

Fig. 5. FAK activation in HUVECs adhered to recombinant cell adhesive proteins. HUVECs cultured in serum-containing EGM-2 media on collagen-coated plates were harvested and held in suspension for 2 h at 37 °C, then plated onto precoated plates with 10 µg/ml of Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, or Attachin. After 2 h incubation at 37 °C, cell lysates were prepared using RIPA buffer. The cell extracts were analyzed for the phosphorylation (A) and protein levels of focal adhesion kinase (B) by Western blotting. The amount of proteins detected was analyzed using MultiGauge software (C). Lane 1: before harvesting; lane 2: after suspension; lane 3: BSA; lane 4: Fibronectin; lane 5: Pronectin F; lane 6: Pronectin F PLUS; lane 7: Pronectin L; lane 8: Retronectin; lane 9: Attachin; lane 10: tissue culture-treatment dish. Similar results were obtained in two independent experiments. Representative blots are shown.

they are expected to be put into clinical use in the near future. Serum-free culture supplemented with recombinant proteins produced without using animal-derived materials would be optimal in order to avoid contamination by animal-derived materials and transmissible agents. This work was carried out as a step in demonstrating the feasibility of recombinant cell adhesion proteins in serum-free cultures of human endothelial cells, which might form the basis of future cellular therapies. HUVECs were used as a model of tissue-derived differentiated endothelial cells.

One of the uses of endothelial cells in cellular therapy is for the vascularization of engineered tissue. Vascularization of a tissue-engineered construct is mediated primarily through the ingrowth of surrounding vessels from the peri-implant tissue. However, this process is quite slow and is not sufficient to achieve appropriate vascularization of large defects [32]. The incorporation of endothelial cells or vascular-like structure in the tissue-engineered constructs before transplantation is a possible solution of this problem. Both human embryonic stem cell-derived endothelial cells and tissue-derived endothelial cells such as HUVECs have been shown to incorporate successfully into engineered skeletal muscle tissue [33–35].

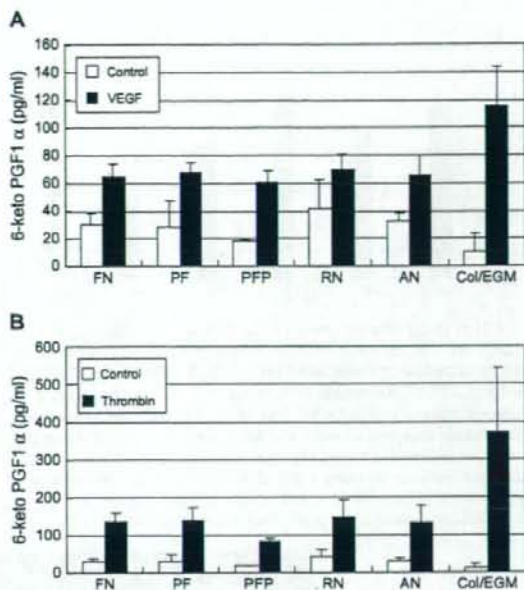


Fig. 6. Effects of cell adhesive proteins on the VEGF- or thrombin-stimulated production of Prostaglandin I₂. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PPF), Retronectin (RN), or Attachin (AN). After culturing for 1 day, the cells were stimulated with 30 ng/ml of VEGF (A) or 1 U/ml of thrombin (B). One hour later, the conditioned media were collected and 6-keto PGF₁α concentration was determined by enzyme immunoassay. Results are expressed as picograms of PGF₁α/ml of the supernatant and represent the mean ± S.D. of triplicate determinations. 6-Keto PGF₁α production from HUVECs cultured in serum-containing growing media (EGM-2) plated on a collagen-coated dish was also examined (Col/EGM).

Tissue-derived human endothelial cells are also used for the endothelialization of artificial artery grafts [36]. Although serum-free media for human endothelial cells has been developed, being supplemented with a cell adhesion protein such as fibronectin is necessary [26]. Since plasma fibronectin is known to bind to several pathogens including viruses [37,38], use of a recombinant cell adhesion protein would be desired in order to avoid the contamination of infectious agents.

Another use of endothelial cells in cellular therapy is for therapeutic neovascularization, an important adaptation to rescue tissue from critical ischemia. In a rat model of myocardial infarction, transplantation of HUVECs has been shown to contribute to increased neovascularization [39]. Recently, much attention has been paid to outgrowth endothelial cells (OECs) obtained by *in vitro* culture of blood mononuclear cells [40–46]. OECs show a marked similarity to fully differentiated endothelial cells with respect to their cellular morphology, marker expression and potential to form capillary-like structures [43,44,47]. Because OECs can be obtained from blood mononuclear cells, they have the advantage that autologous endothelial cells could be obtained without invasive procedures. The potential use of OECs for therapeutic neovascularization has been shown in murine ischemia models

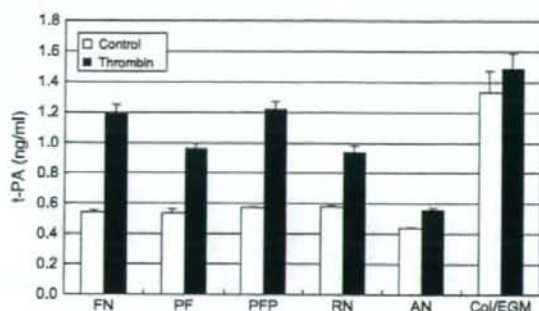


Fig. 7. Effects of cell adhesive proteins on the thrombin-stimulated production of tissue plasminogen activator. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFF), Retronectin (RN), or Attachin (AN). After culturing for 1 day, the cells were stimulated with 1 U/ml of thrombin. After 24 h incubation, the conditioned media were collected and t-PA concentration was determined by enzyme immunoassay. Results are expressed as nanograms of t-PA/ml of the supernatant and represent the mean \pm S.D. of triplicate determinations. t-PA production from HUVECs cultured in serum-containing growing media (EGM-2) plated on collagen-coated dish was also examined (Col/EGM).

[47–50]. The culture conditions for OECs and for tissue-derived endothelial cells such as HUVECs are the same: endothelial basal media supplemented with fetal calf serum, VEGF, basic FGF, IGF-1, EGF and ascorbic acid. Fibronectin is the cell adhesion protein most commonly used to obtain OECs. Therefore, examining the usefulness of recombinant cell adhesion proteins as a substitute for plasma fibronectin in serum-free culture would lead to the establishment of a serum-free culture for OECs as well.

The cell culture method chosen has a direct influence on the quality and safety of cellular therapy products. The composition of media and serum are considered to affect the features of the cells, and animal-derived materials are known to carry the risk of infection or allergy. Thus, developing appropriate culture methods and ways of evaluating these methods are key to assuring the safety of cellular therapy products [11,12]. For example, when culturing human embryonic stem cells that are anticipated to be a useful source for cellular therapy products, non-human-type sialic acid (*N*-glycolyl neuraminic acid) derived from animal serum is incorporated into the sugar chain on the cell surface, which could induce an immune response

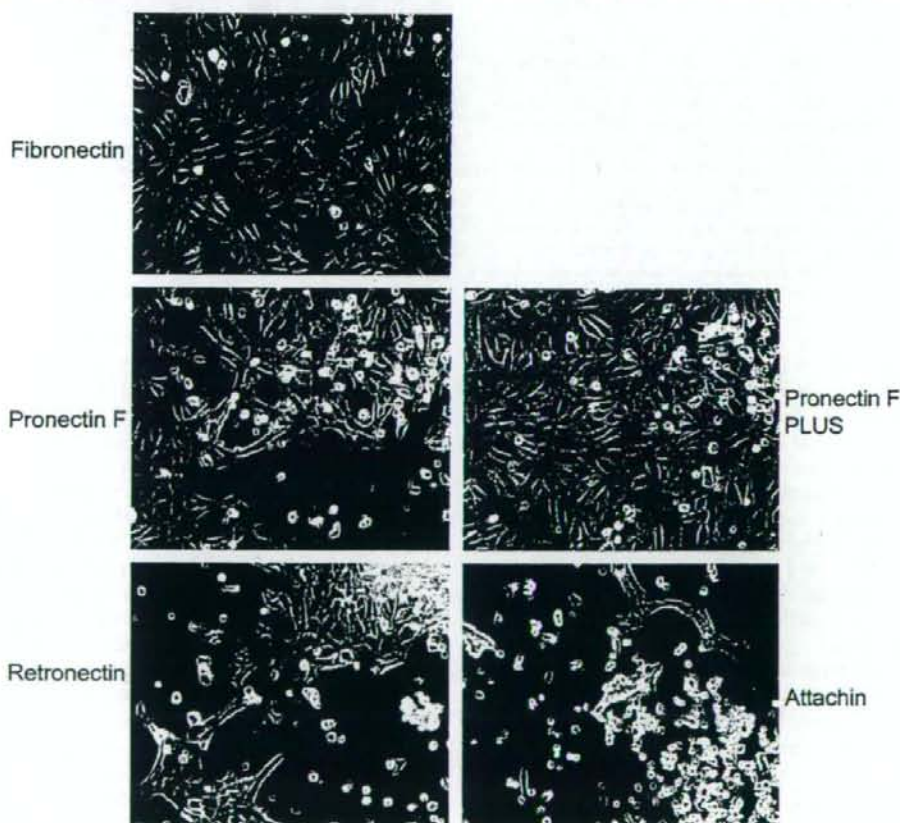


Fig. 8. Detachment of HUVECs cultured in the presence of thrombin. Cells were plated on the plates previously coated with Fibronectin, Pronectin F, Pronectin F PLUS, Retronectin, or Attachin, and cultured for 24 h in the presence of 1 U/ml of thrombin. The cells were photographed before harvesting the supernatant that is provided for the measurement of the t-PA concentration.

upon transplantation [51]. In addition, the transmission of unknown viruses from rodent feeder cells is also a concern [8]. Therefore, establishing cell culture methods that assure safety is an important goal in the development of cellular therapy products.

Because recombinant proteins can be produced without using animal or human-derived materials, providing the protein factors that are necessary to cell culture as recombinant proteins can contribute to improving the safety of cellular therapy products. Recombinant proteins also have an advantage in that they can provide consistent performance from lot to lot, and their function can be modified by molecular design. Artificial recombinant proteins have the possibility of being superior to naturally occurring proteins; for example, Retronectin, a deletion mutant of fibronectin that unfortunately showed less activity as an adhesive protein in this study, is much more effective in supporting gene transfer by retrovirus vectors than fibronectin [23]. If there is a concern in using artificial recombinant proteins, the residual artificial protein has the potential to show immunogenicity.

In our experiments to test cell adhesion, Pronectin F and Pronectin F PLUS were superior to fibronectin when they were used in lower concentrations (Fig. 1). This might be because both Pronectin F and Pronectin F PLUS have 13 repeats of the RGD sequence in one molecule, whereas fibronectin has only two. Pronectin F and Pronectin F PLUS are also structurally stable, and thus the coated substrates retain their performance for at least 2 years at room temperature, which is another advantage over fibronectin [52]. Pronectins were produced without using any animal-derived components (Dr. Kurokawa, Sanyo Kasei Kogyo, personal communication).

Pronectin F is not soluble in water since the silk-like protein sequence forms strong hydrogen bonds intermolecularly; thus, it must be dissolved in LiClO_4 [21]. Therefore, Pronectin F PLUS, a water-soluble variant of Pronectin F, was developed by chemical modification of the serine residues of Pronectin F [22]. As shown in Fig. 3, a higher amount of Pronectin F was absorbed on polystyrene dishes than Pronectin F PLUS. Since the surface of polystyrene plates is hydrophobic, hydrophobic Pronectin F might have been absorbed well. Pronectin L, which has a similar core sequence to that of Pronectin F, was also absorbed well on the dish, although no cells adhered on it.

In spite of their differences in absorption on the polystyrene dish, Pronectin F PLUS and Pronectin F showed similar efficiency with respect to the adhered cell number, growth support, induction of FAK phosphorylation, and production of PGI_2 and t-PA. Moreover, Pronectin F PLUS was more potent than Pronectin F in its support of cell adhesion under stimulation with thrombin (Fig. 9). The cells on Pronectin F PLUS were resistant to cell detachment due to the thrombin-induced cytoskeletal changes. This might be because that Pronectin F PLUS is positively charged by chemical modification. Since the cell surface possesses a negative charge, the adhesive intensity between the cells and Pronectin F PLUS was increased due to the static electrical interaction, resulting in the maintenance of cell adhesiveness after the cytoskeletal change

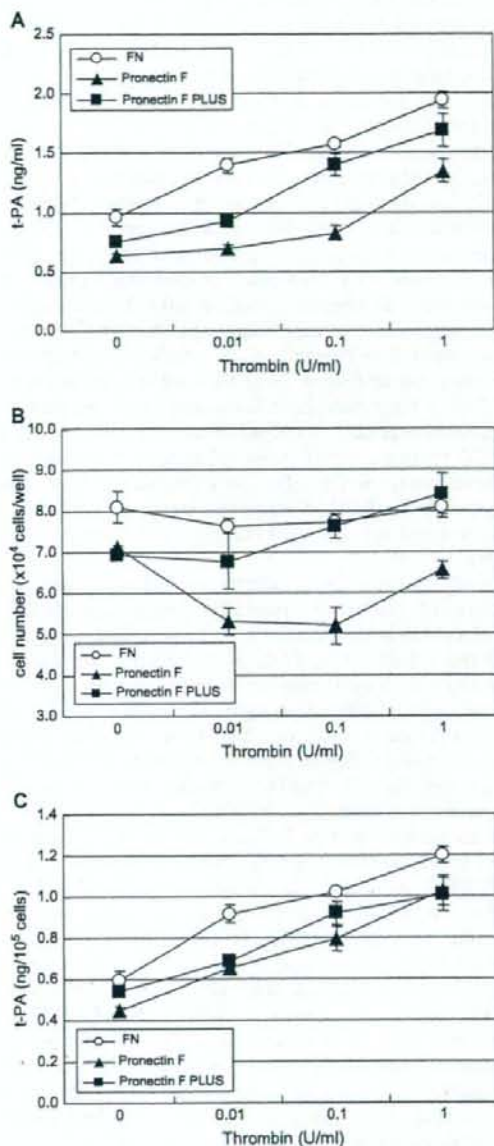


Fig. 9. Thrombin-induced production of t-PA and decrease in viability of HUVECs adhered on Pronectin F. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin, Pronectin F or Pronectin F PLUS. After culturing for 1 day, the cells were stimulated with thrombin at the indicated concentrations. After 24 h incubation, the conditioned media were collected and t-PA concentration was determined by enzyme immunoassay (A). The cell numbers were determined using a Cell-Counting Kit-8 (B). Production of t-PA per 10^5 cells was calculated (C). Each data point represents the mean \pm S.D. of triplicate determinations.

induced by thrombin. To our knowledge, this is the first observation to reveal the difference in biological properties between Pronectin F and Pronectin F PLUS. The retention of human endothelial cells on the lumen in the presence of physiological stimuli is an important prerequisite for artificial artery grafts to be successful, and, therefore, Pronectin F PLUS could also be suited for use in this type of graft.

In the present study, the usefulness of Pronectin F PLUS as a substitute for fibronectin was indicated; however, Pronectin F PLUS and Pronectin F were less effective than fibronectin in long-term cell cultures. We were able to culture HUVECs up to around 6 passages using the serum-free media with fibronectin. However, when Pronectin F or Pronectin F PLUS was used instead of fibronectin, the cell number did not increase beyond the second passage. Although Pronectin F PLUS worked better than Pronectin F, the efficiency of both was lower than that of fibronectin in the long-term maintenance of HUVECs. Since fibronectin has several functional domains that can bind to heparin, fibrin and collagen, signals other than the RGD-integrin interaction would be important in the long-term maintenance of the cells. The incorporation of domains other than that of RGD of fibronectin into Pronectin F PLUS would be necessary to improve its efficacy in long-term cell cultures.

For cell cultures, porcine trypsin is often used when cells are harvested. The use of trypsin may lead to the contamination of animal-derived materials or transmissible agents. In serum-free cultures of HUVECs, we were able to harvest cells for passage using a protease free of animal-derived materials such as TrypLE Select (Invitrogen, Carlsbad, CA, USA) or AccuMax (Chemicon, Billerica, MA, USA); therefore, trypsin is not necessarily used for the maintenance of HUVECs, which means that it is possible to eliminate the potential for contamination of animal-derived proteins.

In summary, Pronectin F PLUS was shown to have the closest activity to plasma fibronectin in the serum-free culture of HUVECs by examining VEGF- or bFGF-induced cell proliferation, FAK phosphorylation, and thrombin-stimulated production of PGI₂ or t-PA. The cell adherent activity of Pronectin F PLUS was superior to fibronectin at concentrations of less than 1 µg/ml. Although Pronectin F PLUS has the same amino acid sequence as Pronectin F, only HUVECs on Pronectin F PLUS were resistant to thrombin-induced cell detachment. Pronectin F PLUS is thus thought to be a promising substitute for plasma fibronectin in the serum-free culture of endothelial cells, although modification would be necessary to improve its efficiency in long-term cell cultures.

Acknowledgements

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Research Paper

Significance of Local Mobility in Aggregation of β -Galactosidase Lyophilized with Trehalose, Sucrose or Stachyose

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Purpose. The purpose of this study is to compare the effects of global mobility, as reflected by glass transition temperature (T_g) and local mobility, as reflected by rotating-frame spin-lattice relaxation time ($T_{1\rho}$) on aggregation during storage of lyophilized β -galactosidase (β -GA).

Materials and Methods. The storage stability of β -GA lyophilized with sucrose, trehalose or stachyose was investigated at 12% relative humidity and various temperatures (40–90°C). β -GA aggregation was monitored by size exclusion chromatography (SEC). Furthermore, the $T_{1\rho}$ of the β -GA carbonyl carbon was measured by ^{13}C solid-state NMR, and T_g was measured by modulated temperature differential scanning calorimetry. Changes in protein structure during freeze drying were measured by solid-state FT-IR.

Results. The aggregation rate of β -GA in lyophilized formulations exhibited a change in slope at around T_g , indicating the effect of molecular mobility on the aggregation rate. Although the T_g rank order of β -GA formulations was sucrose < trehalose < stachyose, the rank order of β -GA aggregation rate at temperatures below and above T_g was also sucrose < trehalose < stachyose, thus suggesting that β -GA aggregation rate is not related to ($T-T_g$). The local mobility of β -GA, as determined by the $T_{1\rho}$ of the β -GA carbonyl carbon, was more markedly decreased by the addition of sucrose than by the addition of stachyose. The effect of trehalose on $T_{1\rho}$ was intermediate when compared to those for sucrose and stachyose. These findings suggest that β -GA aggregation rate is primarily related to local mobility. Significant differences in the second derivative FT-IR spectra were not observed between the excipients, and the differences in β -GA aggregation rate observed between the excipients could not be attributed to differences in protein secondary structure.

Conclusions. The aggregation rate of β -GA in lyophilized formulations unexpectedly correlated with the local mobility of β -GA, as indicated by $T_{1\rho}$, rather than with ($T-T_g$). Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

KEY WORDS: β -galactosidase; global mobility; local mobility; lyophilized formulation; solid-state stability.

INTRODUCTION

Close correlations between storage stability and molecular mobility have been demonstrated for various lyophilized formulations of peptides and proteins (1,2). Aggregation between protein molecules is a degradation pathway commonly observed in lyophilized protein formulations. The rate of protein aggregation is generally considered to depend on the translational mobility of protein molecules, which is related to structural relaxation (α -relaxation) of the formulation. Correlations between aggregation rates and structural relaxation have been shown in various protein systems in visible ways, such as enhancement of aggregation associated with decreases in glass transition temperature (T_g) (3–6) and

changes in the temperature dependence of aggregation rates around T_g (7–10). However, recent studies have suggested that molecular mobility with a length scale shorter than structural relaxation (β -relaxation or local mobility), rather than structural relaxation, is critical to protein aggregation (11,12).

The rate of protein aggregation in lyophilized formulations is also affected by the degree of change in protein conformation produced during the freeze-drying process (1). Greater changes in protein conformation are considered to lead to enhanced aggregation during subsequent storage.

In this study, the significance of local mobility in aggregation of lyophilized β -galactosidase (β -GA), a model protein, is discussed in comparison with the significance of structural relaxation and conformational changes. β -GA underwent significant inactivation during freeze drying with dextran, thus suggesting that significant conformational changes occurred during the process (13). When freeze dried with polyvinylalcohol or methylcellulose, inactivation was not observed during freeze drying (10). However, the time

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courses of aggregation during subsequent storage were describable with the empirical Kohlrausch-Williams-Watts (KWW) equation, thus leading to speculation that there were protein molecules having different conformations resulting from stresses during the freeze-drying process, each aggregating with a different time constant (10,14). The temperature dependence of the aggregation rate measured at the initial stage changed around T_{mc} (T_g determined by NMR relaxation measurement (15)), showing an apparent correlation between aggregation rate and structural relaxation (10). In this study, the significance of local mobility in aggregation of lyophilized β -GA was examined in comparison with that of structural relaxation. The aggregation rates of β -GA lyophilized with trehalose, sucrose or stachyose were measured at temperatures near T_g , and correlations were examined between aggregation rate and local mobility (as measured by solid-state NMR), T_g (a primary parameter of structural relaxation), or protein conformational changes (as measured by FT-IR).

MATERIALS AND METHODS

Preparation of Lyophilized β -GA Formulations

β -GA from *Aspergillus oryzae* (10 U/mg; molecular weight: 105,000; isoelectric point:4.6) was kindly provided by Amano Enzyme Inc. (Nagoya, Japan) and purified by dialysis against 2.5 mM sodium phosphate solution of pH 4.5 (adjusted with HCl). After concentrated by ultrafiltration, trehalose (203-02252, Wako Pure Chemical Ind. Ltd, Osaka, Japan), sucrose (S-9378, Sigma Chemical Co., St. Louis, MO, USA) or stachyose (S-4001, Sigma Chemical Co., St. Louis, MO, USA) solution was added to make a 3.3 mg/ml β -GA solution with various weight fractions of excipient. Two hundred microliters of the solution was frozen in a polypropylene sample tube (2.0 ml) by immersion in liquid nitrogen for 10 min, and then dried at a vacuum level below 5 Pa for 23.5 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo, Japan). The shelf temperature was between -35 and -30°C for the first 10 h, 20°C for the subsequent 10 h, and 30°C for the last 3.5 h.

Determination of Water Sorption and T_g of Lyophilized β -GA Formulations

Water vapor absorption isotherms were measured gravimetrically at 25°C for lyophilized β -GA formulations containing trehalose, sucrose or stachyose using the automated sorption analyzer (MB-300 G system, VTI Corp., FL, USA). Samples were dried at 25°C under a vacuum level below 0.1 Pa until changes in weight were less than $1 \mu\text{g}$ per 10 min. Water contents of the samples at partial vapor pressures of 0.10 and 0.20 (corresponding to 10 and 20% relative humidity (RH), respectively) were determined based on equilibrated sample weight (changes in weight of less than $1 \mu\text{g}$ per 10 min).

The T_g of lyophilized β -GA formulations was determined by modulated temperature differential scanning calorimetry (2920; TA Instruments, DE, USA). Before T_g measurements, samples were stored at 15°C for 24 h in a desiccator with a saturated solution of LiCl \cdot H_2O (12% RH).

The conditions were as follows: modulation period of 100 s, a modulation amplitude of $\pm 0.5^\circ\text{C}$, and an underlying heating rate of $1^\circ\text{C}/\text{min}$. Samples were put in a hermetic pan. Temperature calibration was performed using indium.

Determination of $T_{1\rho}$ of β -GA Carbonyl Carbon by ^{13}C Solid-state NMR

The rotating-frame spin-lattice relaxation time ($T_{1\rho}$) of β -GA carbonyl carbon in lyophilized formulations containing various weight fractions of trehalose, sucrose and stachyose was determined at 25°C using a UNITY plus spectrometer operating at a proton resonance frequency of 400 MHz (Varian Inc., CA, USA). Lyophilized samples were pre-equilibrated at 12% RH. Spin-locking field was equivalent to 19 kHz. The rotor size was 7 mm and spinning speed was 4 kHz. Peak height at approximately 180 ppm due to β -GA carbonyl carbon was followed with delay times of 1, 5, 10, 20, 30, 50 and 80 ms. Similar measurement of $T_{1\rho}$ was performed for lyophilized β -GA alone.

Fourier Transform Infra Red (FT-IR) Spectroscopy Measurements

FT-IR spectroscopy was performed using a JASCO FT/IR-6300 spectrometer (JASCO, Tokyo, Japan). A mixture of 100 mg KBr and 1–2 mg lyophilized β -GA formulation was pressed into a pellet under vacuum. A total of 256 scans and a resolution of 4 cm^{-1} were used for each spectrum. The second-derivative spectra were obtained from intact spectra without smoothing using Spectra Manager software version 2 (JASCO, Tokyo, Japan). The area of spectral absorbance was calculated using a baseline drawn between 1,600 and $1,700 \text{ cm}^{-1}$, and normalized for comparison between the formulations containing different excipients.

Determination of β -GA Aggregation Rate in Lyophilized Formulations

Lyophilized β -GA formulations containing trehalose, sucrose or stachyose, pre-equilibrated at 12% RH, were stored with a tight screw-cap at a constant temperature (40 – 90°C), removed at various times, and stored in liquid nitrogen until assayed. Samples were reconstituted in 1.7 ml of 200 mM phosphate buffer (pH 6.2), and injected into a size exclusion chromatography as described previously (10). The column (Tosoh G3000SW, 30 $\text{cm} \times 7.5 \text{ mm}$, Tokyo) was maintained at 30°C , and 200 mM phosphate buffer (pH 6.2) was used as the mobile phase. The detection wavelength was 280 nm. Monomeric β -GA was determined based on the peak height of its chromatogram.

Reconstitution of lyophilized β -GA formulations after storage was also carried out using reconstitution mediums of 200 mM phosphate buffer (pH 6.2) containing 0.5% additives (dextran sulfate (197-08362, Wako Pure Chemical Ind. Ltd, Osaka, Japan), 2-hydroxypropyl- β -cyclodextrin (C-0926, Sigma Chemical Co.), poly-L-lysine (P-7890, Sigma Chemical Co.), or pluronic (F-68, P-7061, Sigma Chemical Co., St. Louis, MO, USA).

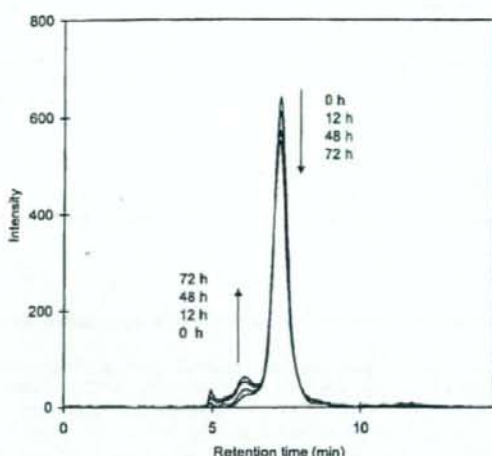


Fig. 1. Size-exclusion chromatograms of β -GA lyophilized with trehalose after various periods of storage at 80°C and 12%RH. The weight fraction of trehalose : 0.5.

RESULTS AND DISCUSSION

Aggregation of β -GA during Storage of Lyophilized Formulations

Figure 1 shows representative size-exclusion chromatograms of β -GA in lyophilized formulations, indicating that monomeric β -GA aggregates to larger sizes during storage. Tables I and II show the effects of reconstitution medium on the amount of monomeric β -GA remaining after storage of the formulation containing trehalose at temperatures below and above T_g , respectively. The amount of monomeric β -GA was not significantly affected by the addition of dextran sulfate or poly-L-lysine. Addition of pluronic also had no significant effect on the amount of monomeric β -GA. For lyophilized interleukin-2, the amount of aggregates after reconstitution was decreased by addition of poly-ions with high charge density, such as dextran sulfate

and poly-L-lysine, and increased by addition of surfactants, such as pluronic, into the reconstitution buffer solution (16). This may be explained by assuming that the formation of aggregates from partially unfolded intermediates, as well as the reverse formation of native protein from intermediates, occur during the reconstitution process. In contrast, the lack of the effects of additives observed for β -GA aggregation indicates that neither formation of aggregates nor reverse formation of native protein from partially unfolded intermediates occurs during the reconstitution process. This finding suggests that β -GA aggregation occurs during the storage of lyophilized formulations, even at temperatures below T_g . Because large-scale diffusion of protein molecules is considered to be very limited in glassy solids, β -GA aggregation is considered to occur between protein molecules that are adjacent to each other without large-scale diffusion.

Temperature Dependence of β -GA Aggregation Rate

Figure 2 shows time courses of aggregation of β -GA at 50°C (below T_g) for lyophilized formulations with an excipient fraction of 0.33, and at 80°C (above T_g) for lyophilized formulations with an excipient fraction of 0.5. Similar time courses were obtained for formulations with various excipient fractions and at various temperatures. The wide range of the time courses could be better described by the KWW equation, but the initial stages of aggregation were describable with first-order kinetics. The solid line in Fig. 2 represents the theoretical time course of first-order kinetics.

The time required for 10% degradation (t_{90}) was calculated from the apparent first-order rate constant. Figure 3 shows the temperature-dependence of t_{90} determined for aggregation of β -GA lyophilized with sucrose, trehalose or stachyose at an excipient fractions of 0.33 and 0.5, 12% RH and various temperatures. For the sucrose and trehalose formulations, the temperature dependence of t_{90} exhibited a change in the slope at around T_g , suggesting significant effects of molecular mobility. For the stachyose formulation, the change in the slope was not obvious, because few data were available at temperatures above T_g . The values of t_{90} at T_g largely depended on the excipient; sucrose > trehalose > stachyose. The finding that the t_{90} values at T_g varied significantly between these three formulations suggests that β -GA aggregation rate is not primarily related to $(T-T_g)$.

Table I. Effects of Reconstitution Medium on β -GA Aggregation Below T_g for 0.09 Trehalose Formulation

Additives in Reconstitution Medium	Peak Height for Monomeric β -GA (Relative to Solution Prior to Freeze Drying)			
	After Freeze Drying		After 24 h-storage at 70°C	
None	0.97	(0.01)	0.65	(0.01)
Dextran Sulfate	0.96	(0.00)	0.62	(0.01)
2-hydroxypropyl- β -cyclodextrin	0.95	(0.01)	0.62	(0.00)
Poly-L-lysine	0.94	(0.01)	0.62	(0.00)
Pluronic	0.97	(0.01)	0.66	(0.01)

0.5% additives

Values in brackets represent standard deviation ($n=3$)

Table II. Effects of Reconstitution Medium on β -GA Aggregation Above T_g for 0.33 Trehalose Formulation

Additives in Reconstitution Medium	Peak Height for Monomeric β -GA (Relative to Solution Prior to Freeze Drying)			
	After Freeze Drying		After 9 h-storage at 90°C	
	Value	SD	Value	SD
None	0.99	(0.01)	0.74	(0.03)
Dextran Sulfate	1.00	(0.00)	0.74	(0.01)
2-hydroxypropyl- β -cyclodextrin	0.98	(0.00)	0.73	(0.01)
Poly-L-lysine	0.98	(0.01)	0.74	(0.01)
Pluronic	1.00	(0.01)	0.76	(0.02)

0.5% additives

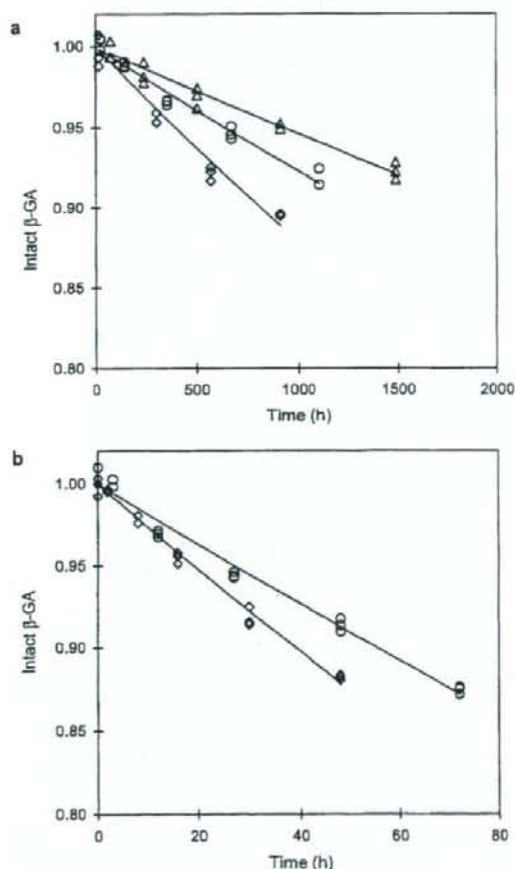
Values in brackets represent standard deviation ($n=3$)

Fig. 2. Time courses of aggregation of β -GA lyophilized with sucrose (Δ), trehalose (O) or stachyose (o). (a) aggregation at 50°C and excipient fraction of 0.33. (b) aggregation at 80°C and excipient fraction of 0.5.

Excipient-Fraction Dependence of β -GA Aggregation Rate

Figure 4 shows the dependence of t_{90} on the weight fraction of excipient at temperatures below T_g (50°C) and above T_g (80°C). As the weight fraction of excipient increased, t_{90} increased for all formulations. The values of t_{90} for the sucrose formulation in the amorphous state could not be determined at fractions above 0.5 at 50°C or above 0.33 at 80°C, because crystallization occurred during storage (crystallization was confirmed by the lack of crystallization peak in DSC thermograms). The t_{90} observed for β -GA aggregation showed a log-linear dependence on the excipient fraction, as reported for other proteins (11,12).

Figure 5 shows the water content and T_g determined for the lyophilized β -GA formulations with various weight fractions of trehalose, sucrose or stachyose. It has often been reported that lyophilized proteins without excipients do not show a distinct change in heat capacity in DSC thermograms. The T_g of lyophilized β -GA alone could not be determined in the dry state, but it could be estimated at 12% RH from small changes in heat capacity (Fig. 6). The T_g value determined at 12% RH depended on the excipient fraction (Fig. 5); T_g decreased with increasing excipient fraction from 0 to 0.3. Only a single T_g was observed in the range of excipient fractions from 0 to 0.3 for all formulations, suggesting that these formulations are a single glassy phase on levels detectable by DSC.

As shown in Figs. 4 and 5, the rank order of t_{90} at a certain excipient fraction was sucrose > trehalose > stachyose, whereas that of T_g of β -GA formulations was sucrose < trehalose < stachyose. The value of t_{90} increased significantly with increasing excipient fraction, even at small excipient fractions, in which T_g decreased significantly with increasing excipient fraction. These findings indicate that β -GA aggregation rate is not primarily related to ($T - T_g$).

Figure 7 shows the amount of monomeric β -GA remaining after freeze drying with sucrose, trehalose or stachyose. Significant changes were observed at an excipient fraction of 0.09 for all formulations. The stachyose formulation exhibited the largest degree of β -GA aggregation during freeze drying. This finding suggests that freeze-drying processes cause changes in protein conformation at differing degrees between excipients, which in turn leads to the differences in β -GA aggregation rate observed between excipients. FT-IR is known to be useful for detecting changes in protein conformation produced during the freeze-drying process (17). Figure 8 compares the second derivative FT-IR

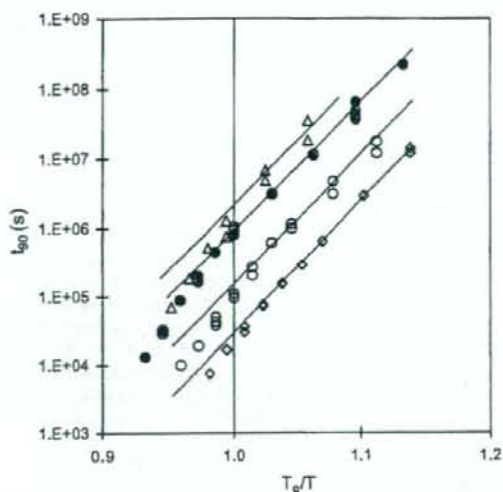


Fig. 3. T/T_g -dependence of t_{90} for aggregation of β -GA lyophilized with sucrose (Δ), trehalose (\circ) or stachyose (\diamond). The weight fraction of excipient: 0.33 ($\Delta\circ$) and 0.5 (\bullet).

spectra between the sucrose, trehalose and stachyose formulations. Significant differences in spectra were not observed between the excipients, and the differences in β -GA aggregation rate observed between the excipients could not be attributed to differences in protein secondary structure. It is known that changes in the tertiary structure of protein molecules created during freeze-drying processes can lead to

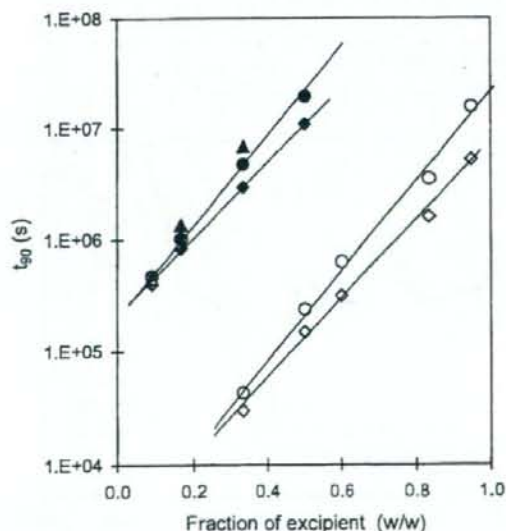


Fig. 4. Dependence of t_{90} on the weight fraction of excipient. The value of t_{90} was determined at 80°C and 12%RH for trehalose (\circ) and stachyose (\diamond), and at 50°C and 12%RH for trehalose (\bullet), sucrose (Δ) and stachyose (\star).

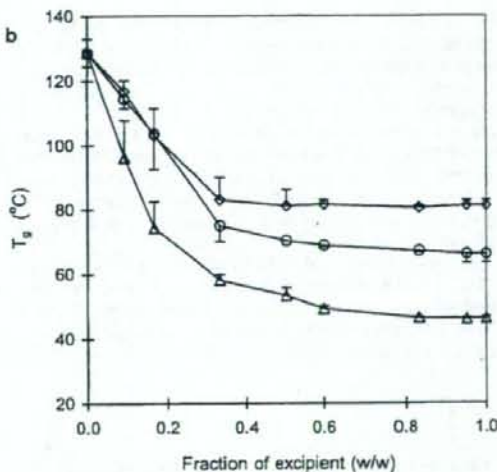
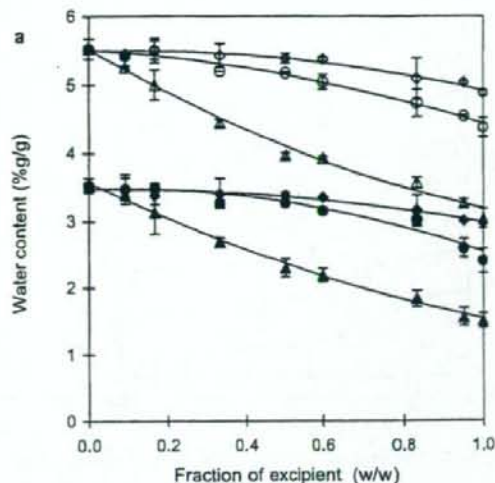


Fig. 5. Water content (a) and T_g (b) of lyophilized β -GA formulations containing trehalose (\circ), sucrose (Δ), or stachyose (\diamond) as a function of the weight fraction of excipient. (a) closed symbols: 10%RH; open symbols: 20%RH. 25°C. (b) 12%RH. sd ($n=3$).

protein aggregation during storage. A possibility that a tertiary structural change is responsible for the differences in β -GA aggregation rate observed between the excipients cannot be excluded.

Significance of Local Mobility, as Determined by $T_{1\rho}$ of β -GA Carbonyl Carbon, and Structural Relaxation in Protein Aggregation

It is generally considered that the rate of protein aggregation, an intermolecular reaction, is mainly determined by structural relaxation that allows for large-scale

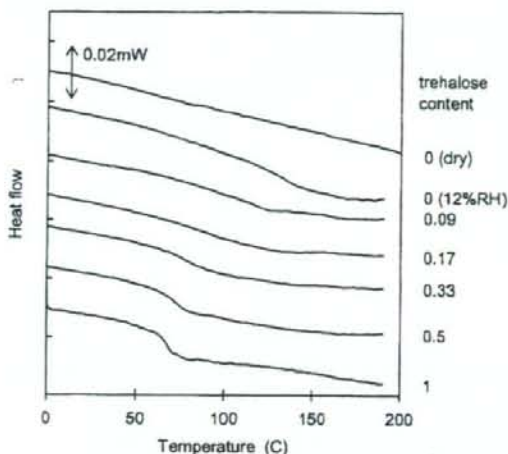


Fig. 6. DSC thermogram for β -GA lyophilized with various weight fractions of trehalose.

diffusion of reactants. From the finding that the t_{90} versus T_g/T plots for the lyophilized β -GA formulations exhibited a change in slope around T_g , β -GA aggregation rate appeared to correlate with structural relaxation. Although β -GA aggregation rate was not related to $(T-T_g)$, this may be explained by assuming that the fragility and fictive temperature of the formulation vary with the excipient. Because the structural relaxation times of the β -GA formulations were not determined in this study, correlations between β -GA aggregation rate and structural relaxation could not be elucidated.

Meanwhile, the local mobility of β -GA was determined by $T_{1\rho}$ of β -GA carbonyl carbon. Figure 9 shows the time course of rotating-frame spin-lattice relaxation at 25°C and 12% RH for the carbonyl carbon of β -GA lyophilized with sucrose, trehalose or stachyose at an excipient fraction of 0.5.

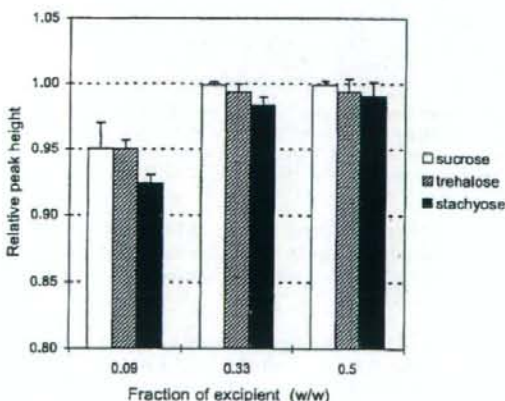


Fig. 7. Ratio of monomeric β -GA remaining after freeze drying with sucrose, trehalose or stachyose. Bars represent standard deviation ($n=3$).

Spin-lattice relaxation was significantly retarded by the addition of excipient. Sucrose resulted in the largest degree of retardation, and there were no significant differences in the degree of retardation between the trehalose and stachyose formulations. The time course of spin-lattice relaxation was describable with a bi-exponential equation including two different $T_{1\rho}$ values. The longer $T_{1\rho}$ value was estimated by curve fitting using a shorter $T_{1\rho}$ of 9 ms and a proportion of 13% for carbonyl carbons with the shorter $T_{1\rho}$. Figure 10 shows the estimates for the longer $T_{1\rho}$ of the dominating proportion, plotted as a function of the excipient fraction. The $T_{1\rho}$ for the sucrose formulation increased significantly with excipient fraction. For the stachyose formulation, in contrast, increases in $T_{1\rho}$ were not significant at an excipient fraction of 0.09, and $T_{1\rho}$ was less than in the sucrose formulation at higher excipient fractions. $T_{1\rho}$ for the trehalose formulation exhibited intermediate behavior when compared to the sucrose and stachyose formulations. The rank order of the ability of excipients to decrease the local

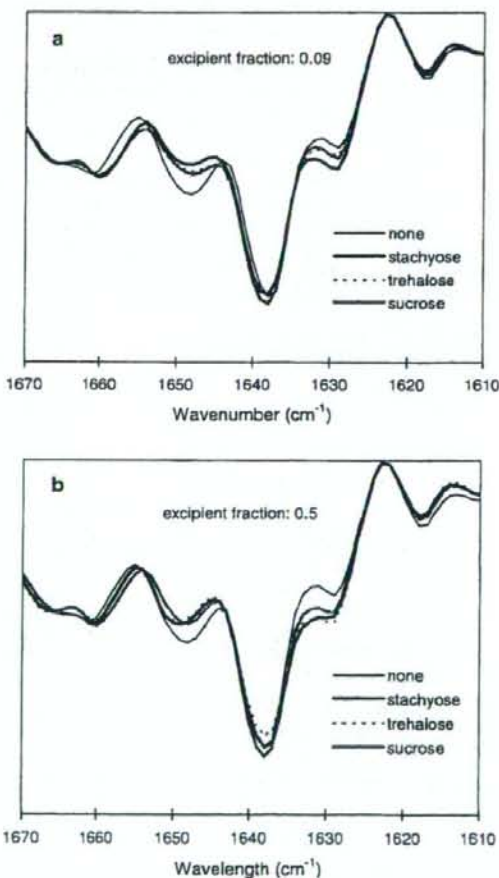


Fig. 8. Second derivative FT-IR spectra for β -GA lyophilized with sucrose, trehalose or stachyose of 0.09 (a) and 0.5 fractions (b).

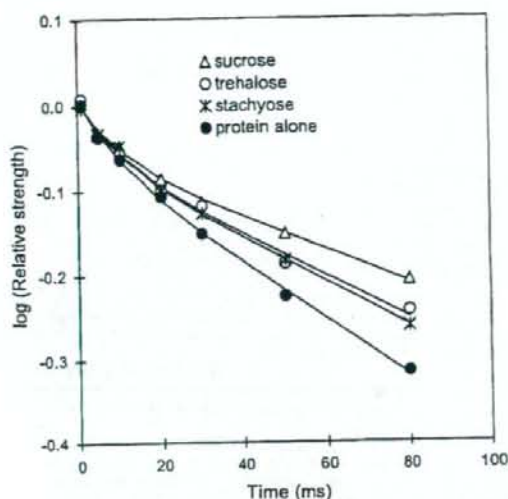


Fig. 9. Time course of spin-lattice relaxation at 25°C and 12%RH for carbonyl carbon of β -GA lyophilized with sucrose, trehalose or stachyose. The weight fraction of excipient : 0.5.

mobility of β -GA appeared to be the same as the rank order of their ability to decrease aggregation rate. This finding suggests that local mobility is a primary factor that affects the stability of lyophilized β -GA formulations; sucrose more potently inhibits local mobility of β -GA, and thus more strongly inhibits β -GA aggregation.

Local mobility is generally considered to follow Arrhenius kinetics. If local mobility is mainly responsible for β -GA aggre-

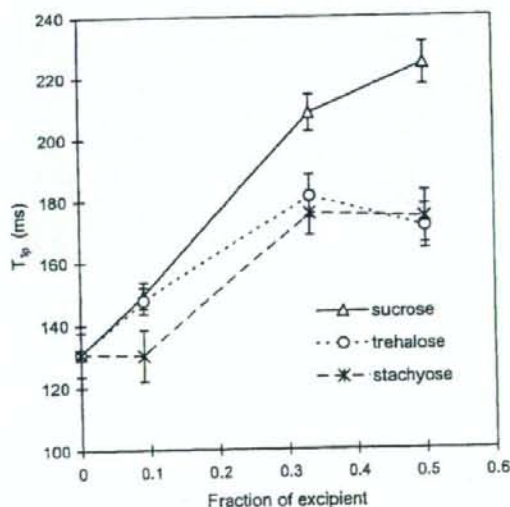


Fig. 10. Effect of weight fraction of excipient on $T_{1\rho}$ of carbonyl carbon at 25°C and 12%RH for β -GA lyophilized with sucrose, trehalose or stachyose

gation, the temperature dependence of t_{90} should not show a change in slope around T_g . The non-Arrhenius temperature dependence observed for the t_{90} of β -GA aggregation, which is considered to be governed by local mobility, may be explained by assuming that local mobility of protein is coupled with structural relaxation. For bovine serum γ -globulin, the local mobility of protein, as measured by the laboratory-frame spin-lattice relaxation time (T_1) of protein carbonyl carbon, exhibited Arrhenius temperature dependence when lyophilized without excipient (18). When lyophilized with dextran, in contrast, the local mobility of protein exhibited a change in the slope of temperature dependence around the T_{mc} (T_g determined by NMR relaxation measurement), as did local mobility of dextran, as measured by T_1 of dextran methine carbon. These findings suggested that the local mobility of protein was coupled with the structural relaxation of lyophilized solids. The same may be said for the local mobility of protein and structural relaxation of β -GA lyophilized with sucrose, trehalose or stachyose. The local mobility of β -GA may exhibit Arrhenius temperature dependence in the absence of excipient. Upon the addition of excipient, local mobility may become to be coupled with structural relaxation, and the temperature dependence of protein local mobility may become to deviate from Arrhenius behavior.

The great increase in t_{90} with increasing excipient fraction observed for β -GA aggregation rate, as indicated by log-linear dependence on the excipient fraction, may be attributed mainly to the effect of excipient inhibiting protein local mobility in addition to the effect of excipient diluting protein molecules.

CONCLUSION

The aggregation rate of β -GA lyophilized with sucrose, trehalose or stachyose unexpectedly correlated with the local mobility of β -GA rather than with ($T-T_g$). An increase in the weight fraction of excipient appeared to increase the effects of excipient decreasing local mobility, resulting in increases in the stability of β -GA. Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

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Influences of the recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells in serum-free culture

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Abstract

To improve the safety of cellular therapy products, it is necessary to establish a serum-free cell culture method that can exclude animal-derived materials in order to avoid contamination with transmissible agents. It would be optimal if the proteins necessary to a serum-free culture could be provided as recombinant proteins. In this study, the influences of recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells (HUVECs) in serum-free culture were examined in comparison with the influence of plasma fibronectin (FN). The recombinant proteins used were Pronectin F (PF), Pronectin F PLUS (PFP), Pronectin L (PL), Retronectin (RN), and Attachin (AN). HUVECs adhered more efficiently on PF or PFP than on FN. No cells adhered on PL. Regarding the VEGF or bFGF-induced cell growth, the cells on PF and PFP proliferated at a similar rate to the cells on FN. RN and AN were less effective in supporting cell growth. Since cell adhesion on PF and PFP induced phosphorylation of focal adhesion kinase, they are thought to activate integrin-mediated intracellular signaling. The cells cultured on PF or PFP were able to produce prostaglandin I₂ or tissue-plasminogen activator in response to thrombin. However, thrombin caused detachment of the cells from PF but not from PFP or FN, meaning that the cells were able to adhere more tightly on PFP or FN than on PF. These data indicate that PFP could be applicable as a substitute for plasma FN.

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Keywords: Cell adhesive protein; Recombinant protein; Fibronectin; Endothelial cells

1. Introduction

The widespread development of cellular therapy products has advanced to the stage of non-clinical and clinical testing [1–3]. Regulatory documents for human somatic cell therapy including instructions for investigational new drug applications have been published [4–6]. To guarantee the safety of both product recipients and the public at large, it is crucial to prevent contamination of cellular therapy products by infectious agents [7].

Serum-free culture is one of the desired methods for manufacturing cellular therapy products when safety issues are a concern [8,9]. Although serum is a very effective additive

for a culture medium that can support cell adhesion, survival, growth, and functions, animal-derived materials such as serum may contain transmissible agents or human allergens [10–13]. Since serum is composed of proteins, sugars, lipids, vitamins, and other ingredients, its quality is affected by the genetic and environmental circumstances of the animal used, which means there are lot-to-lot variations in composition and potency. This variability could reduce the consistency of cellular therapy products, and a serum-free culture that could contribute to improving the consistency of the cell features is thus needed.

Protein factors often need to be added to serum-free culture to substitute for the functions of serum. The proteins used for this purpose must also pose no risk of infection. Because recombinant proteins can be produced without using animal-derived materials, and pose little risk of contaminating human pathogens, they are a useful biomaterial for culturing cells

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when safety issues are a concern. In addition, the functions of recombinant proteins can be improved by modifying their amino acid sequences, which is also an advantage. In this study, using a serum-free culture of human umbilical vein endothelial cells (HUVECs), the usefulness of recombinant artificial cell adhesive proteins as a plasma fibronectin substitute was evaluated.

The extracellular matrix glycoprotein fibronectin consists of two similar polypeptide chains, each with a molecular mass of approximately 250 kDa joined at their respective C termini by disulfide bonds [14,15]. The RGD (arginine–glycine–aspartic acid) recognition sequence located within the molecule was the first amino acid motif shown to mediate cell adhesion [16,17]. Intracellular signaling induced by cell adhesion on fibronectin plays a critical role in cytoskeletal reorganization, cell cycle progression, and cell survival [18,19].

This study defines the influences of five different kinds of recombinant artificial cell adhesive proteins on endothelial adhesion, proliferation, and antithrombotic function in serum-free culture for which plasma fibronectin is needed. The recombinant proteins used were Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, and Attachin (Table 1).

2. Materials and methods

2.1. Recombinant cell adhesive proteins

Pronectin F, Pronectin F PLUS, and Pronectin L were purchased from Sanyo Chemical Industry (Kyoto, Japan), Retronectin from TaKaRa (Shiga, Japan), and Attachin from Bio999 (Taipei, Taiwan). All of these proteins were produced via bacterial fermentation. Pronectin F is a genetically engineered protein containing repeating units of the RGD sequence interspersed with a β -silk peptide for structural stability [20]. It is

comprised of 980 amino acids. Based on its sequence, the molecular weight is estimated to be 72,728. The amino acid sequence is fMDPVVLQRRDWDENPGVTQLNRLAAHPPFASDPMGAGS(GAGAGS)₆GAAVTGRGDSPPASAAGY-[(GAGAGS)₉GAAVTGRGDSPPASAAGY]₁₂-(GAGAGS)₂GAGMDPGRYQLSAGRYHYQLVWCQK. Pronectin F PLUS is a positively charged water-soluble variant of Pronectin F that is produced by chemical modification [21,22]. Pronectin L is a protein polymer that exhibits IKVAV epitopes from the laminin alpha chain with a similar backbone as Pronectin F [21]. It is comprised of 1019 amino acids. The molecular weight is estimated to be 75,639. The amino acid sequence is fMDPVVLQRRDWDENPGVTQLNRLAAHPPFASDPMGAGS(GAGAGS)₆GAAPGASIKVAVSAGPSAGY-[(GAGAGS)₉GAAPGAIKAVSAGPSAGY]₁₂-(GAGAGS)₂GAGMDPGRYQLSAGRYHYQLVWCQK. Retronectin consists of a central cell-binding domain, a high affinity heparin-binding domain II, and a CS1 site within an alternatively spliced type III connecting segment region of human fibronectin [23]. It is comprised of 574 amino acids. The molecular weight is estimated to be 62,631. Attachin is an artificial fusion protein with the molecular weight of 30 kDa that has several functional domains, including a fibronectin-like cell attachment domain [24]. It has reportedly been used to promote the adhesion of several kinds of cell lines, including CHO-K1, MDBK, PK-15, L929, Vero, COS, U373, Swiss 3T3, and MRC-5 [25].

2.2. Cells and materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Sanko Junyaku (Tokyo, Japan) and maintained

Table 1
Fibronectin and recombinant cell adhesive proteins used in this study

Proteins	Structure	Molecular Weight
Fibronectin		250K x 2
Pronectin F	Head-[(GAGAGS) ₉ GAAVTGRGDSPPASAAGY] ₁₂ -Tail ★	73K
Pronectin F Plus	Positively charged, water-soluble variant of Pronectin F	73K
Pronectin L	Head-[(GAGAGS) ₉ GAAPGASIKVAVSAGPSAGY] ₁₂ -Tail ◆	76K
Retronectin	Chimeric protein of human fibronectin fragment NH ₂ -□□□□~□□□□-□ COOH	63K
Attachin	A fusion protein constructed by molecular biotechnology	30K

Fibronectin.

□ Type I module.

○ Type II module.

□ Type III module.

★ cell attachment sequence derived from fibronectin.

◆ cell attachment sequence derived from laminin.

in EGM-2 media (Cambrex, Walkersville, MD) on collagen-coated dishes (Asahi Techno Glass, Tokyo, Japan). EGM-2 media is modified MCDB 131 containing 2% fetal bovine serum, VEGF, bFGF, IGF-1, EGF, heparin, hydrocortisone and ascorbic acid. The cells were kept in a humidified, 5% CO₂ environment at 37 °C. Cells between passages 3 and 5 were used for all experiments.

The serum-free media used was human endothelial SFM (Invitrogen, Carlsbad, CA) [26]. Ten nanograms/ml of epidermal growth factor (EGF) (Invitrogen) and 20 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen) were added as supplements. Fibronectin is recommended for use as a cell attachment factor.

2.3. Cell adhesion assay

Recombinant cell adhesive proteins were diluted with phosphate buffered saline (PBS(-)), and plated on multiwell non-treated polystyrene plates (BD Falcon, Franklin Lakes, NJ). The plates were incubated for 2 h at room temperature, and then the protein solutions were removed and the wells washed with PBS(-). The concentration of the recombinant proteins used was 10 µg/ml (2 µg/cm²), unless the description states otherwise. The amount of absorbed protein was quantified with a QuantiPro BCA Kit (Sigma, St. Louis, MO) using bovine serum albumin as a standard.

The HUVECs were harvested using trypsin and washed twice with PBS(-). The cells were then suspended in serum-free media, and added to each well of 96-well plates previously coated with recombinant cell adhesion proteins at a cell density of 1×10^4 cells/well. After incubating for 60 min under 5% CO₂ at 37 °C, the supernatant was removed, and the wells rinsed with PBS(-) to remove non-adherent cells. Following fixation of the adherent cells by 4% paraformaldehyde for 10 min, the paraformaldehyde was removed and the cells washed once with distilled water. A 0.5% (w/v) solution of crystal violet was then added to the wells. After staining for 25 min, the cells were rinsed five times with distilled water and the crystal violet that was absorbed on the adherent cells was solubilized with 0.5% SDS. The optical density at 595 nm was measured on an EL340 plate reader (BioTek Instruments, Winooski, VT).

2.4. Measurement of cell proliferation

HUVECs were harvested and suspended in serum free media, and plated to each well of the 96-well plates previously coated with recombinant cell adhesive proteins. VEGF (R&D Systems, Minneapolis, MN) or bFGF (Invitrogen, Carlsbad, CA) was added at concentrations of 1–100 ng/ml. After culturing for 2 days under 5% CO₂ at 37 °C, the cell number in each well was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Results are expressed as the mean value \pm S.D. of triplicate determinations.

2.5. Phosphorylation of focal adhesion kinase

The HUVECs were collected by trypsin treatment (Invitrogen), resuspended in serum free medium, incubated for 2 h at 37 °C in suspension, and subsequently plated at 5×10^5 cells/dish on 60 mm diameter dishes pre-coated with cell adhesive proteins or bovine serum albumin (BSA) (10 µg/ml) [27]. After incubation for 2 h under 5% CO₂ at 37 °C, the cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate), and the protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). 3.5 µg of the total cell lysates was resolved by SDS-PAGE, blotted onto Immobilon-P membranes (Millipore, Volketswil, Switzerland), and incubated in 1% BSA with anti-pY397 FAK antibody (Upstate Biotechnology Inc., Lake Placid, NY) followed by incubation with horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology, Danvers, MA). The ECL system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and Luminoimage analyzer LAS 3000 (Fuji Film, Kanagawa, Japan) were used for detection. The membranes were stripped of bound antibody using a Re-Blot Plus Western Blot Recycling Kit (Chemicon, Temecula, CA), and the membrane was reprobed with anti-FAK antibody (Upstate Biotechnology). The labeled bands were quantified using the MultiGauge software program (Science Lab). The quantified value for phosphorylated FAK was normalized with that for total FAK in each sample, and the relative phosphorylation level was then calculated as a ratio against the cell lysate on fibronectin.

2.6. Secretion of Prostaglandin I₂ (PGI₂)

The HUVECs were harvested using trypsin, washed twice, and then suspended in serum-free media. The cells were placed on the 24-well plates coated with recombinant cell adhesive proteins at the density of 7×10^4 /well. After culturing for 1 day, the cells were stimulated with 1 U/ml of thrombin or 30 ng/ml of VEGF. The cells were incubated for 1 h under 5% CO₂ at 37 °C, and then the supernatant was collected. The supernatant was centrifuged for 10 min at 2000 \times g in a microcentrifuge to remove any residual cells. The level of 6-keto Prostaglandin F₁ α , a major metabolite of Prostaglandin I₂, was determined using 6-keto Prostaglandin F₁ α EIA Kit (Cayman Chemical, Ann Arbor, MI). Each culture condition was repeated in triplicate.

2.7. Secretion of tissue-plasminogen activator

The HUVECs were seeded as described above. After culturing for 1 day, the cells were stimulated with 0.01–1 U/ml of thrombin. Twenty-four hours later, the supernatant was collected and spun down for 10 min at 2000 \times g in a microcentrifuge. The level of tissue plasminogen activator was determined using the AssayMax Human Tissue-Type Plasminogen Activator ELISA Kit (Assay Pro, Brooklyn, NY). Each culture condition was repeated in triplicate. Phase contrast images were obtained before the supernatant was collected.

3. Results

3.1. Adhesion of HUVECs onto recombinant cell adhesive proteins

To examine the adhesion onto the recombinant cell adhesive proteins, HUVECs harvested and suspended in serum-free media were applied on plastic wells precoated with Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or fibronectin (Table 1). As shown in Fig. 1, Pronectin F, Pronectin F PLUS, Retronectin, or Attachin showed cell adhesion activity to a similar extent as fibronectin at the concentration of 10 $\mu\text{g/ml}$ (2 $\mu\text{g/cm}^2$). Pronectin F and Pronectin F PLUS were superior to fibronectin when they were used at an amount of less than 1 $\mu\text{g/ml}$. The cells were observed under microscopy to have spread well on the recombinant proteins that they adhered to (Fig. 2). No cells had adhered onto Pronectin L or BSA.

In order to test if these differences are due to the difference in the absorbed amount of each protein, the protein absorbed on the plate was measured (Fig. 3). It was found that Pronectin F and Pronectin L had absorbed to the plate better than other proteins and that the absorbed amount of Pronectin F PLUS was lower than that of Pronectin F and Pronectin L. From the point of view of the efficiency of cell adhesion (Fig. 1) and the absorbed amount of protein (Fig. 3), the number of cells attached was not dependent on the amount of absorbed protein, suggesting that the observed effects were not due to differences in protein absorption but rather due to the character of each cell adhesion protein.

3.2. Influences on cell proliferation

Besides anchorage, cell adhesion to components of the extracellular matrix triggers signaling events affecting diverse

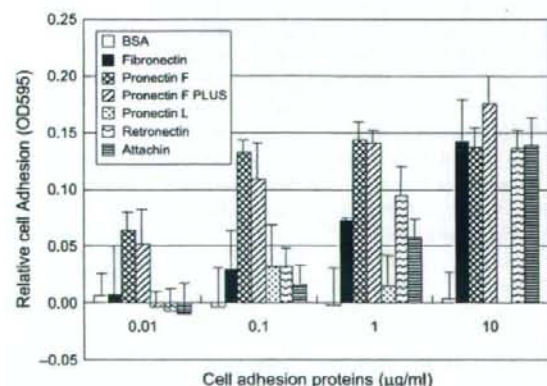


Fig. 1. Adhesion of HUVECs to recombinant cell adhesive proteins. HUVECs were plated into wells previously coated with Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or BSA, and then incubated for 1 h at 37 °C. Attached cells were fixed and stained with crystal violet and quantified by absorbance reading. Results are expressed as mean value \pm S.D. of triplicate determinations. The 0.1 OD corresponded to $0.61 \pm 0.085 \times 10^4$ cells.

cellular traits and activities, including survival, proliferation, and other functions. Therefore, the influences of the recombinant cell adhesion proteins on cell proliferation were examined. The HUVECs in serum-free media were seeded on the plates that were coated with recombinant cell adhesive proteins, and then cultured in the presence or absence of basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) at concentrations from 1 to 100 ng/ml. After culturing for 2 days, the cell numbers in each well were examined (Fig. 4). When HUVECs were cultured in the presence of bFGF, the cells on Pronectin F or Pronectin F PLUS had grown to the same level as that on fibronectin in each concentration of bFGF, showing that Pronectin F and Pronectin F PLUS have the same growth support potency as fibronectin (Fig. 4A). However, the cell numbers on Retronectin or Attachin reached 70% or 40% of that on fibronectin, respectively; showing that the growth support potency of Retronectin and Attachin was lower than fibronectin. When VEGF-stimulated cell proliferation was examined, Pronectin F and Pronectin F PLUS also showed similar growth-supporting potency to fibronectin (Fig. 4B). Retronectin and Attachin were less effective than fibronectin.

3.3. Phosphorylation of focal adhesion kinase

Cell adhesive proteins activate intracellular signaling via cell surface integrins. In regulating the cellular responses to integrin-mediated adhesion, focal adhesion kinase (FAK) has emerged as a key signaling molecule [28–30]. Integrin–ligand engagement promotes FAK tyrosine phosphorylation that promotes FAK signaling activity. The phosphorylation of FAK Tyr-397, the only apparent autophosphorylation site, is known to create a high-affinity binding site for SH2 domains of the Src-family kinases, including c-Src and Fyn [31].

HUVECs cultured in growing media were harvested by trypsin treatment, and incubated in serum-free media for 2 h at 37 °C in suspension. After this incubation, the cells were plated onto the dishes in which recombinant cell adhesive proteins were coated, and then incubated for 2 h under 5% CO_2 at 37 °C. Cell lysates were prepared using RIPA buffer, and the phosphorylation level of FAK on Tyr 397 was then examined by Western blotting. As shown in Fig. 5, FAK Tyr-397 is phosphorylated under an attached condition (lane 1), becomes dephosphorylated upon cell suspension (lane 2), and then becomes rephosphorylated after replating. The FAK phosphorylation level after replating on recombinant proteins differed in each sample (lanes 3–10). Phosphorylation level of cells on BSA (lane 3) or Pronectin L (lane 7), to which cells did not adhere, was still lower after replating. Pronectin F (lane 5) or Pronectin F PLUS (lane 6) induced FAK phosphorylation to the similar extent to Fibronectin (lane 4), suggesting that Pronectin F and Pronectin F PLUS are thought to activate intracellular signaling via integrin as well as fibronectin. Retronectin that has RGD domain of Fibronectin also induced FAK phosphorylation (lane 8).

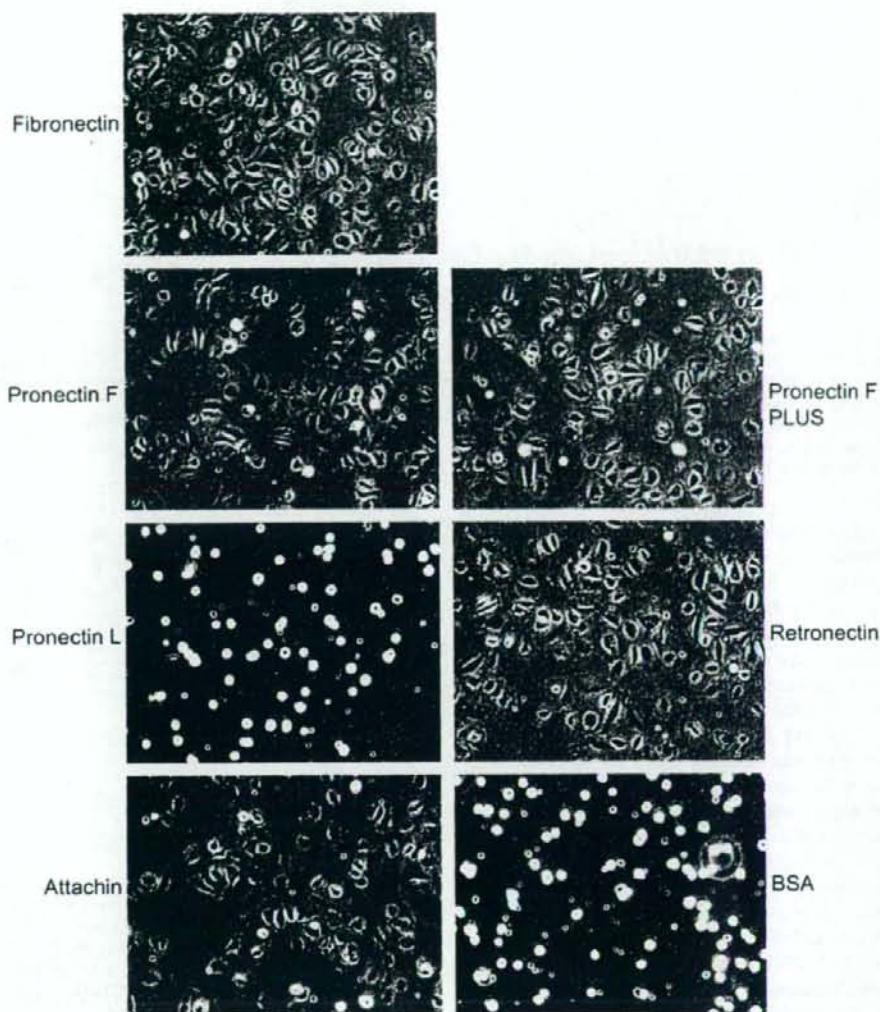


Fig. 2. The phase contrast images of cell attachment and spreading on recombinant cell adhesive proteins. HUVECs were seeded into wells previously coated with 10 $\mu\text{g/ml}$ of Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, Attachin, or BSA. After 1 h incubation at 37 °C, the cells were photographed with an inverted microscope.

3.4. Influences on antithrombotic functions of endothelial cells

In order to examine the influences of recombinant cell adhesive proteins on cellular functions, the production of prostaglandin I_2 (PGI_2) and tissue-plasminogen activator (t-PA), both of which play important roles in the antithrombotic feature of endothelial cells, was tested. Antithrombotic activity is the most important characteristic of endothelial cells that are used for artificial blood vessels or engineered vascularized tissues. PGI_2 inhibits platelet aggregation, thereby inhibiting the formation of thrombus. It is produced quickly after stimulation via the activation of phospholipase A_2 . Since the half

life of PGI_2 is as short as 2 min, the concentrations of its metabolite 6-keto Prostaglandin $\text{F}_{1\alpha}$ were measured. t-PA is produced from endothelial cells via the induction of protein synthesis in response to the extracellular stimuli. It induces the processing of plasminogen to plasmin, and dissolves the fibrin clots.

HUVECs were plated on the recombinant cell adhesion protein-coated dishes and cultured for 1 day, then stimulated with VEGF or thrombin. As shown in Fig. 6A, PGI_2 was produced from VEGF-stimulated HUVECs on different kinds of cell adhesive proteins to the same level. In the serum-free media, the basal production of PGI_2 was higher, and the production after the stimulation was less than in the growing

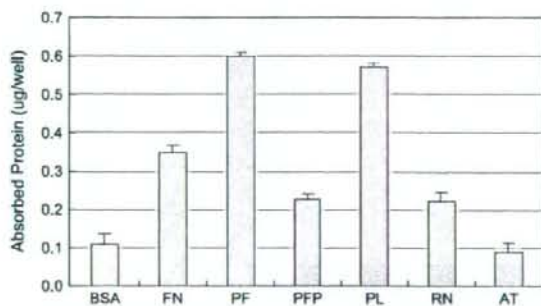


Fig. 3. Protein absorption on non-treated polystyrene plates. Ten $\mu\text{g}/\text{ml}$ of bovine serum albumin (BSA), Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFP), Pronectin L (PL), Retronectin (RN) or Attachin (AN) was added to 96-well plates and incubated at room temperature for 2 h. Each well was washed with PBS(-) and the amount of absorbed protein was quantitated by QuantiPro BCA using bovine serum albumin as a standard. Each data point represents the mean \pm S.D. of triplicate determinations.

media. Similar results were obtained when the cells were stimulated with thrombin (Fig. 6B).

Next, t-PA production from the thrombin-stimulated HUVECs was examined. As shown in Fig. 7, the t-PA concentration in the culture supernatant of HUVECs that adhered on Pronectin F PLUS was the same as that on Fibronectin. However, the concentration was lower in the supernatant on Pronectin F, Retronectin, or Attachin than on fibronectin. The basal production of t-PA in the serum-free media was less than in growing media; therefore, responsiveness to thrombin was readily observed in the serum-free culture.

During these experiments using thrombin, detachment of the thrombin-stimulated cells from Pronectin F, Retronectin, or Attachin was observed (Fig. 8). No detachment was seen in the cells that adhered on fibronectin or Pronectin F PLUS. This result might reflect the possibility that the adhesiveness of the cells to Pronectin F, Retronectin, and Attachin is weaker than to Pronectin F PLUS or fibronectin.

The following data support the results of Fig. 8, which are presented using microscopic images. Since HUVECs are adherent cells, detachment from the dishes leads to a loss of viability. Therefore, changes in the viable cell concentration after thrombin stimulation reflect cell detachment. As shown in Fig. 9, the production of t-PA and changes in the viable cell concentration after culturing in the presence of 0.01, 0.1, or 1 U/ml of thrombin were tested. At all three concentrations of thrombin used, cell detachment was observed only from Pronectin F. Although the t-PA concentration was increased in a dose-dependent manner by thrombin in the cells that adhered on Fibronectin or Pronectin F PLUS (Fig. 9A), there was less of an increase in t-PA production from the cells on Pronectin F when a low dose (0.01 or 0.1 U/ml) of thrombin was used. At those concentrations, the viable cell concentration on Pronectin F was decreased by thrombin stimulation, meaning that cell detachment occurred on Pronectin F (Fig. 9B). At a high concentration of thrombin (1 U/ml), the viable cell concentration decreased slightly even though cell detachment was

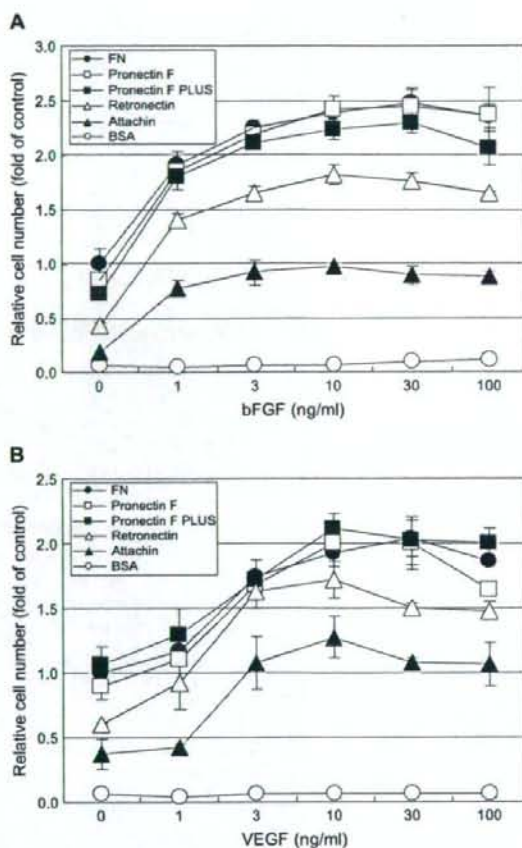


Fig. 4. Effects of recombinant cell adhesive proteins on the VEGF- or bFGF-induced cell proliferation. HUVECs were seeded into wells previously coated with 10 $\mu\text{g}/\text{ml}$ of Fibronectin, Pronectin F, Pronectin F PLUS, Retronectin, Attachin, or BSA, and cultured for 2 days in the presence or absence of bFGF (A) or VEGF (B) at the concentrations indicated. The relative cell number was examined using a cell counting kit-8. The results are expressed as the mean value \pm S.D. of triplicate determinations.

observed. This might be because the cell proliferative effect of thrombin canceled the decrease in cell viability. t-PA production corrected by the viable cell concentration is shown in Fig. 9C. By correcting the t-PA concentration using the viable cell concentration, the dose-dependency of thrombin in t-PA production was similar in Pronectin F to Pronectin F PLUS and fibronectin (Fig. 9C). From these results, in the cells on Pronectin F and Pronectin F PLUS, the signals from thrombin transduced similarly, but the strength of the cell adhesion differed.

4. Discussion

The goal of the present study was to establish a cell culture method that can improve the safety of cellular therapy products. Our focus is now on human endothelial cells, because

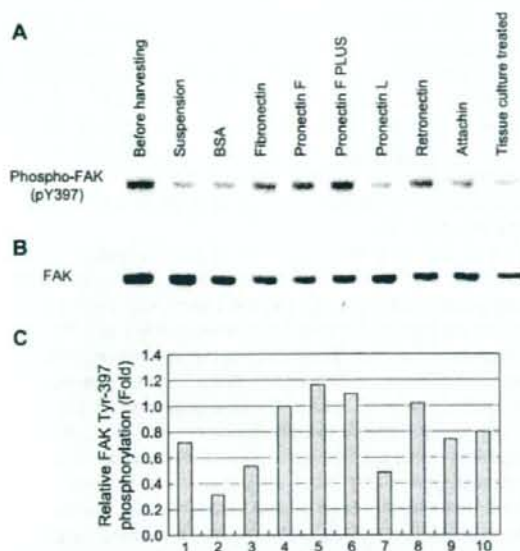


Fig. 5. FAK activation in HUVECs adhered to recombinant cell adhesive proteins. HUVECs cultured in serum-containing EGM-2 media on collagen-coated plates were harvested and held in suspension for 2 h at 37 °C, then plated onto precoated plates with 10 µg/ml of Fibronectin, Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, or Attachin. After 2 h incubation at 37 °C, cell lysates were prepared using RIPA buffer. The cell extracts were analyzed for the phosphorylation (A) and protein levels of focal adhesion kinase (B) by Western blotting. The amount of proteins detected was analyzed using MultiGauge software (C). Lane 1: before harvesting; lane 2: after suspension; lane 3: BSA; lane 4: Fibronectin; lane 5: Pronectin F; lane 6: Pronectin F PLUS; lane 7: Pronectin L; lane 8: Retronectin; lane 9: Attachin; lane 10: tissue culture-treatment dish. Similar results were obtained in two independent experiments. Representative blots are shown.

they are expected to be put into clinical use in the near future. Serum-free culture supplemented with recombinant proteins produced without using animal-derived materials would be optimal in order to avoid contamination by animal-derived materials and transmissible agents. This work was carried out as a step in demonstrating the feasibility of recombinant cell adhesion proteins in serum-free cultures of human endothelial cells, which might form the basis of future cellular therapies. HUVECs were used as a model of tissue-derived differentiated endothelial cells.

One of the uses of endothelial cells in cellular therapy is for the vascularization of engineered tissue. Vascularization of a tissue-engineered construct is mediated primarily through the ingrowth of surrounding vessels from the peri-implant tissue. However, this process is quite slow and is not sufficient to achieve appropriate vascularization of large defects [32]. The incorporation of endothelial cells or vascular-like structure in the tissue-engineered constructs before transplantation is a possible solution of this problem. Both human embryonic stem cell-derived endothelial cells and tissue-derived endothelial cells such as HUVECs have been shown to incorporate successfully into engineered skeletal muscle tissue [33–35].

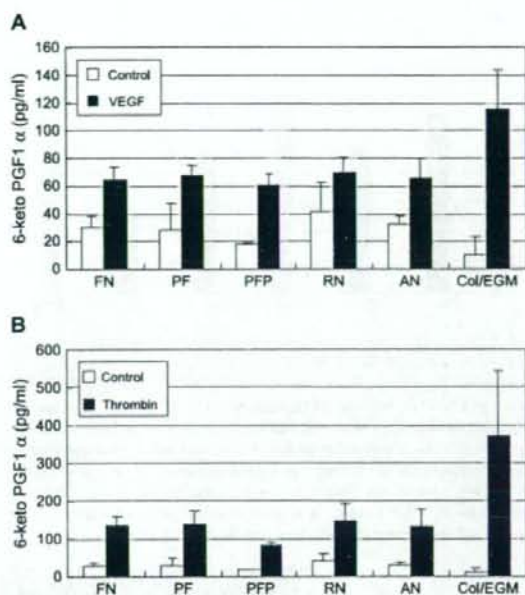


Fig. 6. Effects of cell adhesive proteins on the VEGF- or thrombin-stimulated production of Prostaglandin I₂. HUVECs were seeded at 7×10^4 cells/well on 24-well plates previously coated with Fibronectin (FN), Pronectin F (PF), Pronectin F PLUS (PFP), Retronectin (RN), or Attachin (AN). After culturing for 1 day, the cells were stimulated with 30 ng/ml of VEGF (A) or 1 U/ml of thrombin (B). One hour later, the conditioned media were collected and 6-keto PGF₁α concentration was determined by enzyme immunoassay. Results are expressed as picograms of PGF₁α/ml of the supernatant and represent the mean ± S.D. of triplicate determinations. 6-Keto PGF₁α production from HUVECs cultured in serum-containing growing media (EGM-2) plated on a collagen-coated dish was also examined (Col/EGM).

Tissue-derived human endothelial cells are also used for the endothelialization of artificial artery grafts [36]. Although serum-free media for human endothelial cells has been developed, being supplemented with a cell adhesion protein such as fibronectin is necessary [26]. Since plasma fibronectin is known to bind to several pathogens including viruses [37,38], use of a recombinant cell adhesion protein would be desired in order to avoid the contamination of infectious agents.

Another use of endothelial cells in cellular therapy is for therapeutic neovascularization, an important adaptation to rescue tissue from critical ischemia. In a rat model of myocardial infarction, transplantation of HUVECs has been shown to contribute to increased neovascularization [39]. Recently, much attention has been paid to outgrowth endothelial cells (OECs) obtained by *in vitro* culture of blood mononuclear cells [40–46]. OECs show a marked similarity to fully differentiated endothelial cells with respect to their cellular morphology, marker expression and potential to form capillary-like structures [43,44,47]. Because OECs can be obtained from blood mononuclear cells, they have the advantage that autologous endothelial cells could be obtained without invasive procedures. The potential use of OECs for therapeutic neovascularization has been shown in murine ischemia models