This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK

Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Functional Culture of Human Periodontal Ligament Fibroblast (Hplf) on Polyelectrolyte Complex (Pec)

T. Hamano[a]; H. Suzuki[a]; A. Teramoto[a]; E. Iizuka[a]; K. Abe[a]
[a] Department of Functional Polymer Science Faculty of Textile Science and Technology, Shinshu University, Ueda, Japan

To cite this Article Hamano, T. , Suzuki, H. , Teramoto, A. , Iizuka, E. and Abe, K.(1998) 'Functional Culture of Human Periodontal Ligament Fibroblast (Hplf) on Polyelectrolyte Complex (Pec)', Journal of Macromolecular Science, Part A, 35: 3, 439 — 455
To link to this Article: DOI: 10.1080/10601329808001988
URL: http://dx.doi.org/10.1080/10601329808001988

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J.M.S.—PURE APPL. CHEM., A35(3), pp. 439–455 (1998)

FUNCTIONAL CULTURE OF HUMAN PERIODONTAL LIGAMENT FIBROBLAST (HPLF) ON POLYELECTROLYTE COMPLEX (PEC)

Takaichi Hamano,* Hanako Suzuki,
Akira Teramoto, Eisaku Iizuka, and Koji Abe
Department of Functional Polymer Science
Faculty of Textile Science and Technology
Shinshu University
Ueda 386, Japan

Downloaded At: 13:49 24 January 2011

Key Words: Polyelectrolyte Complex, Human Periodontal Ligament Fibroblast, Cell Adhesion, Cell Growth, Fibronectin Adsorption

ABSTRACT

Cell adhesion onto PEC was mainly controlled by component polyelectrolytes of PEC. In the combination of a synthetic polycation with strong basic groups (2X and PVBMA) and carboxymethyl chitin (CCHN), probably due to the cationic sites of PEC adhesion and growth of human periodontal ligament fibroblast (HPLF) were low and the cell morphology was a round form. Using chitosan (CS100) as a polycation, cell adhesion was scarcely decreased but cell growth was reduced, and cell aggregates were observed. Using sulfated chitin (SCHN) as a polyanion, in spite of a species of polycation, adhesion, growth and spreading form of HPLF were almost the same as those on tissue culture dish (TCD). Cell adhesion onto PEC containing CS100 was not affected by divalent cations. But the amount of adsorbed fibronectin onto such PECs depended upon a kind of polyanion; that onto sulfated chitin's PEC was 2-3 times as much as that onto carboxymethyl chitin's PEC. So it was suggested that cell adhesion was controlled by non specific interaction between PEC and cell, but cell growth was done by adsorbed fibronectin onto PEC. Moreover, cell mor-

Copyright © 1998 by Marcel Dekker, Inc.

phology was seemed to be controlled by functional groups of polyanion.

INTRODUCTION

It is well known that the properties of organized molecular assemblies, such as micelles, vehicles, interpolymer complexes, etc., are different from those of the component molecules in the bulk phase. This may be an important factor for governing the specific functionalities of these assemblies. We have already investigated the properties and applications of polyelectrolyte complex (PEC), which is one of interpolymer complexes [2, 3].

In recent years, biomedical polymers have been drastically developed for many medical and industrial applications, such as artificial organs, biosensers, bio-separaters, bioreactors and biosimulators. One of the most important and essential characteristics for biomedical polymers is the biocompatibility (tissue- and blood-compatibilities). In the case of a tissue-compatibility, it is necessary that the contact of polymer surface with tissue cells does not have a harmful influence on the cell functions *in vivo* and *in vitro*. Up to now, many cell culture methods *in vitro* have been reported [4-6]. The main culture method is a two dimensional one, in which cells attach onto the polymer surface to form a monolayer through cell growth. But in this method, it is difficult to maintain the original cell functions; namely, cultured cells survive and grow but lose their original functions due to rapid dedifferentiation. Therefore, in order to reconstruct tissues *in vitro* so as to maintain cell functions, functional culture methods have been developed. Cells *in vivo* are actually surrounded by hydrogels so called Extra-Cellular Matrix (ECM) composed of such biopolymers as proteins, polysaccharides, proteoglycans, etc., and form three-dimensional ordered cell assemblies to exhibit tissue-dependent functions. Many studies have been made about the reconstruction of tissue *in vitro* using the same components of ECM as a culturing matrix. For example, Kleinman *et al.* reported preparation and characterization of a gel-like structure from a mixture of mouse EHS tumor components including laminin, type IV collagen, heparan sulfate proteoglycan, and entactin/nidogen, which stimulated the growth and differentiation of certain cells [7]. On the other hand, it was reported that such co-factor as L-ascorbic acid 2-phosphate (vitamin C) activated the collagen synthesis of hepatocytes to form the three-dimensional cell assemblies [9]. In these reports, it was found that the cell specific functions were fulfilled in cell aggregates rather than in monolayers. In contrast, only a few studies about the relation between the formation

Downloaded At: 13:49 24 January 2011

of cell aggregates and the maintenance of cell specific functions by using artificial materials as matrices were reported. Akaike *et al.* cultured hepatocyte using artificial polymers (poly-N-p-vinylbenzyl-D-lactonamide) including saccharide residues on there side chains [10]. Ichiba *et al.*, reported the *in vitro* culture of radiation-induced, rat islet cell tumor (RINr) [11] on PECs and it was found that the secretion of insulin from the cells of their aggregates was far more than that from the cells in monolayer forms [12]. Moreover, it was also clarified that the cell morphologies were controlled by a kind of PEC.

HPLF is a dense connective tissue between two hard tissues, the tooth-root cementum and the alveolar bone, and has important functions in anchoring the tooth and in maintaining the structural integrity of those mineralized tissues. A primary objective in the treatment of periodontal diseases is the regeneration of the mineralized and soft connective tissue components of the attachment apparatus [13].

In this report, cell adhesion, growth and morphology of HPLF and fibronectin adsorption onto PEC composed of artificial polyelectrolytes and natural ionic polysaccharides derivatives will be discussed.

Downloaded At: 13:49 24 January 2011

EXPERIMENTAL

Polymers

Figure 1 shows the structures of polycations and polyanions. Poly[(dimethyl-imino)ethylene(dimethylimino)methylene-1,4-phenylenemethylene dichloride] (2X) [14], and poly(vinylbenzyltrimethylammonium chloride) (PVBMA) were prepared by the same manner in our previous reports. Chitosan (the degrees of deacetylation of CS70 and CS100 were 70% and 100%, respectively) as cationic polysaccharides were purchased from Katokichi Co., Ltd. and used without further purification. In order to clarify the effects of anionic substituents on the cell culture, the carboxymethylated and sulfated α-chitin were used as polyanions. The carboxymethylated chitins [CCHNx ; x was a degree of substitution (D.S.); x= 70 and 100 (e.g., 100 meant 1 anionic site/1 saccharide ring)] were purchased from Katokichi Co., Ltd. and used without further purification. Sulfated chitins (SCHNx; x was D.S.) were prepared as follows. 20 ml of distilled N, N-Dimethylformamide (DMF) containing 2g of α-chitin (offered by Shiseido Co., Ltd.) was left overnight with mechanical stirring at room temperature under nitrogen atmosphere. 3.2g of sulfurtrioxide was dropped slowly into 5.3g of DMF in an ice bath. This sulfurtrioxide-DMF complex was added dropwise into the former chitin solution and

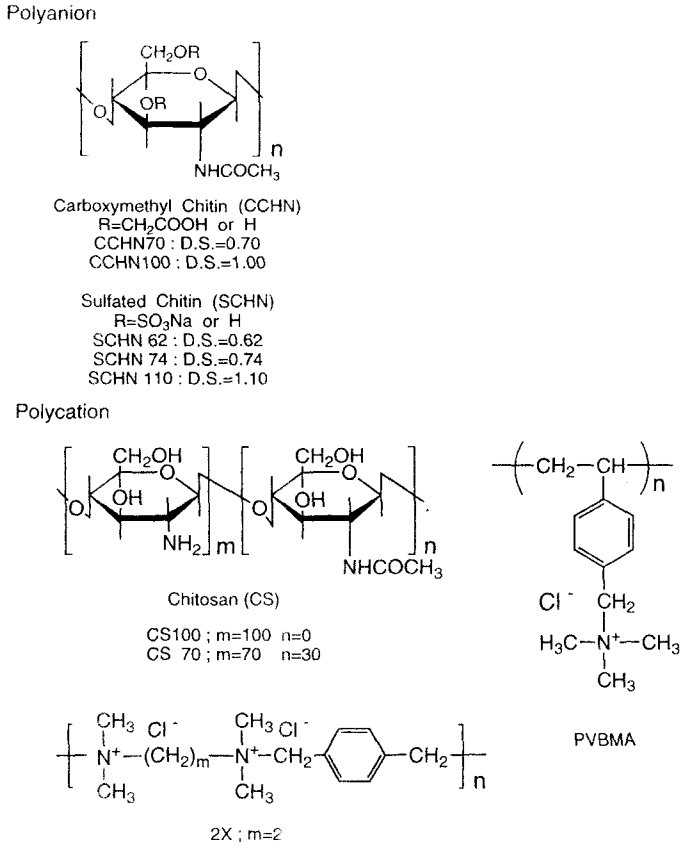

Figure 1. Polymer structures.

stirred for about 4 hours at room temperature. The reaction solution was diluted with 100 ml of distilled water and neutralized by aqueous 1N NaOH. After neutralization, this solution was added to a large quantity of methanol or acetone. The obtained precipitate was filtered with a glass funnel and washed with acetone 2~3 times. Then the precipitate was dissolved in 100 ml-200 ml of distilled water again, and then this solution was dialyzed against distilled water for 1 week and finally lyophilized. The obtained SCHNx was ion-exchanged from H-type to Na type using a cation-exchange resin (AMBERLITE 200C, Rohm and Haas) and then relyophilized. The D.S. of SCHN (D.S. was 62, 74 and 141) was decided by conductometric titration.

Preparation of PEC Coated Dish

The PEC coating method on a TCD was summarized in Figure 2. Both

Downloaded At: 13:49 24 January 2011

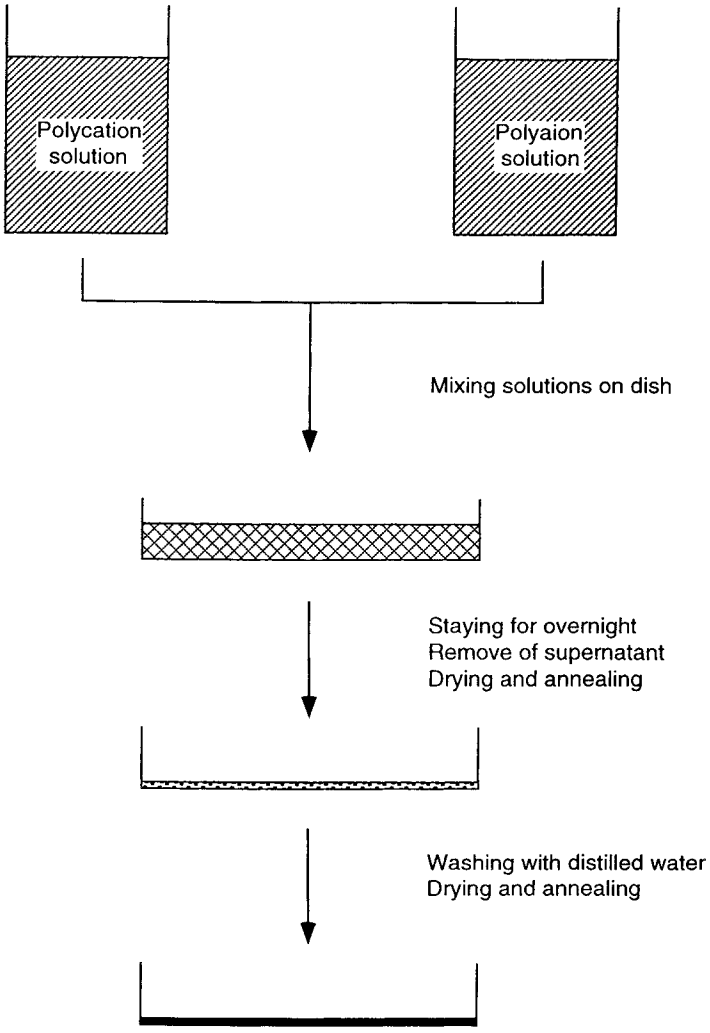

Figure 2. PEC coating method.

Downloaded At: 13:49 24 January 2011

polyanions and synthetic polycations were dissolved individually in distilled water (final concentration = 10^{-2} mol of ionic sites/L) and pH of the solutions were adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosans were dissolved in aqueous 1% acetic acid solution and then pH of the solution was adjusted to 6.0. The mixing ratio of each solution of polyanions and polycations in tissue culture dish was 1:1 by ionic sites in order to make the charge balance of PEC neutral. This mixing solution (1 ml/35 mmϕ TCD; NUNCLON Δ) was stood overnight at room temperature. The supernatant solution was removed and then dish was dried

and annealed at 65°C in the oven. After drying, these dishes were washed with distilled water at once and dried again in the oven forming the PEC coated dish. This dish was sterilized by microwave oven for 3 minutes.

Preculture of HPLF

Human Periodontal Ligament Fibroblast (HPLF) provided by Sanstar Co. Ltd. was cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui-seiyaku Co., Ltd.) containing 0.1g/l streptomycin, 0.1g/l penicillin, 6.9g/l n-2-hydroxy-ethylpiperazin-N'-2-ethanesulfonic acid (HEPES), 1.4g/l $NaHCO_3$, Non-essential amino acid (NEAA) (10ml/l, GIBCO), MEM vitamin (10 ml/l, GIBCO) and 10% fetal bovine serum (FBS, Brockneck) under 5% CO_2 and 95% air at 37°C in the tissue culture flask (25 or 75 cm^2, CORNING). After the cell grew to confluent, DMEM was removed, and then 4 ml or 8 ml of Ca^{2+} and Mg^{2+} free phosphate buffer saline [PBS(-)] including 0.25 w/v% trypsin and 0.02 w/v% ethylenendi-amine-tetraacetic acid disodium salt (EDTA) was added and incubated at 37°C for 5 min.. By means of pipetting with 4 ml of DMEM, the cells were completely detached from the bottom of a flask. The cell was collected from the cell suspension by centrifugation at 1400 rpm for 3 minutes. After removing supernatant, cell was suspended again with 4 ml DMEM. 1 ml of this cell suspension and 3 ml or 11 ml of the culture medium were added to the tissue culture flask (25 or 75cm^2), and then cultured under above mentioned condition until confluent. The culture medium was changed every 4 days.

Downloaded At: 13:49 24 January 2011

Human Fibronectin and Serum

Citrated human serum was obtained from the human blood. The serum was frozen at -20°C and thawed before use. Human recombinant fibronectin (hFN, Funakoshi Co., Ltd.); (500 ng/ml) or human serum (diluted to 1/500; original human serum includes 250 μg/ml fibronectin [15]) solution were prepared in physiological salt solution (PSS; 150mM NaCl).

Precoating of Proteins

After PECs (CS100-CCHN70 AND -SCHN141) and TCD were treated with PSS for 1 hour at room temperature, they were precoated with hFN (10 μg/ml in PSS) or 10% FBS for 1 hour at 37°C. And then they were rinsed three times with PSS to remove excess protein, and stored at room temperature.

Cell Adhesion

Cell suspension (DMEM with and without 10% FBS) was poured into PEC coated dishes ($12x10^4$ cells/dish) and cultured at 37°C under 5% CO_2 for 24

hours. The supernatant solution was retrieved from dishes. Moreover, the dishes were rinsed with 1 ml of PBS(-) which was also retrieved. The number of free cells in these solutions were counted as a non-adhesion cell number by a hemacytometer using a phase contrast microscopy (Nicon, DIAPHOT-TMD). The cell adhesion ratio was calculated from the following equation:

$$\text{Cell adhesion ratio (\%)} = \frac{C_0 - C}{C_0} \times 100 \quad (1)$$

(C_0: seeding cell number, C : non-adhesion cell number)

Effects of Divalent Cations on Cell Adhesion

Cell suspension by PSS in the presence or absence of Ca^{2+} (0.01~5mM) and/or Mg^{2+} (0.01~5mM) was poured into PEC (CS100-CCHN70 and -SCHN141) coating dishes and TCD ($12x10^4$ cells/dish), on which proteins were pre- or non-coated, and they were cultured at 37°C under 5% CO_2 for 2 or 24 hours. The cell adhesion ratio was calculated from the Equation (1).

Downloaded At: 13:49 24 January 2011

Fibronectin Adsorption

The adsorption of fibronectin was detected by means of ELISA. After PECs (CS100-CCHN70, -CCHN100, -SCHN62, -SCHN141) and TCD were equilibrated with PSS for 1 hour at room temperature, PSS solutions of hFN (50ng/well) or diluted human serum (1/500) were poured into them and incubated for 1 or 24 hours at 37°C. After removing supernatant the surfaces were blocked with diluted Block-Ace (1/4, Yukijirushi Nyugyo Co., Ltd.) for 4 hours at 4°C. The antibody [anti-human fibronectin (goat) diluted to 1/5000] to hFN was found by incubation for overnight at 4°C. And they were rinsed twice for 15 minutes with washing buffer [0.05 v/v% of polyoxyethylene (20) sorbitan monolaurate, 10mM $Na_2HPO_4 \cdot 12H_2O$ and 10mM KH_2PO_4], and then with aqueous NaCl, and 10mM phosphate buffer saline (pH=7.4). Then they were incubated with alkaline-phosphatase conjugated secondary anti-goat lgG (H+L) (diluted to 1/3000) to anti-human fibronectin goat IgG for 4 hours at 4°C, and rinsed twice for 15 minutes with the washing buffer and TBS (10mM Tris-HCl and 150mM NaCl, pH 8.2) successively. Finally, the amount of adsorbed hFN was measured by using alkaline phosphatase substrate kit (Bio-Rad Laboratories) at 405nm by EIA READER (Bio-Rad Laboratories, Model 2550).

Cell Growth

Cell suspensions ($5x10^4$ cells/dish in DMEM with 10% FBS) in PEC coated dishes and TCD were cultured under the same conditions described above

for 4 days. After removing the supernatant, the dishes were rinsed with PBS(-). 1ml of PBS(-) containing 0.25 w/v% trypsin and 0.02 w/v% EDTA was poured into the dishes. After incubation at 37°C for 10 minutes and pipetting, the supernatant was retrieved and then the dishes were rinsed and pipetted with 1 ml of PBS(-) to retrieve attached cells on dishes completely. The cells were counted by a hemacytometer using a phase contrast microscopy.

RESULTS AND DISCUSSION

HPLF Adhesion

Figure 3 shows the HPLF adhesion onto PECs in the absence or presence of 10% FBS for 24 hours. Using polysaccharide as component polymers (CCHN, SCHN and CS), cell adhesions were more than 90% in spite of the degree of the substitution ratio. Namely species and substitution ratio of functional groups of polysaccharides scarcely affected the cell adhesion maybe due to the combination of weak polybase with weak or strong polyacid. At physiological pH (7.2), amino groups of CS may not be dissociated, or if they are prolonged, they are neutralized by the opposite ionic sites of CCHN or SCHN. On the contrary, using synthetic strong polybases, 2X and PVBMA, they have positive charges at physiological pH. Only in the systems of 2X- and PVBMA- CCHNx PECs in the absence of serum, cell adhesion was extensively reduced. Since these PECs consisted of strong base and weak acid, the PEC surface were considered to be positive. In general, cell surface has negative charge in the physiological solution, and it is known that weakly positive charged surface enhances cell adhesion more than neutral surface. On the other hand, quaternary ammonium sites show antibacterial character and toxicity against cells [17, 18]. Therefore, it is seemed that there are small amounts of free quaternary ammonium sites on PEC surface and that cell adhesion reduced. On adding FBS, it is well known that serum proteins which are adsorbed on the material surface control the cell adhesion and cell function. Since cationic character of PECs might be shielded by adsorbed serum proteins, cell adhesion onto PEC increased. From these results, it was evidenct that HPLF adhesion onto PEC was scarcely affected by the structure of main chain of polysaccharide and the degree of substitution (D.S.) of ionic sites, while the differences of anionic functional groups and D.S. may affect the hydrophilic/hydrophobic balance in PEC and the structure of PEC.

Figure 4 shows the effect of divalent cations (Ca^{2+} and Mg^{2+}) on cell adhesion for 2 hours culture onto PECs and PECs precoated with human fibronectin

Downloaded At: 13:49 24 January 2011

Downloaded At: 13:49 24 January 2011

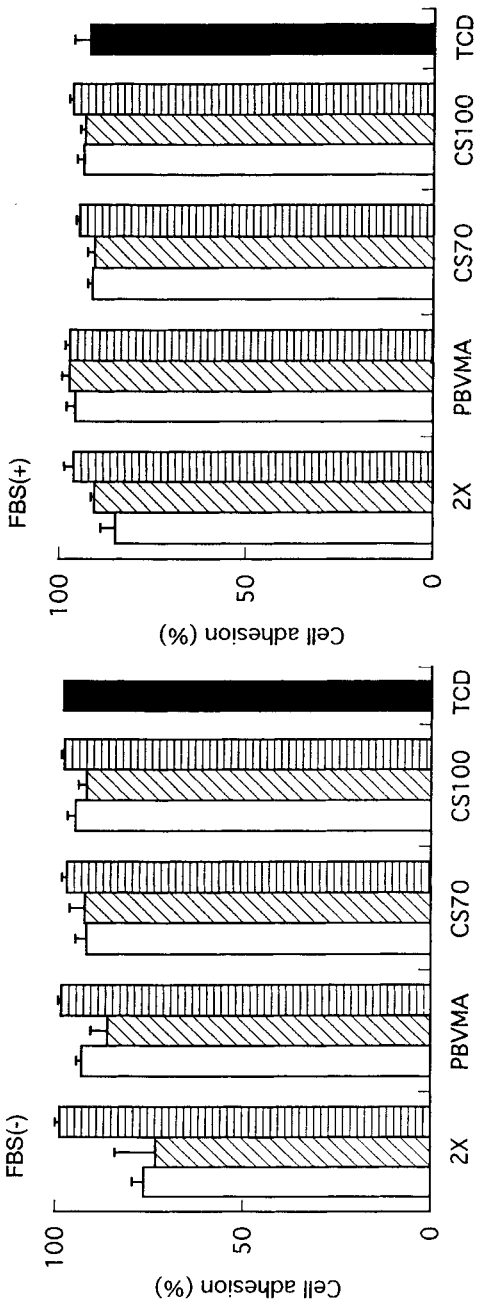

Figure 3. Cell adhesion onto PECs and TCD in the absence or presence of FBS (10%) after 24 hours. Polyanions; ☐ CCHN70, ▨ CCHN100, ▤ SCHN74.

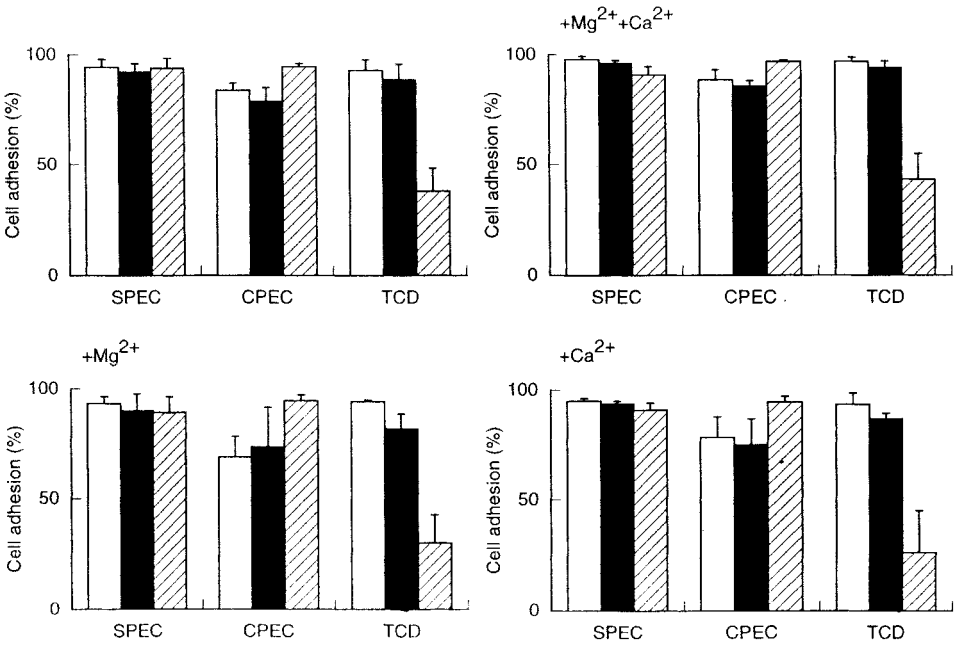

Figure 4. The effect of divalent cations on the cell adhesion onto non- and pre-coated PECs and TCD after 2 hours. Precoating; □ Non, ■ hFN, ▨ Serum.

Downloaded At: 13:49 24 January 2011

(hFN) or serum. Cell adhesion onto SPEC, which contained SCHN74 as a component polyanion, was independent of the presence and absence of divalent cations and the precoating of proteins. Cell adhesion onto non-coated and hFN coated tissue culture dish (TCD) was not inhibited by divalent cations. On the other hand, cell adhesions onto serum coated TCD, CPEC, which contained CCHN as a component polyanion, and hFN-precoated CPEC were decreased in spite of the presence or absence of divalent cations. Moreover, cell adhesions onto PECs and TCD were independent with the concentration of divalent cations. From these results, it was suggested that some factors on CPEC surface might inhibit the cell adhesion and this effect was lost by the precoat of serum. In case of the 24 hours culture on the serum precoated TCD, the cell adhesion was increased in the order of no divalent cations < $+Mg^{2+}$ < $+Ca^{2+}$ <$+Mg^{2+}+Ca^{2+}$. Generally, when adhesive proteins (e.g., fibronectin and vitronectin in serum) are adsorbed to the surface of material, the specific cell adhesive site of such proteins (fibronectin has a tetrapeptide of RGDS [19]) interacts with cell specific receptors for adhesive proteins in the presence of divalent cations. The interactions between them play an important role

on the maintenance and promotion of cell anchorage, polarity, migration, and differentiation. Many of such receptors belong to a family of proteins so called as integrins, which have the tripeptide Arg-Gly-Asp-Ser (RGDS) [19], and the binding of the receptors to their ligands requires the presence of divalent cations such as Ca^{2+}. In the systems of PEC, cell adhesion was hardly dependent with divalent cations and more than 70% of cells were adhered to non-precoated PEC. These results suggest that major driving force of cell adhesion onto PEC, may be non-specific interaction rather than ligand-receptor interaction.

Fibronectin Adsorption

Figure 5 shows the hFN adsorption onto CS100-containing PECs and TCD incubated for 1 and 24 hours at 37°C. The amount of hFN adsorption onto PECs for 24 hours was almost the same as that for 1 hour, but the adsorption onto TCD was decreased with time. The hFN adsorption onto SCHN was approximately three times more than that onto CCHN. In the case of CPEC, the hFN adsorption was independent with D.S., but in the case of SPEC, more hFN was adsorbed with the increase of D.S.. The similar hFN adsorption profiles were observed even in the other PEC systems using 2X and PVBMA instead of CS as a polycation. hFN adsorption from physiological salt solution (PSS) -diluted whole human serum (x500) onto PECs and TCD for 1 and 24 hours indicated the similar tendency as shown in Figure 6. But, the amount of adsorbed fibronectin from serum solution after 1 hour was approximately half of that from hFN solution. After the incubation for 24 hours, the amount of fibronectin adsorbed onto CS100-CCHN70 was scarcely changed, but that onto CS100-CCHN100 was three and four times more than 1 hour's adsorption onto the same PEC and all PECs of CCHN70 (see Figure 5). The amount of adsorbed fibronectin onto CS100-SCHN141 after 24 hours was about double that onto other SPECs. However, adsorption onto TCD from serum solution showed little dependence with time. These results suggested that fibronectin adsorption profiles from hFN and whole human serum solutions were different with each other. The adsorption from hFN solution onto materials was saturated within 1 hour and carboxymethyl residues of CCHN inhibited the adsorption of hFN. Whole human serum contains other proteins (mainly albumin and globulin) in the solution. In general, it was reported that albumin and globulin were firstly adsorbed onto materials and then these proteins were gradually exchanged by other proteins. These exchange reaction might be performed on PECs of CS100-CCHN100 and -SCHN141. Consequently, the cause of these results was not clear, but it was found that the interaction between hFN and PEC

Downloaded At: 13:49 24 January 2011

Downloaded At: 13:49 24 January 2011

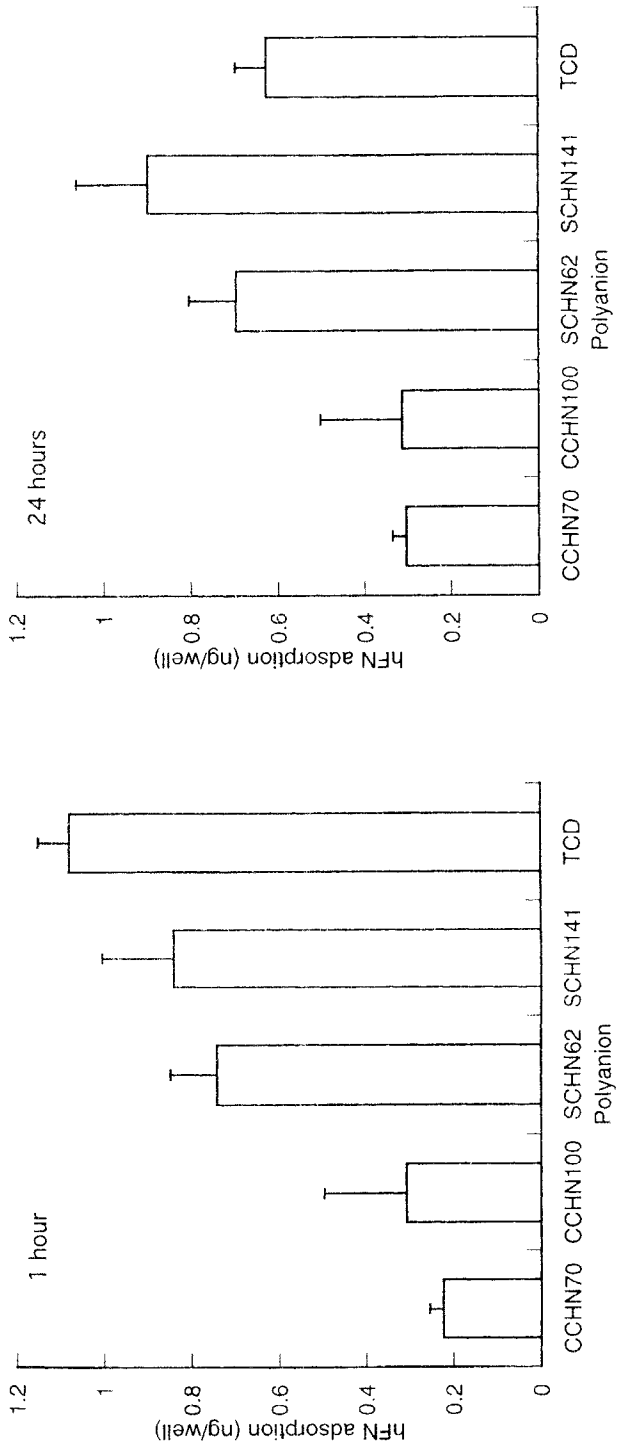

Figure 5. Adsorption of hFN from recombinant figronectin solution (50ng/well) to PEs and TCD incubated for 1 and 24 hours at 37°C. Polycation; CS100.

Downloaded At: 13:49 24 January 2011

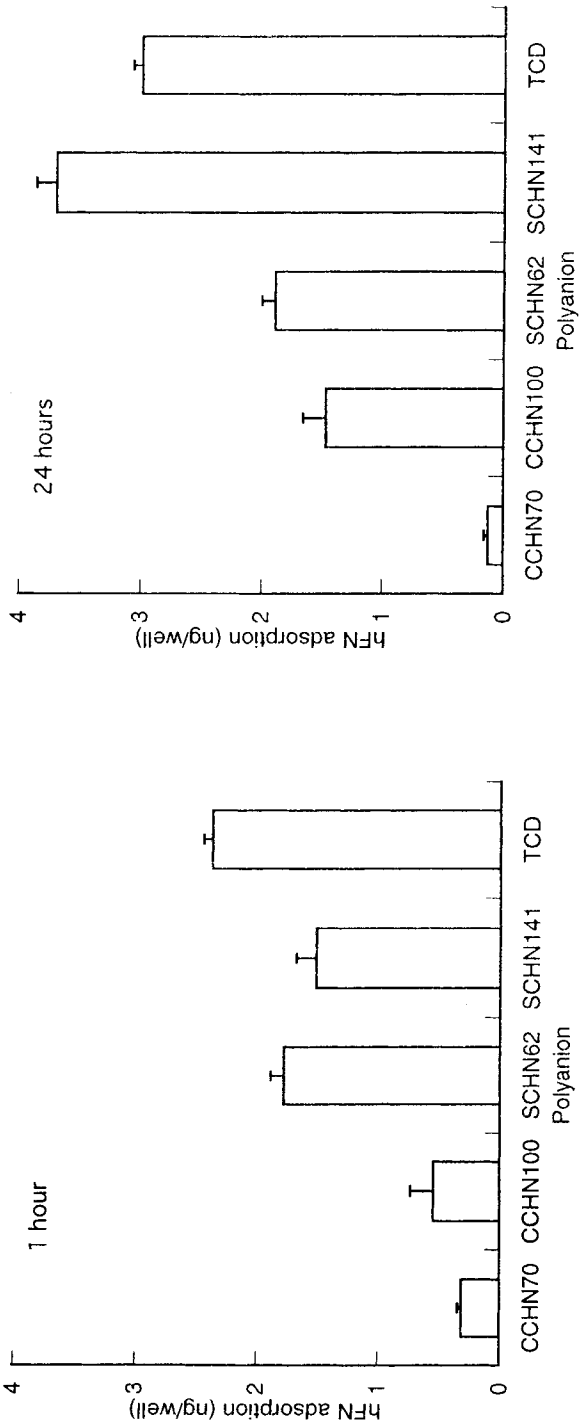

Figure 6. Adsorption of hFN from physiological salt solution-diluted whole human serum (X500) to PECs and TCD incubated for 1 and 24 hours at 37°C. Polycation; CS100.

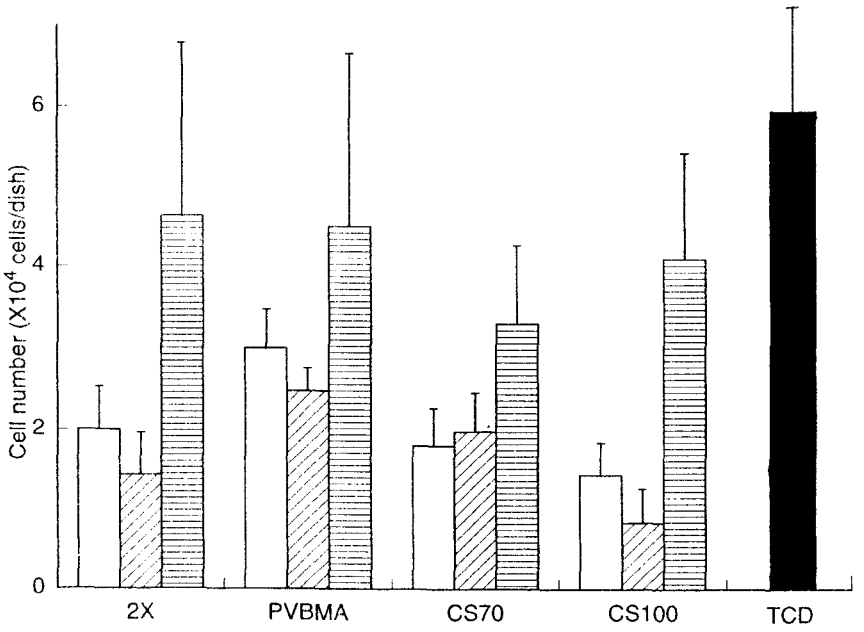

Figure 7. HPLF growth on PECs in the presence of FBS (10%) after culturing 4 days. Polyanions; ☐ CCHN70, ▨ CCHN100, ▤ SCHN74.

Downloaded At: 13:49 24 January 2011

might be controlled by the types of functional group and substitution ratio on polyanion.

Cell Growth

Figure 7 shows the cell number of HPLF cultured for 4 days on PECs and TCD. Cell growth on all of PECs was slower than on TCD. Focusing on functional groups of polyanion, cell growth was enhanced in the order of sulfate (SCHN) > carboxymethyl (CCHN). This tendency was similar to cell adhesion (Figure 3) and fibronectin adsorption (Figures 5 and 6). Cell growth on PECs containing synthetic strong polycations (2X and PVBMA) were controlled not only by cell adhesion but also by fibronectin adsorption, though on PECs of CS100-CCHN70 and -CCHN100, the cell growth was mainly due to fibronectin adsorption. Furthermore, using CS100 as a polycation, cell morphology was different with PEC using 2X and PVBMA as shown in Figure 8. Namely, cell form on 2X-CCHN70 was round, but on CS100-CCHN70 cells were aggregated.

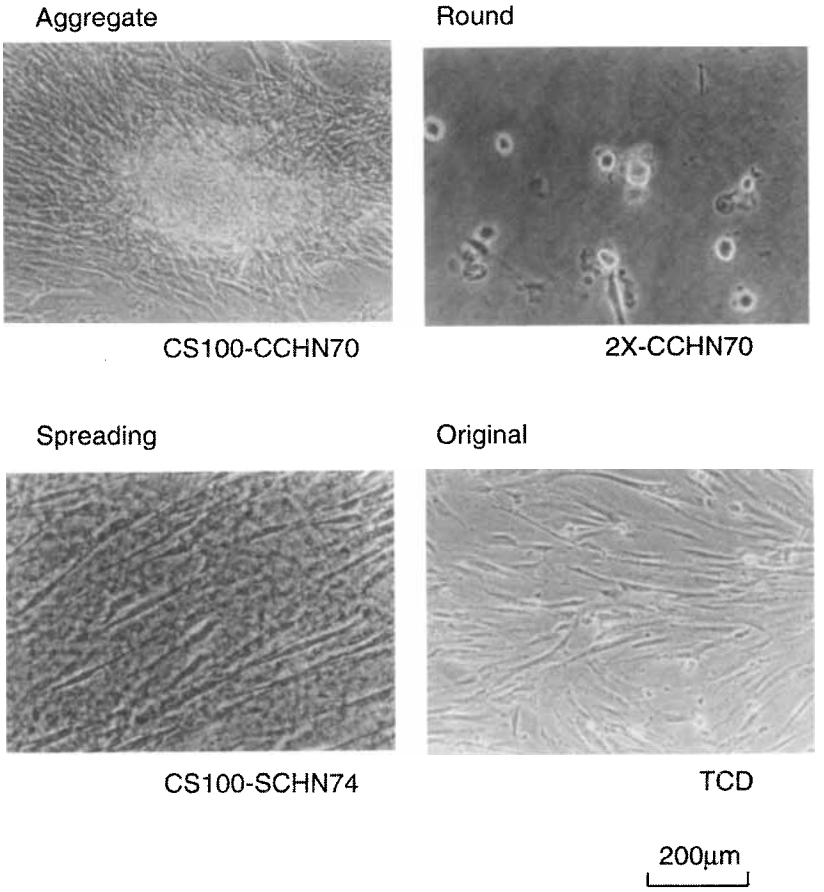

Figure 8. Morphology of HPLF on PECs in the presence of FBS (10%) after culturing 4 days.

On the other hand, using SCHN74 as a polyanion, cells were all spreaded like a fibroblastic form in spite of polycations. These results suggested that cell growth was controlled not only by cell and fibronectin adsorption, but also the characteristics of PECs; the species of functional groups of component polymers, that is, strong or weak polycations, sulfate or carboxylate groups of polyanions. The reason for these morphological changes of HPLF has not been clarified. But they were related to the cell growth. So, it was pointed out that PEC could control cell cycle or cell functions, e.g., growth and differentiation.

Downloaded At: 13:49 24 January 2011

CONCLUSION

The relationship between cell adhesion to PEC, in spite of adding the serum protein and divalent cations, was controlled mainly by non specific interaction of between cell and PEC. Besides, PEC regulated fibronectin adsorption and high amount of fibronectin adsorption onto PEC induced the cell growth and spreading form of HPLF. But HPLF showed aggregate on CPEC.

SYMBOLS

HPLF	Human periodontal ligament fibroblast
PEC	Polyelectrolyte complex
SPEC	CS100-SCHN
CPEC	CS100-CCHN
2X	Poly[(dimethyliminio)ethylene(dimethyliminio)methylene-1,4-phenylenemethylene dichlorides]
PVBMA	Poly(vinylbenzyltrimethylammonium chloride)
CS	Chitosan
SCHN	Sulfated chitin
CCHN	Carboxymethylated chitin
TCD	Tissue culture dish
DMEM	Dulbecco's modified eagle medium
hFN	Human recombinant fibronectin

Downloaded At: 13:49 24 January 2011

REFERENCES

[1] E. Tsuchida and K. Abe, *Advances in Polymer Science, 45*, 1 (1982).
[2] E. Tsuchida and K. Abe, in *Development in Ionic Polymers-2*, 191 (1986).
[3] R. G. Ham, in *Methods and Selected Applications,* Vol. 3, 209 (1984).
[4] D. W. Hopcroft, D. R. Mason, and R. S. Scott, in *Vitro, 21*, 485 (1985).
[5] I. Yamane , M. Kan, and H. Hoshi, *Exp. Cell Res., 134,* 470 (1981).
[6] S. Tobe, Y. Takei, K. Kobayashi, and T. Akaike, *Biochem.- Biophys. -Res.-Commum., 184(1)*, 225 (1992).
[7] H. K. Kleinman, M. L. McGarvey, J. R. Hassell, V. L. Star, F. B. Cannon, G. W. Laurie, and G. R. Martin, *Biochemistry, 25,* 312 (1986).
[8] R. Hata, *Biochemical, 60*, 201 (1988).

[9] S. Tobe, Y. Takei, K. Kobayashi, and T. Akaike, *Biochem.-Biophys.-Res. -Commum., 184(1)*, 225 (1992).

[10] Adi F. Gazder, William L. Chick, Herbert K. Oie, Harvie L. Sims, Dennis L. King, Gordon C. Weir, and Vilma Lauris, *Cell Biology, 77(6)*, 3519 (1980).

[11] Y. Ichiba, J. Ono, T Okeda, R. Takagi, K. Abe, and S. Senoh, *J. Japan Diab. Soc., 32(9),* 635 (1989).

[12] N. Matsuda, N. M. Kumar, P. R. Ramakrishnan, W.-L. Lin, R. J. Genco, and M. I. Cho, *Archs Oral Biol., 38(7)*, 559 (1993).

[13] E. Tsuchida, Y. Osada, and K. Sanada, *J. Polym. Sci. Part A-1, 10,* 3397, (1972).

[14] E. Ruoslahti, E. Engvall, and E. Hayman, *Collagen Res., 1*, 221 (1981).

[15] T. Ikeda and S. Tazuka, *Makromol. Chem., 185*, 869 (1984).

[16] T. Ikeda, H. Yamaguchi, and S. Tazuke, *Antimicrob. Agents Chemother., 26*, 139 (1984).

[17] M. D. Pierschbacher and E. Ruoslahti, *Nature, 309*, 30 (1984).

Received August 20, 1997
Revision received October 20, 1997

Downloaded At: 13:49 24 January 2011