given in Fig. 7, which indicates that the rate of aggregation of FM-treated cells or untreated control cells increased a little with an increase of the concentration of Mg^{2+}. Cells pretreated with CM^{3-4} remained unaffected in any concentrations of Mg^{2+}. In the case of cells pretreated with CM^{5-6} (α), though the rate of aggregation increased gradually with Mg^{2+} concentration, an overall level was lower than untreated control (data not shown).

4. *Surface negativity and aggregation of fixed cells*

It has been known that the treatment of cells by fixativies, such as $KMnO_4$, OsO_4 and formaldehyde increased the surface negativity (Glaeser & Mel, 1964; Mayhew & Nording, 1966). The effect of Ca^{2+} on aggregation was examined with GLA-fixed
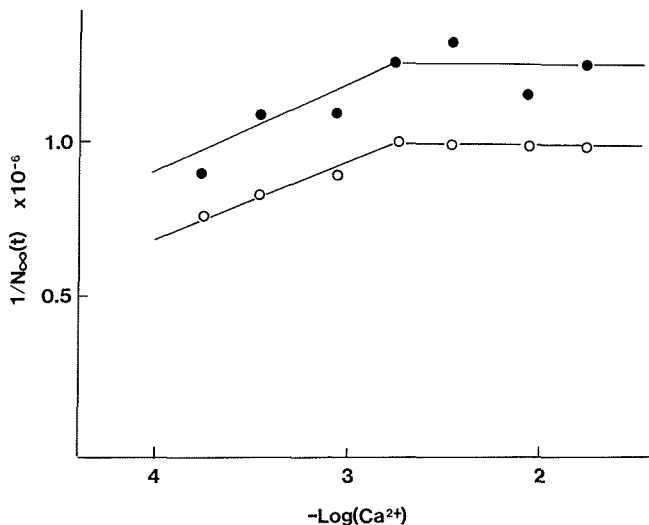
Fig. 6. An influence of Ca^{2+} on cell aggregation. Ordinate: rate of cellular adhesiveness (1/ $N_{\infty(t)}$, see the text) at 30 minutes after the start of cell aggregation. Abscissa: molar concentration of Ca^{2+} expressed by -log(Ca^{2+}). —●—●—, cells pre-treated with CM^{5-6} (a); —○—○—, control cells without the pretreatment.
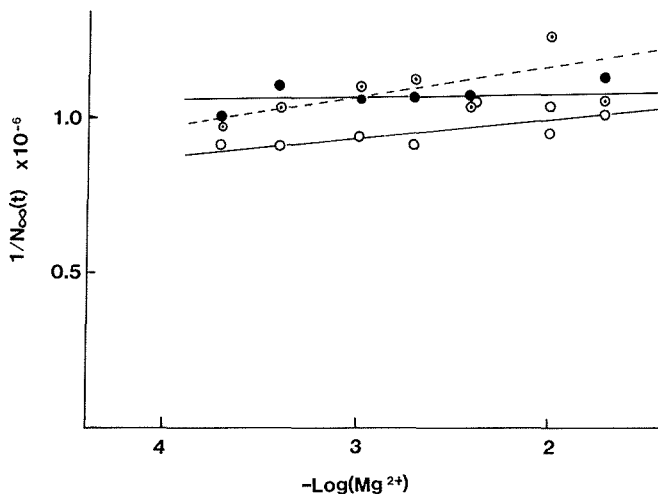

Fig. 7. An influence of Mg^{2+} on cell aggregation. Ordinate: cellular adhesiveness, $1/N_{\infty(t)}$ at 30 minutes after the start of cell aggregation. Abscissa: molar concentration of magnesium ion. —●—●—, cells pre-treated with CM^{3-4}; --⊙--⊙--, cells pre-treated with FM; —○—○—, cells without any treatment.

cells. Comparison of the data given in Fig. 8 with those in Fig. 5 indicates that when cells were fixed with GLA, the binding capacity of PS with cell surfaces was increased, probably due to an increased surface negativity. In fixed cells, gEq. $\times 10^{-7}$ does not linearly increase with the amount of PS (cf. Fig. 5). The reason of such non-linear relation is obscure yet. An increase of the binding capacity of cell surfaces with PS
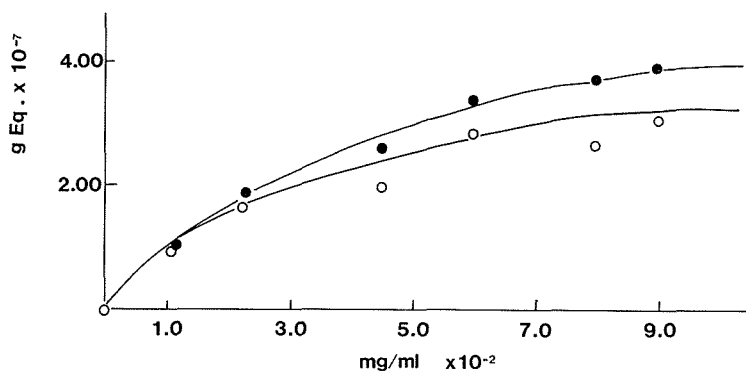
Fig. 8. Kinetics of PS adsorption of GLA-fixed cells with or without pretreatment with CM^{5-6} (a). Ordinate: the binding capacity of PS on cell surfaces expressed by gram equivalents PVSK per test tube containing 9.0×10^6 cells. Abscissa: concentration of PS (mg/ml). —●—●— , fixed cells pre-treated with CM^{5-6} (a); —○—○— , fixed cells without any treatment.

caused by the pretreatment with CM^{5-6} (a) was not so consipicuous in fixed cells as seen in unfixed fresh cells.

In the experiment shown in Fig. 9, fixed cells were incubated with CM^{5-6} (a) for various intervals, and then their binding capacity with PS (1.8 mg/ml) for 10 minutes was measured by titration with PVSK. The results indicate that the binding capacity of CM^{5-6} (a) pre-treated fixed cells with PS is much greater than that of untreated fixed cells. Cells' binding capacity with PS reached to saturation at around 25 minutes after the start the of incubation. The treated cells were 350×10^{-7} gram equivalent per 9.0×10^6 cells and the untreated cells were 200×10^{-7}. A time required to reach saturation was the same in both treated and untreated cells.

The effect of Ca^{2+} on aggregation of fixed cells was examined. Fig. 10 shows that a difference in the concentration of Ca^{2+} affected aggregation of fixed cells with the different manner from that observed in fresh cells. For aggregation of fixed cells, the maximal effect occurred at 1.8 mM of Ca^{2+}, and over this concentration the rate of
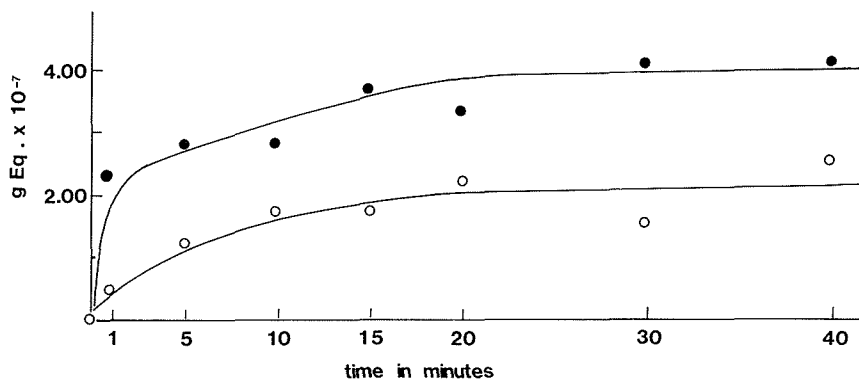

Fig. 9. A typical example of time course of PS adsorption on GLA-fixed cells with CM^{5-6} (a). Ordinate : the binding capacity of PS (1.8 mg/m²) on cell surfaces expressed by gram equivalent PVSK per test tube containing 9.0×10^6 cells. Abscissa : time in minutes. —●—●— , fixed cells pre-treated with CM^{5-6} (a); —○—○— , fixed cells without any treatment.
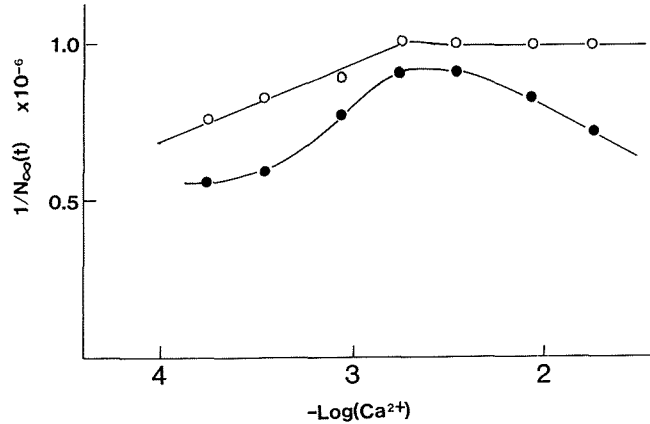
Fig. 10. An example of cell aggregation of GLA-fixed cells in the presence of Ca^{2+}. Ordinate: $1/N_{\infty(t)}$ at 30 minutes after the start of cell aggregation. Abscissa: molar concentration of Ca^{2+}. —●—●—, fixed cells; —○—○—, unfixed cells.

aggregation sharply dropped. An overall level of aggregation of fixed cells was much lower than fresh cells.

Discussion

In the previous paper (Takahashi & Okada, 1971), the presence of a factor to promote cell-substrate as well as cell-to-cell adhesion (aggregation) was shown in fraction a separated from CM through Sephadex G–25 gel. Some data of the previous study suggested that this factor (designated as a) may be adsorbed onto cell surfaces. Therefore, it was supposed that some changes in surface properties should occur by treating cells with a.

The present report, in which the measurement of a rate of cell aggregation based upon a model of colloid stability (Wilkins et al., 1962b) was adopted, first revealed the promotion of aggregation by CM^{5-6}, which contains a. Then, an increase of surface negativity after treatment of cells by CM^{5-6} (a) was shown by measuring the cell's binding capacity with positive colloid (PS) by means of colloid titration technique. Such surface change is considered to be induced rather 'specifically' by adsorption of CM^{5-6} (a), because the treatment of cells by CM^{3-4} which does not contain the aggregation promoting factor (see Takahashi & Okada, 1970; 1971) was not effective to increase surface negativity.

It has been suggested that the fundamental premise of cell aggregation is similar, in theory, to the interaction of colloidal particles which are affected by an electrostatic repulsive energy and van-der-Waals-London attractive energy (Wilkins et al. 1962b; Swift & Friedlander, 1964; Curtis, 1969). The repulsive energy has been thought to be due to surface negative charges of cells. So, it is naturally presumed that highly charged surfaces should be less adhesive than those with less negativity (Curtis, 1960).

Then, the question naturally arises, why cells with increased surface negativity by the adsorption of CM^{5-6} (a) can aggregate with much faster rate than other cells. Fig. 1B shows that promoted aggregation of cells treated with CM^{5-6} (a) is dependent to

the presence of Ca^{2+}. It is speculated that an increase of surface negative charge may render cells more easily to interact with Ca^{2+} to lead to faster aggregation. An important function of divalent cations in cellular adhesion has been explained in terms of its action in stabilizing a surface negativity of cementing materials between cells (Curtis, 1960; Wilkins et al., 1962b; Heard and Seaman, 1960). Wilkins et al. (1962a), however, suggested that high concentration of divalent cations may induce charge reversal effect. So, if the concentration of the cations is beyond the critical point, aggregation of cells will continue until a complete reversal of surface negativity. It seems, therefore, that Ca^{2+} promotes aggregation by way of neutralizing surface negative charges.

In cases of cells fixed with GLA, high concentrations of Ca^{2+} ion act inhibitory to aggregation (Fig. 10). Qualitative differences in the surface property between unfixed and fixed cells cannot be overlooked. In fact, in cases of unfixed cells a linear relation exists between the amount of PS adsorbed upon cells and the amount of PS added, whereas it was not observed in fixed cells (cf. Fig. 5 and 8). The reason to cause this difference is not clear.

To sum up, the following process can be speculated as to the action of aggregation promoting factor contained in α-fraction of CM. An increase of surface negativity is induced in cell surfaces to which the factor is adsorbed. Such surfaces now with the factor interact with Ca^{2+} in the medium in such a manner as to result the neutralization of surface charge, which resulted in aggregation with faster rate.

References

Becker, F. F. & Green. H. (1960) The effects of protamines and histones on the nucleic acids of ascites tumor cells. *Exptl. Cell Res.* 19: 361–375.

Curtis, A. S. G. (1960) Cell contacts: Some physical considerations. *Amer. Na.* 94: 37–56.

Curtis, A. S. G. & Greaves, M. F. (1965) The inhibition of cell aggregation by a pure serum protein. J. Embryol. *Exp. Morph.* 12: 309–326.

Curtis, A. S. G. (1967) In: *The Cell Surface; Its Molecular role in Morphogenesis*, Logos-Academic Press, London.

Curtis, A. S. G. (1969) The measurement of cell adhesiveness by an absolute method. *J. Embryol. Exp. Morphol.* 22: 305–325.

Fisher, H. W., Puck, T. T. & Sato, G. (1958) Molecular growth requirements of single mammalian cells: The action of fetuin in promoting cell attachment to glass. *Proc. Natl. Acad. Sci., USA*, 44: 4–15.

George, J. N., Weed, R. I. & Reed, C. F. (1971) Adhesion of human erythrocytes to glass: The nature of the interaction and the effect of serum and plasma. *J. Cell. Physiol.* 77: 51–59.

Glaeser, R. M. & Mel, H. C. (1964) The electrophoretic behavior of osmium tetroxide-fixed and potassium permanganate-fixed rat erythrocytes. *Biochim. Biophys.Acta.* 79: 606–617.

Heard, D. H. & Seaman, G. V. F. (1960) The influence of pH and ionic strength on the electrokinetic stability of the human erythrocyte membrane. *J. gen. Physiol.* 43: 635–654.

Mayhew, E. & Nordling, S. (1966) The electrophoretic mobility of fixed tissue culture cells: studies with formaldehyde and osmium tetroxide. *Exptl. Cell Res.* 43: 72–76.

Oellermann, R. A. & Miller, E. M. (1969) The influence of conditioned media and non essential amino acid supplementation of the growth of cells *in vitro. J. Cell. Physiol.* 74: 229–306.

Orr, C. H. & Roseman, S. (1969a) Intercellular adhesion. I. A quantitative assay for measuring the rate of adhesion. *J. Membrane Biol.* 1: 109–124.

Orr, C. H. & Roseman, S. (1969b) Intercellular adhesion. II. The purification and properties of a horse serum protein that promotes neural retina cell aggregation. *J. Membrane Biol.* 1: 125–143.

Perterson, J. A. & Rubin, H. (1969) The exchange of phospholipids between cultured chick embryo fibroblasts and their growth medium. *Exptl. Cell Res.* 58: 365–378.

Rubin, H. (1969) A substance in conditioned medium which enhances the growth of small number of chick embryo cells. *Exptl. Cell Res.* 41: 138–148.

Steinberg, M. S. (1958) On the chemical bonds between animal cells. A mechanism for type-specific association. *Amer. Nat.* 92: 65–82.

Swift, D. D. & Friedlander, S. K. (1969) The coaggulation of hydrosols by Brownian motion and laminar shear flow. *J. Colloid Sci.* 19: 621–647.

Takahashi, K. & Okada, T. S. (1970) An analysis of the effect of 'conditioned medium' upon the cell culture at low density. *Develop. Growth & Differ.* 12: 65–77.

Takahashi, K. & Okada, T. S. (1971) Separation of two factors affecting the aggregation kinetics from the conditioned medium. *Develop. Growth & Differ.* 13: 15–24.

Terayama, H. (1952) Method of colloid titration (A new titration between polymer ions). *J. Poly. Sci.* 8: 243–253.

Virolainen, M. & Deffendi, V. (1967) Dependence of macrophage growth *in vitro* upon interaction with other cell types. In: V. Deffendi & M. Stoker (eds.) *Growth Regulating Substances for Animal Cells in Culture.* The Wister Institute Press, New York. 67–85.

Wilkins, D. J., Ottewill, R. H. & Bangham, A. D. (1962a) On the flocculation of sheep leucocytes: I. Electrophoretic studies. *J. Theoret. Biol.* 2: 165–175.

Wilkins, D. J., Ottewill, R. H. & Bangham, A. D. (1962b) On the flocculation of sheep leuco-cytes: II. Stability studies. *J. Theoret. Biol.* 2: 176–191.

著者：高橋　敬，〒693　島根県出雲市　島根医科大学生理学教室.