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Adsorption of Fibronectin, Albumin and Fibroblast Growth Factor-Basic onto Polyelectrolyte Complex (PEC), Which Controls Human Periodontal Ligament Fibroblast (HPLF) Proliferation and Differentiation

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ADSORPTION OF FIBRONECTIN, ALBUMIN AND FIBROBLAST GROWTH FACTOR-BASIC ONTO POLYELECTROLYTE COMPLEX (PEC), WHICH CONTROLS HUMAN PERIODONTAL LIGAMENT FIBROBLAST (HPLF) PROLIFERATION AND DIFFERENTIATION

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Key Words: Polyelectrolyte Complex, Human Periodontal Ligament Fibroblast, Fibronectin, Albumin, Fibroblast Growth Factor-Basic, Proliferation

ABSTRACT

Cells *in vivo* are surrounded by many types of extracellular matrices (e.g., collagen, proteoglycans, and adhesion proteins), vitamins, growth factors, and hormones which control cell functions. Poly-electrolyte complex (PEC), composed of polysaccharides, has a structure similar to that of glycosaminoglycans. In order to assess the importance of the adsorption of fibronectin (hFN), albumin (Alb), and fibroblast growth factor-basic (FGF-b) onto PECs in effecting cell proliferation, the hFN and Alb adsorbed onto the surface had a charge balance which decreased with the increasing negative charge on CS100-SCHNx (SPECs). However, in the case of using CCHN70 as a polyanion, the amount of their adsorption was at minimum when the charge balance = 0. Moreover, Alb adsorption onto PECs increased with an increase in the degree of substitution of anionic functional groups of polyanion.

On the other hand, the amount of FGF-b adsorption onto CPEC70 (CS100-CCHN70) was higher than that onto SPECs, although cell proliferation was more pronounced on SPECs than on CPEC70. As has been reported previously [1], cell morphologies showed an aggregate on CPEC70 and a spreading on SPECs. Therefore, the high amount of FGF-b adsorption onto CPEC70 might be attributable to induce cell aggregation and differentiation.

INTRODUCTION

It is well known that components of the extracellular matrix (ECM) are important in the development, maintenance, and regulation of cellular organization *in vivo*. Most cells *in vivo* are in contact with ECM during their development, however, their composition and structure are variable and differ for different cells and different locations. ECM components themselves may induce the proliferation and differentiation of primary and established cell lines. For example, heparin, which is a component of proteoglycan, has the binding capability of fibronectin [2] as well as fibroblast growth factors [3]. Chemically-modified chitin derivatives (carboxymethylated and sulfated) and chitosan, component polymers of polyelectrolyte complex (PEC), have a structure and properties similar to those of glycosaminoglycans of ECM, including properties such as the adsorption of blood components [4], antigenicity [5], anticoagulant activity, antiplatelet aggregation activity [6], antiviral activity [7], biodegradability, low toxicity, and biocompatibility [8, 9]. The formation and characteristics of polyelectrolyte complexes (PEC) are governed by the characteristics of the individual polyelectrolyte components (e.g., properties of ionic sites, position of ionic sites, charge density, rigidity of macromolecular chains), and the chemical environment (e.g., concentration, solvent, ionic strength, pH, temperature, and others) [10]. As previously reported, the fibronectin adsorption onto PEC is controlled by the degree of substitution of sulfate groups in chitin derivatives, and this adsorption affects human periodontal ligament fibroblast (HPLF) morphologies [1]. HPLF is a dense connective tissue between two hard tissues, the tooth-root cementum, and the alveolar bone, and is important in maintaining the structural integrity of those mineralized tissues by anchoring them to each other. The characterization of HPLF *in vitro* has demonstrated that these fibroblasts have osteoblast-like properties, including the synthesis and expression of alkaline phosphatase activity, osteopontin, and osteocalcin, responsiveness to parathyroid hormone, and a capacity to produce mineralized nodules [11, 12-19].

In this report, we will discuss the adsorption of fibronectin, albumin, and fibroblast growth factor-basic onto PEC, as well as the effects that fibroblast growth

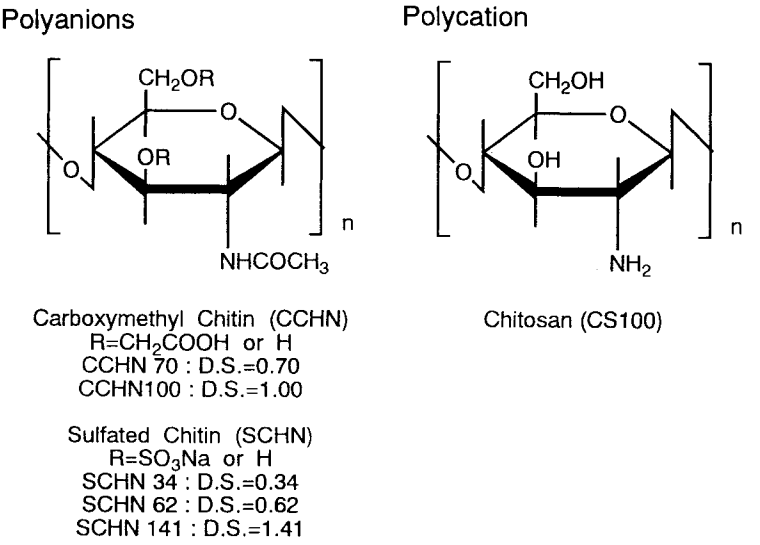

Figure 1. Polymer structures

factor-basic adsorbed onto the PEC surface might have on HPLF proliferation and differentiation.

EXPERIMENTAL

Materials and Methods

Polymers

Figure 1 shows the chemical structures of polycations and polyanions. Chitosan (CS100) and carboxymethylated chitins (CCHNx) were purchased from Katokichi Co., Ltd. and used without further purification. The degree of deacetylation of CS100 was 100% and x was a degree of substitution (D.S.); for CCHNx, x= 70 and 100 (e.g., 100 meant 1 anionic site/1 saccharide ring). Sulfated chitins (SCHNx ; x= 34, 62, and 141) were previously reported [1].

Preparation of PEC Coated Dish

The polyanions were dissolved in distilled water (final concentration $=10^{-3}$ mol of ionic sites/L). The pH of the solution was adjusted to 7.4 by adding aqueous HCl or NaOH. CS100 was dissolved in aqueous 1% acetic acid solution, and the pH of this solution was adjusted to 6.0. The mixing ratios of polyanions and polycation in each solution in the tissue culture plates (TCP) were 3:7, 5:5, and 7:3,

and were arranged this way in order to investigate the effect of the charge balance of PEC when it is "+", "0", and "-". TCPs were 96 well (NUNC) for proteins adsorption, and 12 well (IWAKI) for cell proliferation. Each solution was poured into a TCP and stood overnight at room temperature. The supernatant solution was removed, then the 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 to complete the PEC coating on the dish.

Human Fibronectin, Albumin, Fibroblast Growth Factor-Basic

The Human recombinant fibronectin (hFN, 500ng/ml, Funakoshi Co. Ltd.), human albumin (Alb, 500ng/ml, Organon Teknika Corp.), and human recombinant fibroblast growth factor-basic (FGF-b, 500ng/ml, Pepro Tech EC LTD) solutions were prepared by physiological salt solution (PSS ; 150mM NaCl).

Primary and Secondary Antibody

The primary and secondary antibodies of hFN use an anti-human fibronectin goat IgG (Organon Teknika Corp.) and an alkaline-phosphatase conjugated anti-goat IgG IgG (Organon Teknika Corp.), respectively. The primary and secondary antibodies of Alb use an anti-human albumin rabbit IgG (Organon Teknika Corp.) and an alkaline-phosphatase conjugated anti-rabbit IgG IgG (Organon Teknika Corp.), respectively. The primary and secondary antibody of FGF-b use an anti-human fibroblast growth factor basic rabbit IgG (AUSTRAL Biologicals) and the alkaline-phosphatase conjugated anti-rabbit IgG IgG, respectively.

Proteins Adsorption

The adsorption of antigens was detected by means of ELISA. After PECs [CS100-CCHN70 (CPEC70), CS100-CCHN100 (CPEC100), CS100-SCHN34 (SPEC34), CS100-SCHN62 (SPEC62) and CS100-SCHN62 (SPEC141)] and TCP were equilibrated with PSS for 1 hour at room temperature, PSS solutions of antigens (50ng/well) were poured into them and incubated for 1 or 24 hours at 37°C. After removing the supernatant, the surfaces were blocked with diluted Block-Ace [1/4 with PBS (10mM $Na_2HPO_4 \cdot 12H_2O$ and 10mM KH_2PO_4), Yukijirushi Nyugyo Co. Ltd.] for 4 hours at 4°C. The primary antibodies to antigens were found by incubation overnight at 4°C. They were reacted with the alkaline-phosphatase-conjugated secondary antibodies for 4 hours at 4°C. Finally, the amount of adsorbed antigens was measured by absorbance at 405nm (Microplate reader; Bio-Rad, Model 550) using an alkaline phosphatase substrate kit (Bio-Rad Laboratories).

Preculture of Human Periodontal Ligament Fibroblast (HPLF)

HPLF, provided by Sanstar Co. Ltd., was precultured in the tissue culture flask (80cm^2, IWAKI) using Dulbecco's modified Eagle's medium (DMEM, Nissui-seiyaku Co. Ltd.) supplemented with 0.1g/l streptomycin, 0.1g/l penicillin, 6.9g/l n-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), 1.4g/l $NaHCO_3$, 10ml/l non-essential amino acid solution, 10ml/l MEM vitamin solution, 10ml/l MEM sodium pyruvate solution, and 10% fetal bovine serum (FBS, GIBCO) under 5% CO_2 at 37°C. The above-mentioned culture medium was changed every 3 days. Cells that grew to confluence were detached using trypsin-EDTA and used in the following experiments.

HPLF Proliferation

Each well of TCP was coated with a concentration of FGF-b solution overnight at 4°C. The contents of each well were rinsed three times with Ca^{2+} and Mg^{2+} free phosphate buffer saline [PBS(-)]. The cell suspension was poured into SPEC141, CPEC70, and TCP ($2x10^4$ cells/well) in ASF301 (Cosmo•Bio Co., Ltd.), which is a serum-free medium and was cultured under the same conditions as those described above. The number of cells were counted by a hemacytometer under phase contrast microscopy (Nicon, DIAPHOT-TMD).

Statistical Analysis

Data were subjected to analysis of variance, using StatView and SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS AND DISCUSSION

Human Recombinant Fibronectin Adsorption

Figure 2 shows the effects of the charge balance of PECs on the adsorption of human recombinant fibronectin (hFN) from hFN solution over 1 hour. The amount of hFN adsorbed onto SPEC decreased as the negative charge on the PEC increased. Since the isoelectric point of hFN is 5.5~6.2, it has a negative charge in its physiological condition (pH7.4). Therefore, it was possible that the surface net charge of the SPEC controlled the hFN adsorption onto the SPEC, by being stronger than the interaction between the heparin binding site [2] of hFN and the SCHN of PEC. Moreover, we reported that hFN adsorption onto SPECs increased with an increase in the D.S. of sulfonic groups of the polyanion [1]. Mainly, hFN adsorbed onto SPEC through an electrostatic interaction and through physiological

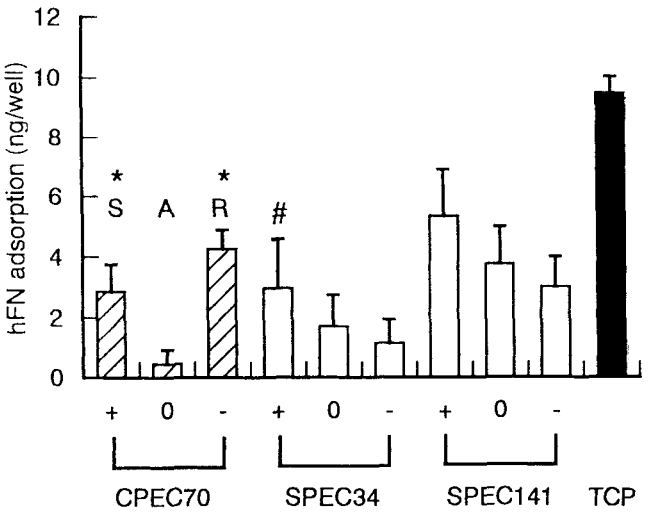

Figure 2. Adsorption of fibronectin from physiological salt solution-diluted human recombinant fibronectin (hFN, 50ng/well) to various charge balance PECs and TCP for 1 hour. Cell morphologies: S; spreading, A; aggregate, R; round. *; $P<0.05$ (vs. CPEC70 0), # ; $P<0.05$ (vs. SPEC34 0, -).

binding of fibronectin, although this might be weaker. On the other hand, in the case of CPEC70, the amount of hFN adsorption was at a minimum when the charge balance = 0. The specific binding sites of hFN might interact with carboxymethyl groups of CCHN in CPEC70 when the charge balance is negative. Besides, in the case of a positive charge balance, the main force of the hFN adsorption would be electrostatic interaction, since the PEC would be rich in polycations. Consequently, in the case of a zero charge balance, since the free carboxymethyl groups and the excess cationic charges were reduced, the hFN adsorption may be low. Therefore, CCHN differed from SCHN not only in acidity but also in the specific interaction between their ionic groups and biocomponents. Such different properties would affect PEC structure and the physiological activities of fibronectin. The aggregate formation of HPLF was observed only when CPEC70 had a charge balance of 0. A detailed discussion follows below. These results suggest that the inhibition of protein adsorption onto CPEC70 might be one of the reasons for HPLF aggregation.

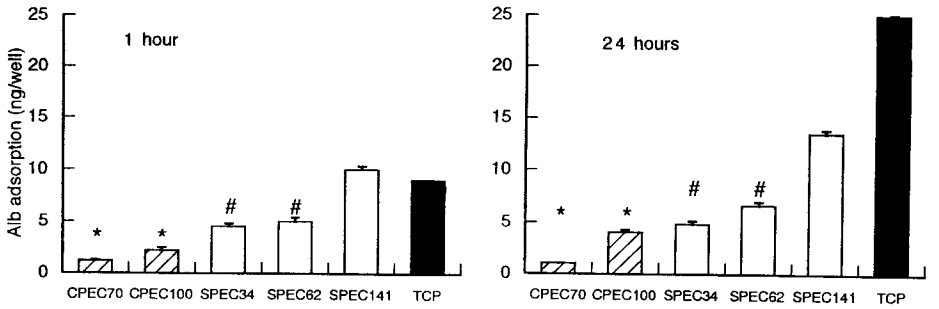

Figure 3. Adsorption of albumin from physiological salt solution-diluted human recombinant albumin (Alb, 50ng/well) to various PECs and TCP for 1 and 24 hours. *; P<0.05 (vs. SPECs), # ; P<0.05 (vs. SPEC141).

Albumin Adsorption

Figure 3 shows the Albumin (Alb) adsorption onto PECs and TCP over periods of 1 and 24 hours. The amount of Alb adsorption onto PECs over 24 hours was almost the same as that over 1 hour, but the adsorption onto TCP increased significantly with time. Alb adsorption onto PECs increased with the increase of the D.S. of anionic functional groups of polyanion. Abe *et al.* reported that the drug binding site II of Alb was bound to anionic sites of polyanions even at pH 7.4, and that the interaction force of poly(sodium styrenesulfonate) was stronger than that of poly(methacrylic acid) [20]. Considering these facts, it was suggested that binding site II of Alb recognized and bound the anionic groups on the PEC surface. Therefore, more Alb was adsorbed onto PECs at a higher D.S. because of the high charge density of the polyanions in PEC. These results suggested that changes in the kind and D.S. of functional groups of polyanions could control the Alb adsorption onto PEC.

Figure 4 shows the effects of the charge balance of PECs on Alb adsorption over 1 hour. The amount of Alb adsorbed onto SPEC decreased as the negative charge in the PEC increased. The isoelectric point of Alb is 5~6, and so it has negative charge in its physiological condition (pH7.4). It was concluded that it was mainly the surface charge of SPEC that controlled Alb adsorption onto SPEC. On the other hand, in the case of CPEC, the amount of Alb adsorption was at a minimum when the charge balance was 0. The binding site II of Alb might be

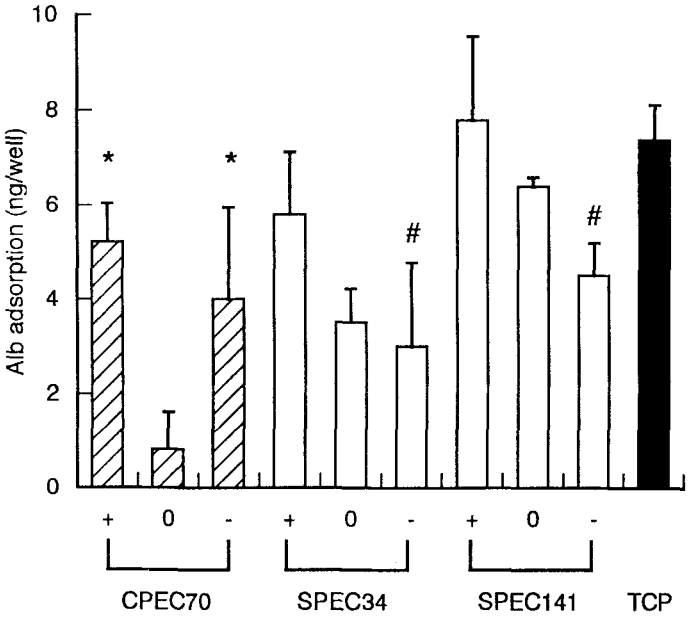

Figure 4. Adsorption of albumin from physiological salt solution-diluted human recombinant albumin (Alb, 50ng/well) to various charge balance PECs and TCP for 1 hour. *; $P<0.05$ (vs. CPEC70 0), # ; $P<0.05$ (vs. SPECx +).

bound to the carboxymethyl groups on the surface of CPEC in the case of a negative charge balance. Besides, in the case of a positive charge balance, the main force behind the Alb adsorption would be an electrostatic interaction. Such a tendency of protein adsorption onto CPEC was observed in the hFN adsorption described above. Kataoka *et al.* have investigated the function of the platelet that adheres to Alb-adsorbed PECs under various charge balances [21].

Figure 5 shows the human recombinant fibroblast growth factor-basic (FGF-b) adsorption onto PECs and TCP over 1 hour. The amount of FGF-b adsorption onto various SPECs was the same, but the FGF-b adsorption onto CPEC differed between CPEC70 and CPEC100. The amount of FGF-b adsorption onto CPEC70 was about two-fold that of FGF-b adsorption onto CPEC100. The highest FGF-b adsorption among all PECs was found in CPEC70. These protein adsorption results showed trends opposite those of hFN and Alb in their adsorption onto PECs as described above. FGF-b has specific binding sites for heparin and heparan sulfate, and can bind them at the cell surface or in the extracellular matrix *in*

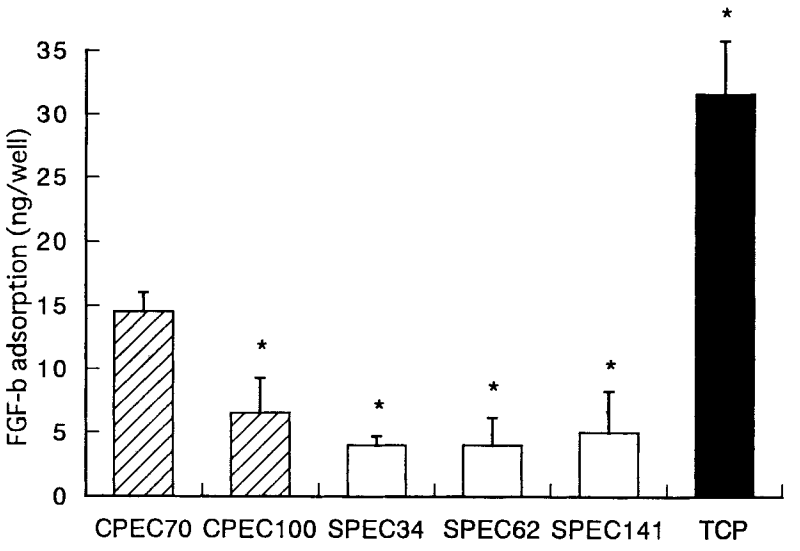

Figure 5. Adsorption of fibroblast growth factor-basic from physiological salt solution-diluted human recombinant fibroblast growth factor-basic (FGF-b, 50ng/well) to various PECs and TCP for 1 hour. *; $P<0.05$ (vs. CPEC70).

vivo. Furthermore, the specific binding sites for heparin and heparan sulfate have hexasaccharide structures. SCHN141 has a structure similar to heparin, but the amount of adsorption did not increase in association with the increase of the D.S. of sulfate groups in polyanions because FGF-b recognized the area to be specific to heparin or heparan sulfate. On the other hand, CPEC70 did not have these sites, but FGF-b adsorbed onto it. This phenomenon is not fully understood, but it was observed that the adsorbed FGF-b stimulated a human periodontal ligament fibroblast (HPLF), which in turn made an aggregate on CPEC70. FGF-b exhibits and inhibits cell proliferation and differentiation in association with its concentration, and has been found to promote the binding process of the periodontal ligament in rats [22], the skin of mice and pigs [23, 24], and tumors in mice and rats [25, 26].

Effects of Precoating FGF-b on HPLF Proliferation

Figure 6 shows proliferation curves for HPLF cultured on FGF-b precoated and noncoated CPEC70, SPEC141, and TCP for 5 days in a serum-free medium. HPLF proliferation on TCP was not affected by the precoat of FGF-b, in spite of the high adsorption value of the culture materials. HPLF proliferation on

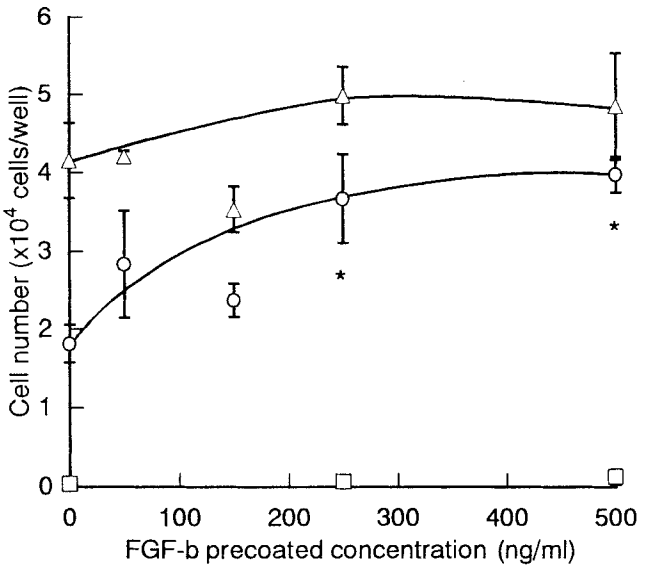

Figure 6. Effects of human recombinant fibroblast growth factor-basic precoating on HPLF proliferation for 5 days culture in serum free medium. □; CPEC70, ○; SPEC141, Δ; TCP. *; $P<0.05$ (vs. 0ng/ml precoating FGF-b concentration on SPEC141).

precoated SPEC141 was promoted by the over 250ng/ml FGF-b concentration, but HPLF proliferation on noncoated SPEC141 was not promoted in comparison with that of TCP. We reported that HPLF grew on both SPECs and TCP in DMEM in the presence of 10% FBS [1], but these results suggest that the signal of SPEC to HPLF functions differed from that of TCP. Moreover, the mediated FGF-b in the serum-free medium stimulated the HPLF growth in a dose-dependent manner (1~10ng/ml), as previously reported [27]. Although FGF-b fixed on SPEC141 in our experiments, a precoated of over 250ng/ml FGF-b stimulated HPLF growth. These mechanisms were similar to a dual receptor system for the FGF-b receptor [28]. Consequently, SPEC141 may play a role similar to that of the heparan sulfate chain in the heparan sulfate proteoglycans of the extracellular matrix. On the other hand, HPLF proliferation on CPEC70 was not promoted by the precoat of FGF-b in spite of the higher adsorption attained in comparison with SPEC141. We reported that HPLF did not grow and made an aggregate on CPEC70 in DMEM in the presence of 10% FBS. HPLF on CPEC100 did not make aggregates in spite of the inhibition of HPLF proliferation [1]. FGF has the ability to form a colony of

normal cells in a dose-dependent manner (50~200ng/ml) in soft agar [29, 30]. In addition, the number of colonies increased with an increase of the FGF concentration. The amount of FGF-b adsorption onto CPEC70 was about twice that of FGF-b adsorption onto CPEC100 (see Figure 5). These results suggest that HPLF aggregation on CPEC70 was caused by the difference between the amount of FGF-b adsorbed and the low amount of hFN adsorbed (see Figure 2). Consequently, the effect of the interaction between adsorbed FGF-b and CPEC70 on HPLF functions was different from these brought about by the interaction between adsorbed FGF-b and SPEC141. The interaction between these proteins and PECs kept HPLF proliferation and differentiation under control. These results suggest that CPECs and SPECs have different abilities in controlling the cell functions (proliferation and differentiation).

CONCLUSION

PEC has the ability to control protein adsorption in three ways: through the kind of substitution, the degree of substitution, and the charge balance. In addition, PEC, in combination with the adsorbed proteins, stimulates cell functions indicating that HPLF might be an aggregation from the stimulation by such adsorbed proteins. This experiment used one substituent (either carboxymethyl or a sulfate group) in chitin as a polyanion. If this substituent were substituted in one polysaccharide, the D.S. of the substituent would change and it could be used as a polyanion to PEC, which controls cell functions with adsorbed proteins as effectively as the glycos-aminoglycans *in vivo*. Consequently, if PEC is used as a material in artificial organ, it could play the role of the glycosaminoglycans.

SYMBOLS

CPEC70; CS100-CCHN70

CPEC100; CS100-CCHN100

SPEC34; CS100-SCHN34

SPEC62; CS100-SCHN62

SPEC141; CS100-SCHN141

hFN; Human recombinant fibronectin

Alb; Human albumin

FGF-b; Human recombinant fibroblast growth factor-basic

TCP; Tissue culture plate

HPLF; Human periodontal ligament fibroblast

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