Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblast (HPLF) function. II. Enhancement of HPLF differentiation and aggregation on PEC by L-ascorbic acid and dexamethasone

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Abstract: In addition to many types of extra cellular matrix (ECM) *in vivo,* cells are stimulated by many types of vitamins, hormones, growth factors, etc. In this paper the effects of L-ascorbic acid 2-phosphate (Asc-2P) and dexamethasone (Dex) on proliferation and differentiation of human periodontal ligament fibroblast (HPLF) using polyelectrolyte complex (PEC) as a matrix *in vitro* will be discussed. The PEC was composed of chitosan as a polycation, with carboxymethyl (CPEC) or sulfated chitin (SPEC). Asc-2P (0.2 m*M*) inhibited the growth of HPLF on CPEC, but promoted the growth on SPEC. Moreover, the aggregation of HPLF on CPEC was inhibited by Asc-2P, but that on SPEC was induced in the presence of Asc-2P and Dex. Although Asc-2P reduced an increase in alkaline phosphatase (ALPase) activity of HPLF on CPEC as well, it induced a twofold increase in ALPase activities on SPEC and TCD. Furthermore, in the medium containing Asc-2P and 100 m*M* of Dex, cell growth was inhibited, but ALPase activity was promoted on both SPEC and TCD to form many aggregates on SPEC. ALPase activity increased by twofold over that of HPLF cultured in the medium containing only Asc-2P. Therefore, it is suggested that the cell functions of HPLF are controlled by the combination of PEC and additives. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 41, 270–277, 1998.

Key words: polyelectrolyte complex; human periodontal ligament fibroblast; proliferation; differentiation; alkaline phosphatase

INTRODUCTION

To clarify the mechanisms of cell attachment to biomedical polymer surfaces would provide important information in the areas of biotechnology, artificial organs, and so on. Cellular behaviors, such as adhesion, morphological change, functional alteration, and proliferation, are greatly affected by the physicochemical parameters of polymer surfaces and specific biocomponents, for example, hormones, growth factors, etc.. Such surface properties of polymers as hydrophilic/hydrophobic balance, roughness, charge balance and density, surface free energy, and hardness play important roles in stimulation or prevention of cell adhesion.[1–5] Cells *in vivo* actually are surrounded by many types of extra cellular matrix (ECM), which form a three-dimensional tissue-like a hydrogel. Collagens, adhesive proteins, and proteoglycans are three major constituents of ECM. Proteoglycans are divided into a diverse group of proteins that carry sulfated carbohydrate chains and glycosaminoglycans (GAG) as a common feature.

We already have reported that PECs derived from artificial polymers or natural polysaccharides have a widespread influence on adhesion, growth, and morphology of human periodontal ligament fibroblast (HPLF). Importantly, it has been found that PECs of chitosan (CS) with chitin derivatives [carboxymethyl chitin (CCHN) and sulfate chitin (SCHN)] can control cell growth and morphology; HPLF formed three-dimensional cell aggregates on CPEC (CS-CCHN70), but the cells were spread and well proliferated on SPEC (CS-SCHN74) in Dulbecco's modified Eagle's medium (DMEM).[6,7]

It was reported that a cofactor, such as L-ascorbic acid 2-phosphate, activates the collagen synthesis of hepatocytes to form a multilayer.[8] Glucocorticoids modulate a variety of cell-specific factors as well as other hormones. For example, it has been shown that dexamethasone, a potent synthetic glucocorticoid, stimulates proliferation of osteoprogenitor cells selec-

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tively and induces mammalian bone marrow stromal cells to differentiate into osteoblasts.[9–12]

In this report, we demonstrate that the proliferation, alkaline phosphatase activity, and morphological pattern of HPLF are controlled by a combination of additives (Asc-2P and Dex) and PECs. Also, we discuss the ability to control HPLF function in an *in vitro* culture on PECs as a biomaterial.

MATERIAL AND METHOD

Polyanions and a polycation

Figure 1 shows the chemical structures of polyanions and a polycation. In order to estimate the effects of the anionic substituents on cell functions, carboxymethyl and sulfate residues were introduced to chitin as polyanions. The carboxymethylated chitin [CCHNx; x was a degree of substitution (D.S.); x = 70 (0.7 anionic site / 1 saccharides ring)] was purchased from Katokichi and used without further purification. Sulfated chitin (SCHN110) was prepared as reported previously.[13] Chitosan (CS) as a cationic polysaccharide was purchased from Katokichi and used without further purification.

PEC coating method

Polyanions were dissolved individually in distilled water (final concentration = 10^{-2} mol of ionic sites/L), and the pH of the solutions as adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 1% acetic acid solution and the pH adjusted to 6.0. The mixing ratio of the solutions of polyanions and polycation in a tissue culture dish was 1:1 for ionic site concentration to neutralize the charge balance of PEC. This mixing solution [1 mL/35 mmϕ tissue culture dish (TCD); NUNCLON Δ] stood overnight at room temperature. The supernatant solution was removed, the dish was dried and annealed at 65°C in an oven. After drying, these dishes were washed with distilled water and oven dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven.

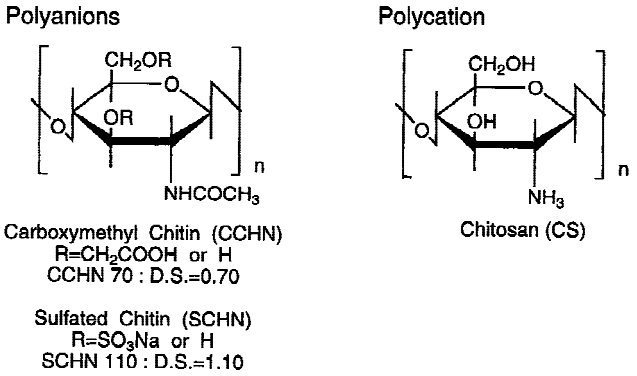

Figure 1. Polymer structures.

Preculture of HPLF

HPLF provided by Sanstar Co. Ltd. was precultured in the tissue culture flask (75 cm^2, CORNING) using Dulbecco's modified Eagle's medium (DMEM, Nissui–Seiyaku Co. Ltd.) supplemented with 0.1 g/L streptomicin, 0.1 g/L penicillin, 6.9 g/L n-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), 1.4 g/L $NaHCO_3$, 10 mL/L nonessential amino acid solution, 10 mL/L MEM vitamin solution, 10 mL/L MEM sodium pyruvate solution, and 10% fetal bovine serum (FBS, Brockneck) under 5% CO_2 at 37°C. The abovementioned culture medium was changed every 4 days. Cells that grew to confluence were detached using trypsin-EDTA and used in the following experiments.

Cell growth

For cell growth experiments, 1.5 mL of the cell suspension was poured into PEC-coated dishes or tissue culture dishes (TCD, 35 mmϕ), NUNCLON Δ {5×10^4 cells/dish in DMEM, DMEM-A [DMEM supplemented with 0.2 m*M* L-ascorbic acid 2-phosphate (Asc-2P, Wako pure chemical industries, Ltd.)]}, and DMEM-D [DMEM supplemented with 0.2 m*M* Asc-2P, 10 m*M* β-glycerophosphoric acid disodium salt (β-GPO_4, Nacalai tesque), 100 n*M* dexamethasone (Dex, Nacalai tesque)] and cultured in the same conditions as described above. Cells were counted with a hemacytometer under phase contrast microscopy (Nicon, DIAPHOT-TMD).

Alkaline phosphatase activity assay

For assay in alkaline phosphatase (ALPase) activity, cells cultured as above were rinsed three times with PBS(−) (Ca^{2+} and Mg^{2+} free phosphate buffer saline, pH 7.4), and 2 mL of nonidet solution [0.2% nonidet P-40 (Nacalai tesque): PBS(−) = 1:4] was poured into the dishes. The dishes were incubated for 10 min at 37°C and then homogenated with a homogenizer. These suspensions were centrifuged at 1000 rpm for 10 min at 4°C, and the ALPase activity of these supernatants was measured. The supernatant and substrate (16 m*M* p-nitrophenylphosphate disodium salt hexahydrate, Wako Pure Chemical Industries, Ltd.) were mixed and then incubated for 30 min at 37°C. After that, aqueous 0.2*N* NaOH was added to the mixture to stop the reaction, and the absorbance at 410 nm was measured using a spectrophotometer (HITACHI 100-10). The amount of total protein also was measured by the Bio-Rad protein assay method (protein assay, Bio-Rad Lab.) and calibrated from the standard curve of albumin (Bovine Albumin Fraction V; GIBCO). Therefore, ALPase activity of HPLF was calculated according to the following equation.

$$\text{Specific activity (U/mg protein)} = \frac{OD_{410} \times 100}{0.873 \times 0.2\ \text{mL} \times 30\ \text{min} \times \text{total protein (mg/mL)}} \quad (1)$$

Statistical analysis

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

RESULTS

Cell growth

Figure 2 shows growth curves of HPLF in DMEM (a), DMEM-A (b), and DMEM-D (c) on PECs. Table I summarizes the results of the morphological changes of HPLF on PECs time dependently. Figure 3 shows photographs of HPLF cultured for 21 days on PECs. HPLF growth rates on PEC-coated dishes were slower than those on TCD in DMEM. Focusing on functional groups of polyanion, irrespective of the medium, HPLF growth rates were in the order of sulfate > carboxymethyl. Asc-2P slightly induced HPLF growth on TCD, but Dex depressed cell growth. However, such additives did not induce similar effects on the growth of HPLF on PECs directly: HPLF growth on CS-CCHN70 (CPEC) was very low even in DMEM-A. On the other hand, after 4 days on CPEC, HPLF made aggregates in all mediums, and those aggregates were detached from CPEC to float in the medium at 5 days. But in the case of DMEM-A on CPEC, the second aggregate, which was observed for over 14 days in DMEM,[7] was not constructed, and most of the remaining HPLF showed round forms and easily were detached from the PEC surface by every medium change. Asc-2P did not affect the cell morphology of HPLF cultured on CS-SCHN110 (SPEC), but it promoted HPLF growth, just as on TCD as a control; that is, the difference in the final cell numbers in the absence or presence of Asc-2P was about 8×10^4 cells/dish. The cell numbers in the presence of Asc-2P on TCD in a stationary phase was decreased since the cells are not completely separated by a trypsin–EDTA solution. HPLF in DMEM-D on CPEC showed the same behaviors as in DMEM. In contrast, the cell growth of HPLF in DMEM-D on both SPEC and TCD

TABLE I
Time-Dependent Morphological Change of HPLF on PECs

Culture Days		1	3	5	7	10	14	21
DMEM	CPEC	R	R,S	R,S	R,S	S	S,A	S,A
	SPEC	S	S	S	S	S	S	S
	TCD	S	S	S	S	S	S	S
DMEM-A	CPEC	R	R	R,S	R	R,S	R,S	R,S
	SPEC	S	S	S	S	S	S	S
	TCD	S	S	S	S	S	S	S
DMEM-D	CPEC	R,S	R,S	R,S	R,S	R,S	R,S,A	R,S,A
	SPEC	S	S	S,A	S,A	S,A	S,A	S,A
	TCD	S	S	S	S	S	S	S

R = Round; S = Spreading; A = Aggregate; CPEC = CS-CCHN70; SPEC = CS-SCHN110; TCD = Tissue culture dish. The letters (R, S, A) indicate the existence of these forms in 1 mm^2 at several random areas under the phase contrast microscopy. S and R in one column indicate HPLF morphology is 100% spreading and round forms, and R, S indicate 50% round and 50% spreading form, respectively. The symbol ''A'' has a different meaning compared with the other symbols: The HPLF aggregate was very big compared to the other forms, and so we marked ''A'' when they were observed on PEC. Approximately 50 aggregates are observed on a 35 mmø dish.

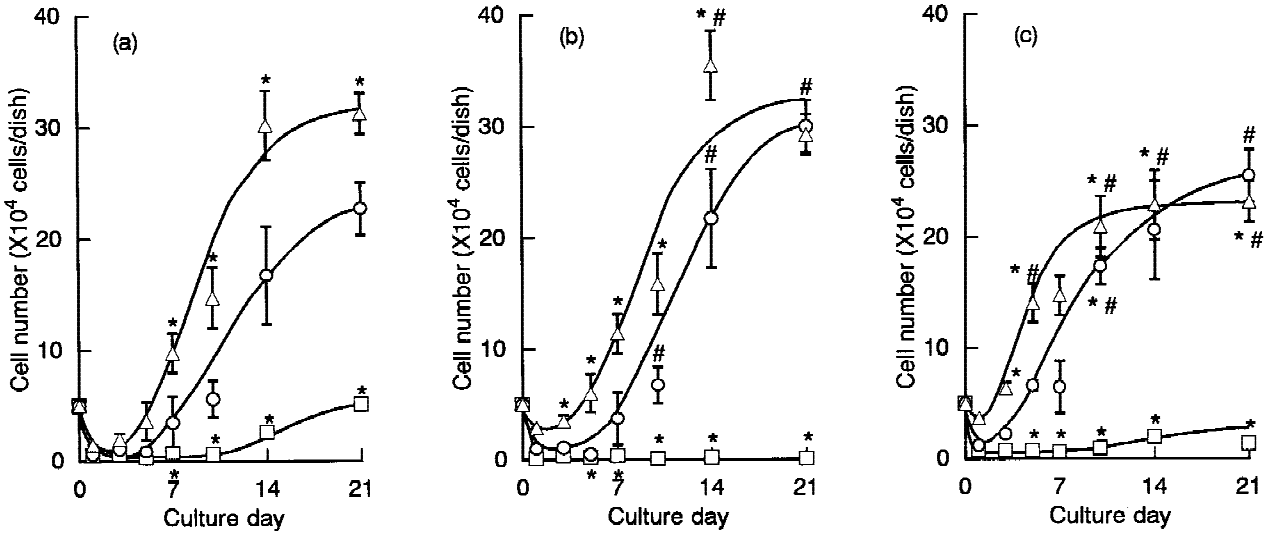

Figure 2. Growth curves of HPLF cultured on PECs in DMEM (a), DMEM-A (b), and DMEM-D (c). □: CPEC (CS-CCHN70); ○: SPEC (CS-SCHN110), and △: TCD. Each value is the mean ± SD of six dishes. *: $P < 0.05$ (vs. SPEC); # in (b): $P < 0.05$ (vs. DMEM); # in (c): $P < 0.05$ (vs. DMEM-A).

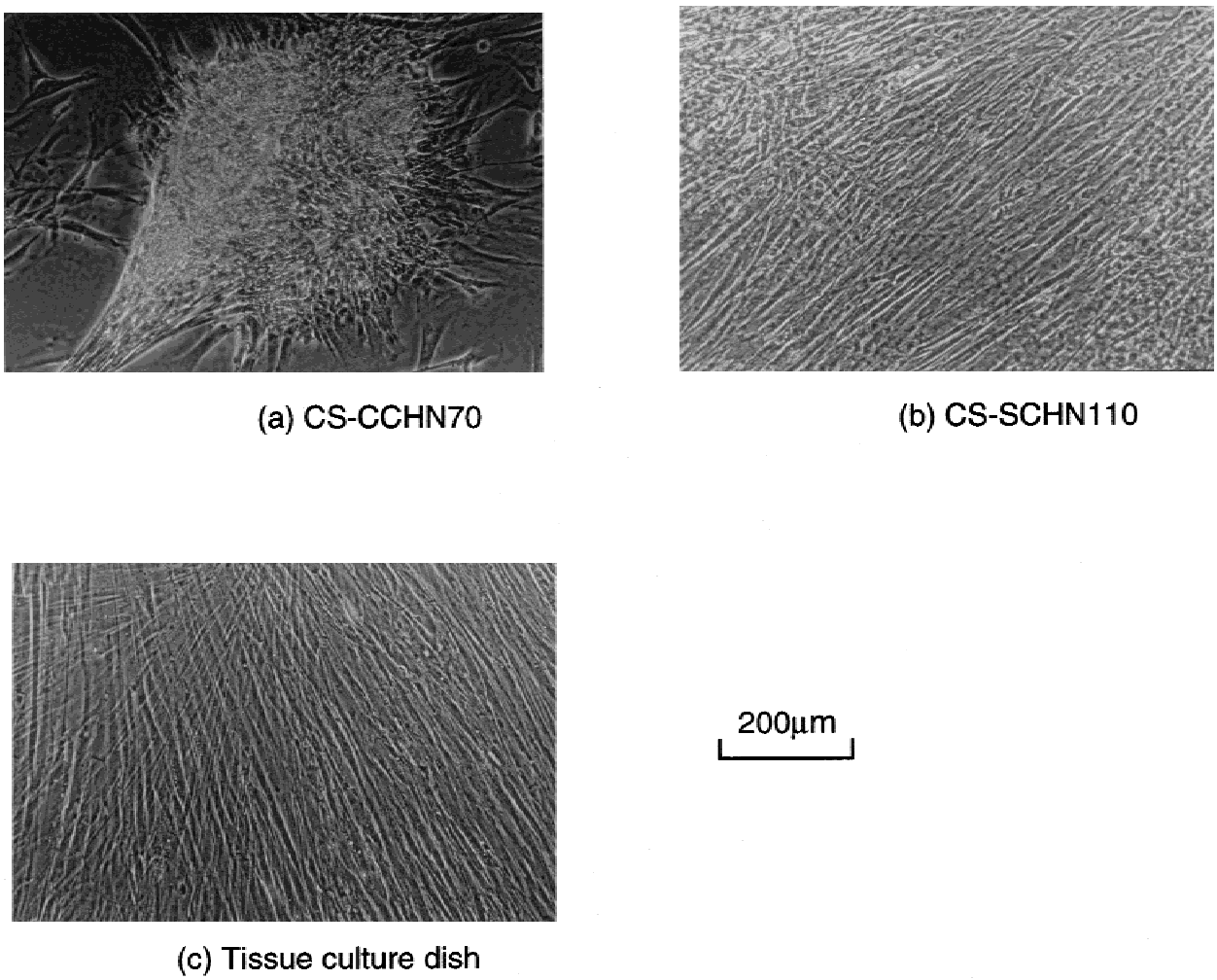

Figure 3. Morphology of HPLF cultured for 21 days on PECs and TCD.

obviously was reduced compared with DMEM and DMEM-A. In DMEM-D, the cell numbers on SPEC were a little greater than on TCD after 21 days of culture because HPLF on SPEC made an aggregate at that time. The cell form on SPEC in the absence of Dex was a fibroblast-like spreading form just as on TCD, but in the presence of Dex HPLF aggregated after 4 days of culture. Furthermore, these aggregates were similar to the cell form cultured on CPEC in DMEM for over 2 weeks, they kept their form for 21 days in culture, and, at the same time, cell growth was stopped. The cells that had no connection with the aggregate in DMEM-D on SPEC showed spreading forms and grew, but then they gradually turned to an aggregate form after a certain period of time. Therefore, in this system, the aggregate number increased time dependently. On the contrary, HPLF on TCD in DMEM-D did not show the aggregate.

Thus it is suggested that there are ligand-like groups on the PEC surface, similar to Dex for the HPLF surface receptor, or there is a nonspecific (nonphysiological) mechanism (similar interaction between SPEC or Dex and HPLF surface) that stimulates differentiation of HPLF directly.

Alkaline phosphatase activity assay on CPEC

Figure 4 shows the time dependence of ALPase activities of HPLF cultured on CPEC and TCD in DMEM, DMEM-A, and DMEM-D. It is well known that ALPase activity increases when cells contact each other.[14,15] Therefore, the seeding number of HPLF was adjusted to 10^6 (a) and 3×10^5 (b) cells/dish in DMEM, respectively. Although in the case of 3×10^5 cells/dish, HPLF did not make an aggregate, ALPase activity initially increased temporarily for a few days on CPEC and then decreased gradually. In the case of 10^6 cells/dish, the ALPase activity also increased on CPEC after 2–4 days in culture, and gradually increased again after 8 days in culture. The decrease of ALPase activity after that was caused by the fact that the aggregate was detached from CPEC and floated into the me-

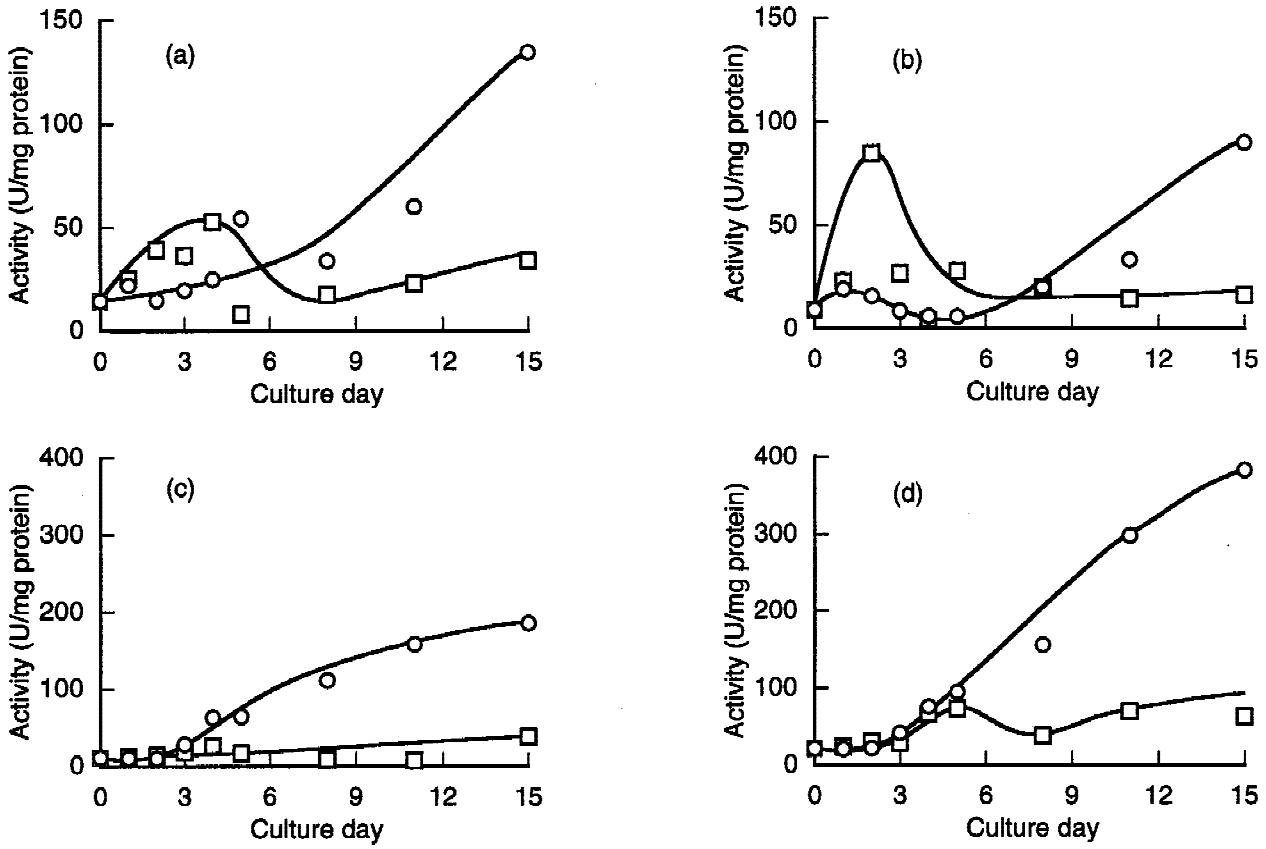

Figure 4. ALPase activity of HPLF cultured on CPEC and TCD: (a) in DMEM (10^6 cells/dish), (b) in DMEM (3×10^5 cells/dish), (c) in DMEM-A (10^6 cells/dish), and (d) in DMEM-D (10^6 cells/dish). □: CPEC; ○: TCD.

dium. The increase of ALPase activity after 8 days was coincident with the formation of cell aggregates on CPEC, but that, in the initial days, did not relate to the formation of aggregates. ALPase activity of HPLF on TCD (b) also increased after the cells grew to confluence, which was consistent with an increase of ALPase activity, as reported previously.[11,12] In the case of 10^6 cells/dish in DMEM (a), ALPase activity on TCD was increased earlier than that for 3×10^5 cells/dish. Therefore, it is suggested that the increase of ALPase activity is caused by cell–cell contact, and initially the high ALPase activity on CPEC for several days seems to reflect an increase of cell–cell contact due to the formation of aggregates. Addition of Asc-2P scarcely affected ALPase activity on CPEC [Fig. 4 (c)]. However, as mentioned previously, the cell aggregates were not formed in this system in a whole period of time, and so a specific increase of ALPase activity correlative to aggregates was not observed. On the contrary, ALPase activity on TCD in the presence of Asc-2P was much increased in comparison with Figure 4 (a). HPLF cultured on CPEC in DMEM-D [Fig. 4 (d)] showed morphological change (see Table I). ALPase activity increased in accordance with the formation of aggregates (aggregates were not formed after 4 days of culture), and the maximum value of ALPase activity, observed after 21 days of culture, was similar to that after 21 days in the absence of Asc-2P. ALPase activity on TCD in the presence of Dex also was much higher than that in the presence of only Asc-2P. The cell number in this system was about 50% less than that in DMEM.

Alkaline phosphatase activity assay on SPEC

Figure 5 shows ALPase activities of HPLF cultured on SPEC and TCD in DMEM and DMEM-A under different cell densities. In the case of 10^6 cells/dish in DMEM [Fig. 5 (a)], an increase of ALPase activity upon initial culture was not observed, which was different from that on CPEC [Fig. 4 (a)]. However, when cells were seeded in a 3×10^5 cells/dish [Fig. 5 (b)], ALPase activity showed a peak at about 2 days. PEC did not affect cell condition as a similar peak was observed on TCD. In the case of the 3×10^5 cells/dish, cells might not contact each other initially in culture because there is enough space for cell growth, and thus ALPase activity is low. But as cells grow close to a confluent condition, ALPase activity increases gradually. Therefore, when large numbers of cells were seeded in the dish, cell culturing duration until ALPase activity started to increase was shorter. The

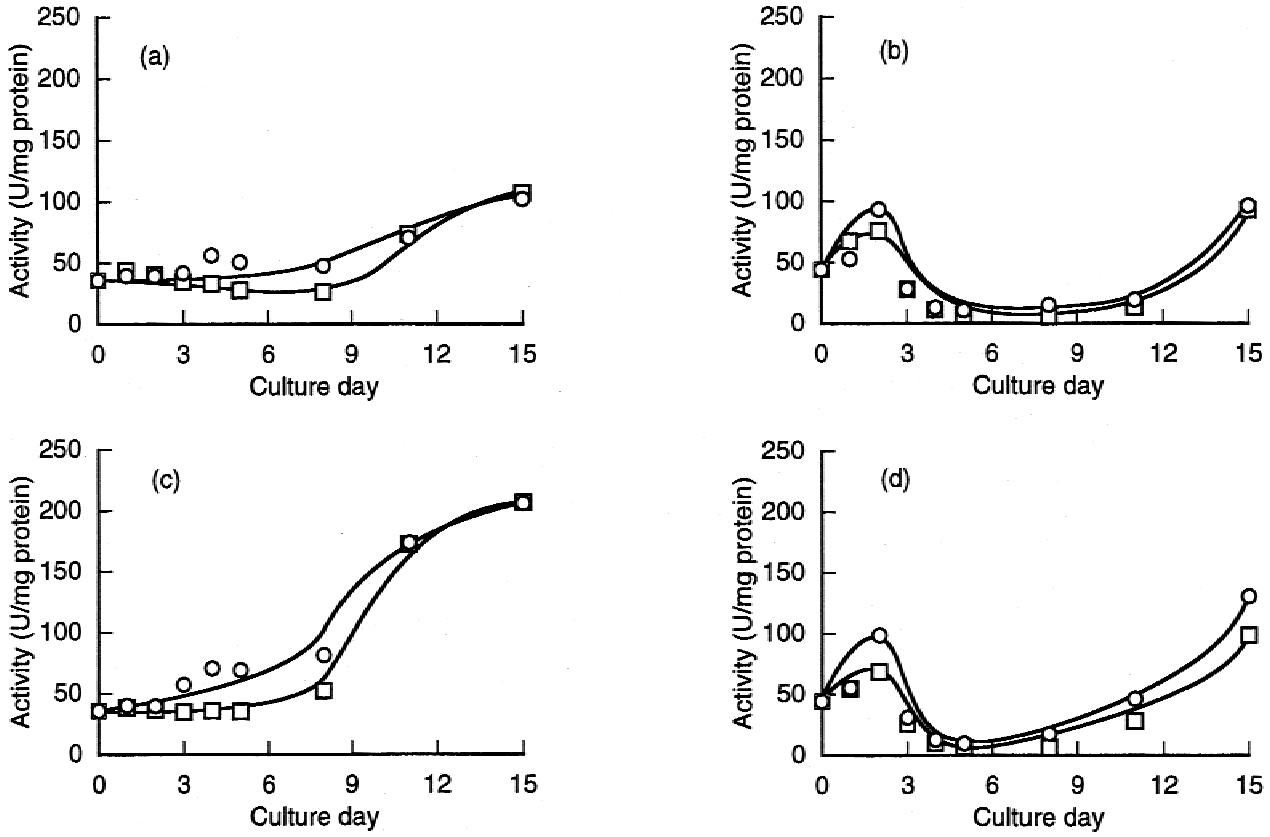

Figure 5. ALPase activity of HPLF cultured on SPEC and TCD: (a) in DMEM (10^6 cells/dish), (b) in DMEM (3×10^5 cells/dish), (c) in DMEM-A (10^6 cells/dish), and (d) in DMEM-A (3×10^5 cells/dish). □: SPEC; ○: TCD.

profile of ALPase activity on SPEC and TCD showed almost identical changes, indicating that SPEC had almost the same effect on HPLF as on TCD. ALPase activity in DMEM-A (10^6 cells/dish) [Fig. 5 (c)], indicated similar behavior as that shown in Figure 5 (a), but the final ALPase activity in DMEM-A was about twofold larger than that in DMEM. Moreover, the length of time before ALPase activity started to increase on SPEC was greater from that on TCD because the number of cell–cell contacts on SPEC was fewer than on TCD (SPEC reduced the cell growth rate). In the case of 3×10^5 cells/dish in DMEM-A [Fig. 5 (d)], in spite of the presence of Asc-2P, the change of ALPase activity showed a similar profile to that shown in Figure 5 (b).

Figure 6 shows the ALPase activity of HPLF (10^6 cells/dish) cultured on SPEC and TCD in DMEM-D. Although ALPase activity shows almost the same tendency as if cultured in DMEM and DMEM-A at the same seeding cell number, the activity value in DMEM-D was much higher than when the HDLF was cultured in DMEM and DMEM-A. And final ALPase activity showed about a fourfold value to DMEM. As mentioned above, ALPase activity increased about twice in the presence of only Asc-2P, and so Dex affected HPLF directly and controlled not only cell growth but also ALPase activity.

DISCUSSION

The detailed mechanisms of the effects of L-ascorbic acid (vitamin C) on cell proliferation *in vivo* have not been clarified yet, but whether or not L-ascorbic acid promotes collagen synthesis is being investigated. Murad et al. reported that collagen synthesis of human fibroblasts cultured under low L-ascorbic acid conditions is increased about eightfold by adding 0.25

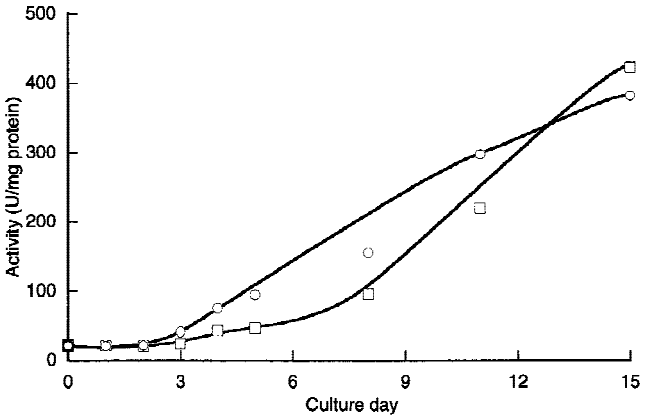

Figure 6. ALPase activity of HPLF cultured on SPEC and TCD in DMEM-D (10^6 cells/dish).

m*M* of L-ascorbic acid to the medium.[16] On the other hand, Hata et al. reported that L-ascorbic acid under usual culture conditions (37°C, in neutral solution) is completely degraded by oxidation with 24 h, and an excess of L-ascorbic acid in the medium was found to inhibit cell growth. It is well known that Asc-2P is more stable in culture conditions and that it is fit for evaluation of the effects of L-ascorbic acid on cell functions. It was determined that fibroblasts continued to grow in multilayers just by adding more than 0.1 m*M* of Asc-2P to the medium.[8] With this in mind, in this study the effects of Asc-2P on cell functions were evaluated in connection with a kind of matrix (CPEC, SPEC, and TCD). Asc-2P indicated a little cell proliferation and did not form a multilayer on TCD. On CPEC, Asc-2P inhibited cell growth and made aggregates. These results suggested that Asc-2P certainly affects HPLF functions when cultured on PEC *in vitro* and that HPLF also receives different signals from the PEC surface. Moreover, Asc-2P and PEC affected the ALPase activity of HPLF. In the absence of Asc-2P, ALPase activity on CPEC increased in parallel with the formation of aggregates. Inside HPLF, aggregate cells contacted each other in a manner similar to contact inhibition conditions in confluence. In general, the ALPase activities of HPLF and osteoblasts are increased when these cells grow to confluence[17,18] and, at that time, cell proliferation is stopped and the cells enter a differentiation phase. Many investigators have reported that the ALPase activities of these cells increase at an initial differentiation stage and that they are suitable for one of the initial differentiation markers.[17] On the other hand, using DMEM-A, HPLF did not construct aggregates on CPEC at 4 days, and ALPase activity did not increase. In order to increase ALPase activity on CPEC, it is necessary that HPLF forms aggregate and contact each other. However, the ALPase activities of HPLF cultured in DMEM-A on SPEC and TCD were twofold higher than those in DMEM. Moreover, in these systems the HPLF layer was not completely dispersed by the trypsin–EDTA solution. This fact suggests that collagen is synthesized and secreted to spaces between the cells and that cell–cell contact becomes stronger. Differences between SPEC and CPEC were only a kind of substituent of polyanion, sulfate, and carboxylate. Since PEC is formed mainly through an electrostatic interaction between a polycation and a polyanion, characteristics of PEC are controlled by a kind of dissociative group of component polyelectrolytes.[19] Therefore, SPEC and CPEC are different in conformation, charge balance, charge density, hydrophobic/hydrophilic balance, microdomain structure, roughness, etc. of the PEC surface. We already have reported that adsorption of fibronectin onto the PEC surface also is controlled by a kind of substituent of polyanion.[6] To follow up this line of research, we investigated whether or not HPLF growth, function, and morphology are controlled not only by the physicochemical properties of PEC but also by adsorbed proteins onto PEC.

Generally DMEM-D is used as a medium for the *in vitro* culture of osteoblasts. We used dexamethasone (Dex) as a glucocorticoid (a steroid hormone involved in the regulation of glucose metabolism). Although the effects of Dex on the proliferation of cells derived from human donors differ with a variety of cell types, detailed mechanisms of the effects of Dex on cell proliferation have not been clarified yet. Thus several factors, such as cell type, donor age, species, and culture conditions, play important roles in determining whether cellular proliferation is stimulated or inhibited in the presence of Dex.[20] In this experiment, Dex inhibited HPLF proliferation on SPEC and TCD. Moreover, in the presence of Dex, HPLF on a SPEC constructed aggregate forms, but such aggregates were not observed on TCD under the same conditions. On the other hand, HPLF on CPEC in DMEM-D again formed aggregates that were not observed in the presence of Asc-2P alone. Many workers have reported the beneficial effects of glucocorticoids on the aggregate-forming ability of both normal and transformed cells.[21–23] Moreover, it has been found that primary HPLF forms aggregates when cultured in normal medium and in the presence of L-ascorbic acid and Dex on TCD after confluence.[24] Although, in general, normal cells lose their own functions gradually when cultured on TCD under usual conditions, HPLF cultured on PEC kept its own functions through cell growth. On the other hand, the ALPase activity of HPLF cultured in DMEM-D was twofold higher than that in DMEM-A. Glucocorticoids play a role in the differentiation and proliferation of cells in many organs and tissues.[25–27] We already have reported that the aggregates on CPEC were stained by alizarin red. It was found that these aggregates were mineralized in the presence or absence of Asc-2P and/or Dex. In conclusion, it is suggested that cell culture conditions on PEC are better than on TCD.

CPEC has the ability to differentiate HPLF even in the absence of Asc-2P and Dex. Although SPEC alone does not have the capability to differentiate HPLF in the absence of the same additives, HPLF is differentiated by combination with such supporting additives. These results suggest that PEC has the ability to differentiate and maintain cell function *in vitro.* Although the mechanisms by which PEC affects cellular functions are not clear in detail, polysaccharides as components of PEC play an important role in the maintenance and activation of cellular function *in vitro.* Living cells *in vivo* exist in three-dimensional hydrated extracellular matrices (ECM)[28,29] that are composed of an insoluble network of collagen, proteins, and proteoglycans (PG). PG consists of core protein and glycosaminoglycans (GAG). GAG contain substituents of

carboxylic and sulfonic acid on the side-chain of polysaccharide, for example, chondroitin sulfate, heparin, dermatan sulfate, and hyaluronic acid. CCHN and SCHN are similar structures of GAG, and cells seem to recognize PEC as a component of ECM. Therefore, it has been determined that PEC composed of polysaccharides is a good material for cell functional culture *in vitro.*[1] Moreover, these studies are considered to be a suitable model for using GAG *in vivo* to determine cell proliferation and differentiation.

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