Evaluation of Additives Required for Periodontal Disease Formulation Using Basic Fibroblast Growth Factor

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To design a suitable periodontal disease formulation using basic fibroblast growth factor (bFGF), legally available thickeners were evaluated focusing on their viscosity, extrusive force from a syringe, flow property and inertness to bFGF. Thirteen candidate thickeners showed appropriate viscosity (about 1×10^4 mPa·s), and further evaluations were conducted on them. Flow property was evaluated by the tilting test tube method. As a result, most thickener solutions with the optimum viscosity showed appropriate flow time (about 100 s) and the flow time did not depend on thickener concentration, whereas the extrusive force from a syringe depended on thickener concentration despite the thickener type and grade. Thickener solutions of 2—3% showed ideal result (10—20 N) and thickener solutions prepared outside of the concentration range (2—3%) were found to show unsuitable extrusive force. Consequently, to obtain required properties for a dental drug formulation, thickener solutions needed to show adequate viscosity (about 1×10^4 mPa·s) at 2—3% thickener concentration. In addition, several types of cellulose derivatives showed inertness to the bFGF because of their structure, without strong ionic dissociable groups, and neutral pH. Overall, the present work demonstrates that some water-soluble cellulose derivatives, such as hydroxypropylcellulose (HPC) and hydroxyethylcellulose (HEC), were suggested to have required properties for a dental drug formulation including bFGF.

Key words periodontal disease; basic fibroblast growth factor; selection of thickener

Recently it has become clear in *in vitro* experiments that basic fibroblast growth factor (bFGF) had a stimulation effect on the proliferation, chemotaxis, and differentiation of various types of cells, such as vascular endothelium cells, vascular smooth muscle cells, and epithelial cells, in addition to the proliferation effect on fibroblasts.[1—5] Furthermore, an *in vivo* study clearly demonstrated that bFGF had a strong neovascularization effect.[6] Since bFGF has these pharmacological effects, rhbFGF [recombinant human basic fibroblast growth factor (rhbFGF), General name: trafermin] has been promoted in clinical development as a medical agent for intractable skin ulcer. Its excellent therapeutic effects and safety have been confirmed in clinical studies, and it is now on the market. Furthermore, bFGF was also reported to stimulate the proliferation of periodontal membrane-derived cells in periodontal tissues.[7] Thus, bFGF is attracting a lot of attention and has been researched in various research fields recently.[8—11] However, rhbFGF has not been applied to periodontal disease as a pharmaceutical product and there are no findings about the optimization of pharmaceutical dental formulation including rhbFGF.

In our previous study, the requirements for drug formulations for periodontal disease were revealed.[12] A formulation should be designed that can be used in surgical treatment (flap operation), that is inhibited from flowing out of the application site, that can be administered to the upper lateral disease area, and has adequate resistance when administered in a small amount less than 1 ml with a syringe. To satisfy these requirements, we found that the formulation should be viscous as a formulation concept and a viscous formulation was expected to improve the drug remaining at the application site. To investigate selecting requirements of thickener; additive to obtain viscosity, essential formulation properties were revealed as viscosity, flow properties, and extrusive force by an *in vitro* release test using hydroxypropylcellulose (HPC) as a model thickener.

It was also reported that viscosities were correlated with flow times, and thickeners with the optimum viscosity showed the desired flow properties in the previous study.[12]

In the present study, to find elements necessary for thickeners used for a periodontal disease formulation including rhbFGF, various thickeners were evaluated. Thickeners were evaluated from the viewpoint of whether 1) they were available legally as dental drug excipients, 2) they met the requirements of dental formulations (viscosity, flow property and extrusive force) revealed in the previous study,[12] and 3) they did not affect the stability of rhbFGF. As the first evaluation, fifty-nine thickeners described in the Japanese pharmaceutical excipients dictionary published in 2007 were surveyed whether they were described in the representative regulatory documents, such as Japanese Pharmacopoeia 15th Edition (JP15), as available dental drug excipients. Also, since rhbFGF is a protein, it would be denatured in an oily or organic solvent, so thickeners were required to be water soluble. The candidate thickeners given from the first evaluation above were performed the further investigations: viscosity, flow property and so on. Especially, viscosity is considered to be one of the most important properties of the periodontal disease formulation and the selected candidate thickeners were screened from the standpoint of viscosity at first.

Experimental

Materials The thickeners used in this study are listed in Table 1 and their abbreviations are also shown in the table. HPC, hydroxyethylcellulose (HEC) and methylcellulose (MC) have several grades according to their polymerization degree, and the degree is in proportion to viscosity. rhbFGF (number of amino acid residues: 153—154, molecular weight: 17 kDa) was obtained from Kaken Pharmaceutical Co., Ltd. (Shizuoka, Japan). All solutions were prepared using water, about 6.8 pH value, manufactured by reverse osmosis membrane method. All other chemicals were of reagent grade and used without further purification.

Preparation of Thickener Solution Thickeners were dissolved in water

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Table 1. Investigated Thickeners

Thickeners (abbreviation)	Manufacturer	Grade
Sodium alginate	Foodchemifa Co., Ltd.	—
Carboxyvinyl polymer (CVP)	Noveon	CarbopolNF980
Carboxymethylcellulose sodium (CMCNa)	Dai-ichi Kogyo Seiyaku Co., Ltd.	Cellogen
D-Sorbitol	Wako Pure Chemical Industries Ltd.	—
Concentrated glycerin (glycerin)	Kozakai Pharmaceutical Co., Ltd.	—
Hydroxyethylcellulose (HEC)	Hercules	HHX, HX, M[a)]
Hydroxypropylcellulose (HPC)	Nippon Soda Co., Ltd.	H, M, L, SL, SSL[b)]
Propylene glycol (PG)	Maruishi Pharmaceutical Co., Ltd.	—
Sodium polyacrylate (PAA-Na)	Wako Pure Chemical Industries Ltd.	—
Polysorbate 80	Junsei Chemical Co., Ltd.	—
Polyvinyl alcohol (PVA)	Nippon Synthetic Chemical Industry Co., Ltd.	EG-40
Povidone (PVP)	BASF	K90
Macrogol (PEG)	Wako Pure Chemical Industries Ltd.	PEG20000
Methylcellulose (MC)	Shin-Etsu Chemical Co., Ltd.	SM8000, M1500, SM100, SM25[c)]

a) Polymerization degree: HHX>HX>M. *b*) Polymerization degree: H>M>L>SL>SSL. *c*) Polymerization degree: SM8000>SM1500>SM100>SM25.

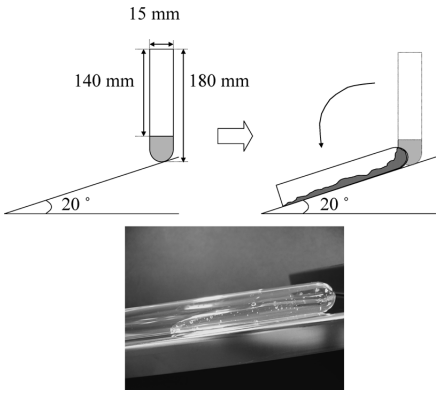

Fig. 1. Measurement of Flow Time by Tilting Test Tube Method

at various concentrations, which were 10% or less because they were required to have sufficient viscosity at lower concentrations for this formulation.

Measurement of Viscosity The viscosity of each thickener solution was measured using a cone-flat plate-type rotational viscometer (RE550H; Toki Sangyo Co., Ltd., Japan) at room temperature (about 25 °C). Based on viscosity data obtained from the measurement, a concentration–viscosity curve was established for each thickener and the concentration that showed the optimum viscosity (about 1×10^4 mPa·s)[12] was researched.

Evaluation of Flow Property The flow property of each thickener solution was evaluated by the tilting test tube method[13] described in Fig. 1. About 7 ml of the prepared thickener solution was poured into a glass test tube (ϕ15 mm, length; 180 mm) and the sample was left to stand for more than 1 h. After air bubbles had disappeared, the experiment was carried out. The flow distance was set at 140 mm. The test tube was quickly tilted at an angle of 20 degrees and the time for the solution to reach the lip of the test tube was measured at room temperature (about 25 °C). The data obtained from the measurement were evaluated to identify the optimum value (about 100 s).[12]

Evaluation of Extrusive Force The extrusive force of each thickener solution was measured by EZ TEST (Shimadzu Co., Ltd., Japan) using the same method as in the previous study.[12] Each thickener solution filled a 1 ml syringe (Terumo Co., Ltd., Japan, for tuberculin testing, ϕ; 6 mm) and was attached to a 27 gauge needle (Nipro Co., Ltd., Japan, ϕ; 0.4 mm), and then the average load was measured by pressing the plunger of the syringe at 50 mm/min. The measured average load was regarded as an extrusive force when the viscous formulation was extruded from the syringe. This measurement was conducted at room temperature (about 25 °C) and the data obtained from the measurement were evaluated to identify the optimum value (10—20 N).[12]

Preparation of rhbFGF Lyophilized Product A 1 ml sample of rhbFGF solution, adjusted to 4 mg/ml, was used to fill a 5 ml glass vial and lyophilized by a freeze-dryer (SF-08; Okawara Co., Ltd.). The lyophilized product included citric acid as a pH regulator and sucrose as a filler.

Evaluation of rhbFGF in Thickener Solution Stability rhbFGF lyophilized product was dissolved with 1 ml of each thickener solution, and viscous formulations, including rhbFGF, were prepared. A control formulation was also prepared by dissolving the lyophilized product with 1 ml water. Each viscous formulation, including rhbFGF, was stored for 24 h at room temperature (about 25 °C). The purity of rhbFGF remaining in the formulation was measured at 0, 6 and 24 h after storage. The purity of rhbFGF was measured by chromatography and the analytical conditions were previously reported by our laboratory[14] using HPLC.

Results and Discussion

Viscosity of the Thickener The concentration–viscosity curves of the thickeners, listed in Table 1, are shown in Fig. 2, and thickeners showing the optimum viscosity (about 1×10^4 mPa·s)[12] are listed in Table 2. As a result of this determination, all samples of 3% HPC(H), 6% HPC(M), 1.75% carboxyvinyl polymer (CVP), 10% carboxymethylcellulose sodium (CMCNa), 2.3% MC(SM8000), 4% MC(SM1500), 6% MC(SM100), 9% MC(SM25), 1.25% HEC(HHX), 2% HEC(HX), 2.4% HEC(M), 5% sodium alginate and 1.15% sodium polyacrylate (PAA-Na) showed the optimum viscosity. Meanwhile, the other thickener solutions showed low viscosity. These results were attributed to the polymerization degree and molecular weight of thickeners. Thus, subsequent evaluations were conducted using these thirteen thickener solutions with optimum viscosity.

Flow Property of the Thickener The flow properties of the selected thirteen thickener solutions were evaluated by the tilting test tube method and the results are shown in Table 3. The relationship between the flow time and concentration is shown in Fig. 3. As a result, most thickener solutions with optimum viscosity showed appropriate flow times as expected and the flow times did not depend on the thickener concentration. All samples of 3% HPC(H), 6% HPC(M), 1.75% CVP, 1.25% HEC(HHX), 2% HEC(HX), 2.4% HEC(M), 10% CMCNa and 5% sodium alginate showed appropriate flow times (80—185 s), close to the optimum flow time (about 100 s).[12] In the interests of following the complex shapes of diseased tissues and avoiding drips from the application site, these thickeners were considered to be useful. In contrast, four MC solutions had relatively long flow times (297—364 s), despite optimum viscosity. The cellulose derivatives such as MC and HPC are partially ether-substituted cellulose to achieve water solubility. It is known that the substituent make cross-linking each other by hydrophobic

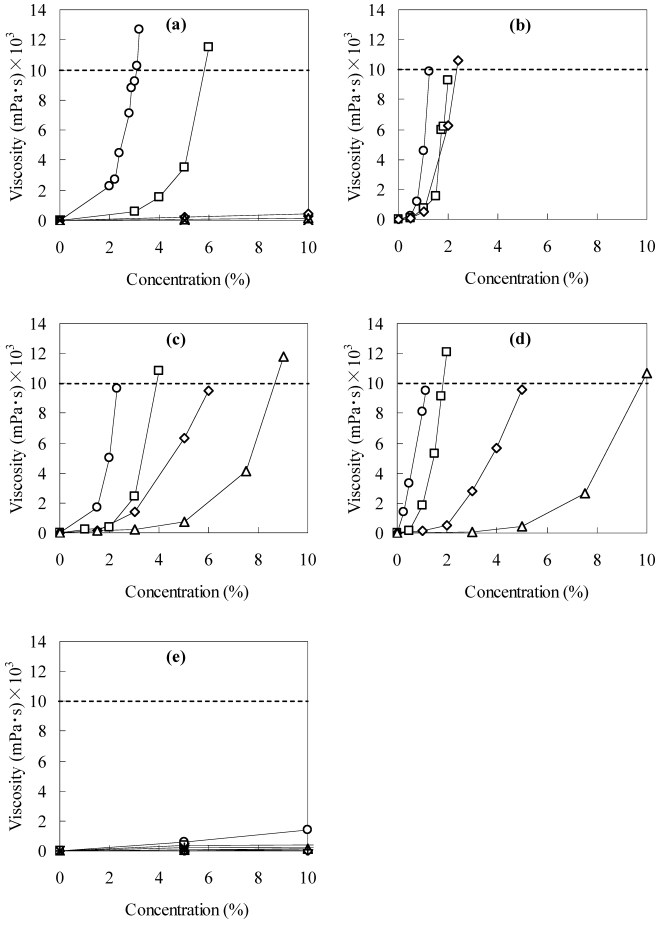

Fig. 2. Concentration–Viscosity Curve of Various Thickeners ($n=1$)

(a) (○): HPC(H), (□): HPC(M), (◇): HPC(L), (△): HPC(SL), (×): HPC(SSL). (b) (○): HEC(HHX), (□): HEC(HX), (◇): HEC(M). (c) (○): MC(SM8000), (□): MC(SM1500), (◇): MC(SM100), (△): MC(SM25). (d) (○): PAA-Na, (□): CVP, (◇): Sodium Alginate, (△): CMCNa. (e) (○): PVA, (△): PVP, (◇): PEG, (□): Glycerin, (×): PG, (✕): Polysorbate 80, (+): D-Sorbitol. The dotted line shows the optimum viscosity (10000 mPa·s).

Table 2. Viscosity of Various Thickener Solutions at Concentrations with about 1×10^4 mPa·s

Sample	Grade	Concentration (%)	Viscosity (mPa·s)×10^3 (mean±S.D.[a])
HPC	H	3.00	10.8±0.2
	M	6.00	10.9±0.0
MC	SM8000	2.30	9.5±0.1
	SM1500	4.00	11.0±0.3
	SM100	6.00	10.9±0.3
	SM25	9.00	10.7±0.3
HEC	HHX	1.25	9.1±0.1
	HX	2.00	11.0±0.1
	M	2.40	10.2±0.1
CMCNa	PR-S	10.0	9.8±0.1
CVP	NF980	1.75	10.3±0.0
Sodium alginate	—	5.00	10.3±0.0
PAA-Na	—	1.15	9.9±0.0

a) $n=3$; S.D.: standard deviation for three experiments.

bonding in the solution state and form three-dimensional structure, which holds water in the network and makes gel when temperature increase.[15] It is also known that a polymer which has substituent with short molecular chain, such as

Table 3. Flow Time of Various Thickener Solutions at Concentrations with about 1×10^4 mPa·s

Sample	Grade	Concentration (%)	Flow time (s) (mean±S.D.[a])
HPC	H	3.00	108.3±0.6
	M	6.00	83.7±1.2
MC	SM8000	2.30	336.7±19.3
	SM1500	4.00	364.3±38.6
	SM100	6.00	361.0±33.4
	SM25	9.00	296.7±6.7
HEC	HHX	1.25	185.3±4.2
	HX	2.00	142.0±3.0
	M	2.40	100.3±1.2
CMCNa	PR-S	10.0	140.7±13.3
CVP	NF980	1.75	125.3±12.7
Sodium alginate	—	5.00	80.0±1.0
PAA-Na	—	1.15	986.3±116.5

a) $n=3$; S.D.: standard deviation for three experiments.

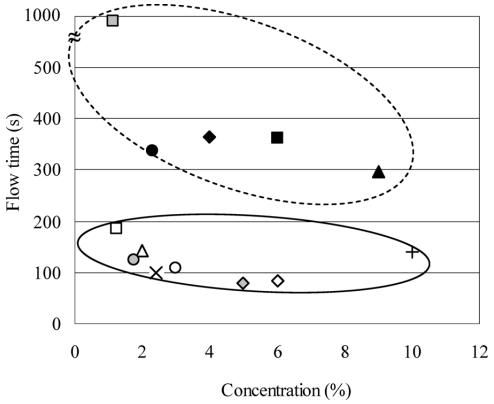

Fig. 3. Correlation Diagram between Concentration and Flow Time

Thickener Type: (○): HPC(H), (◇): HPC(M), (□): HEC(HHX), (△): HEC(HX), (×): HEC(M), (●): MC(SM8000), (◆): MC(SM1500), (■): MC(SM100), (▲): MC(SM25), (+): CMCNa, (●): CVP, (◆): Sodium Alginate, (■): PAA-Na. Continuous circle shows a group of thickeners with optimum flow properties. Dotted circle shows a group of thickeners with low flow properties. Each point is the mean value of 3 experiments.

MC, easily forms the cross-linking structure.[16] MC is completely dissolved in water at low temperature,[17] but in this study, MC got slightly-turbid and might start to gel at room temperature, about 25 °C, by forming partial cross-linking, and this might cause the decrement of flow property. Thus, MC was considered to be unsuitable as a dental thickener because it would be difficult to follow the complex shapes of dental tissue and would take too long to dissolve the lyophilized product homogeneously using its solution. In addition, a 1.15% PAA-Na solution had a markedly long flow time of 986 s. PAA-Na also formed a three-dimensional network in water and gelled,[18] so it was judged to be unsuitable from the flow property standpoint. It was therefore established that thickeners should not form a three-dimensional network in water.

Extrusive Force of the Thickener When rhbFGF is applied to periodontal disease, the formulation is thought to be administered in small amounts (≤1 ml) using a syringe. In addition, dentists often bend anesthetic syringe needles to tailor them to the shape of the disease site. With formulations for periodontal disease, a thin needle should be selected so

that a dentist can bend it as required. In our previous study, we found that the diameter of dosing needle significantly affected extrusive force. It was also found that 10 to 20 N with 27 gauge needle was optimum for the formulation of periodontal disease from the result of force measurement and sensory evaluation.[12] So the extrusive force of various thickener solutions were measured using the 27 gauge needle thought to be use in clinical practice in this study.

The results of the extrusive force measurement of the selected thirteen thickener solutions are shown in Table 4. The relationship between extrusive force and concentration is shown in Fig. 4. The extrusive forces of the thickener solutions were positively correlated with their concentrations, regardless of the type and grade of thickeners. All samples of 3% HPC(H), 2.3% MC(SM8000) and 2.4% HEC(M) showed the optimum extrusive force (10—20 N),[12] and their concentrations were all in the range 2—3%. Meanwhile, CVP, HEC (HHX and HX) and PAA-Na showed lower forces of 10 N or less and their concentrations were all below 2%. To achieve sufficient force, an increment of the thickener was necessary and therefore increased viscosity and worsening flow properties were expected. Thus, these thickeners were considered to be unsuitable. In contrast, 6% HPC(M), 10% CMCNa, 4% MC(SM1500), 6% MC(SM100), 9% MC(SM25), and 5% sodium alginate showed higher forces of 20 N or more and their concentrations were all above 4%. In general, when a fluid passes through a tube, such as a syringe or needle, it forms a fluid film on the tube wall and resistance of the fluid to flow depends on the thickness of this fluid film. The thickness of a fluid film cannot be measured but it is considered to increase with the number of solute molecules in the fluid. This was thought to be why these thickener solutions showed higher forces; therefore, to achieve the optimum extrusive force of 10—20 N, it was thought that thickener solutions should be prepared in 2—3% concentration and should show the optimum viscosity.

Table 4. Extrusive Force of Various Thickener Solutions at Concentrations with about 1×10^4 mPa·s

Sample	Grade	Concentration (%)	Extrusive force (N) (mean±S.D.[a)])
HPC	H	3.00	131.1±0.7
	M	6.00	28.9±1.6
MC	SM8000	2.30	15.9±0.4
	SM1500	4.00	24.8±1.1
	SM100	6.00	Not measured (≥40 N)
	SM25	9.00	Not measured (≥40 N)
HEC	HHX	1.25	5.5±0.3
	HX	2.00	9.3±0.1
	M	2.40	12.2±0.6
CMCNa	PR-S	10.0	Not measured (≥40 N)
CVP	NF980	1.75	7.0±0.3
Sodium alginate	—	5.00	30.7±0.5
PAA-Na	—	1.15	7.1±0.3

a) $n=3$; S.D.: standard deviation for three experiments.

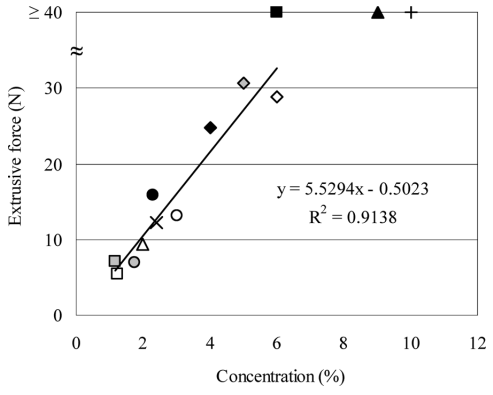

Fig. 4. Correlation Diagram between Concentration and Extrusive Force

Thickener Type: (○): HPC(H), (◇): HPC(M), (□): HEC(HHX), (△): HEC(HX), (×): HEC(M), (●): MC(SM8000), (◆): MC(SM1500), (■): MC(SM100), (▲): MC(SM25), (+): CMCNa, (●): CVP, (◆): Sodium Alginate, (■): PAA-Na. Each point is the mean value of 3 experiments.

Stability of rhbFGF in Thickener Solutions The rhbFGF formulation was designed as a lyophilized product dissolved just before use because of its instability in solution. The lyophilized product of rhbFGF was dissolved in each thickener solution and the stability of rhbFGF in the solution was evaluated. The initial purity measurement reflected the quality just after preparation and 6-h measurement reflected the quality of the formulation prepared in the morning and administered the same day. Twenty-four-hour measurement demonstrated the quality of the formulation administered a day after preparation.

The purity of rhbFGF remaining in the formulation was calculated based on the initial control (rhbFGF solution) value as 100% and rhbFGF in the thickener solution was judged stable if the purity decrement was within 5%. The results of the purity measurement of rhbFGF in the selected thickener solutions are shown in Fig. 5. The purity was more than 98% at all measuring points for 3% HPC(H), 6% HPC(M), 2.3% MC(SM8000), 1.25% HEC(HHX), 2% HEC(HX) and 2.4% HEC(M) formulations. The purity of rhbFGF in CVP formulation could not be measured because

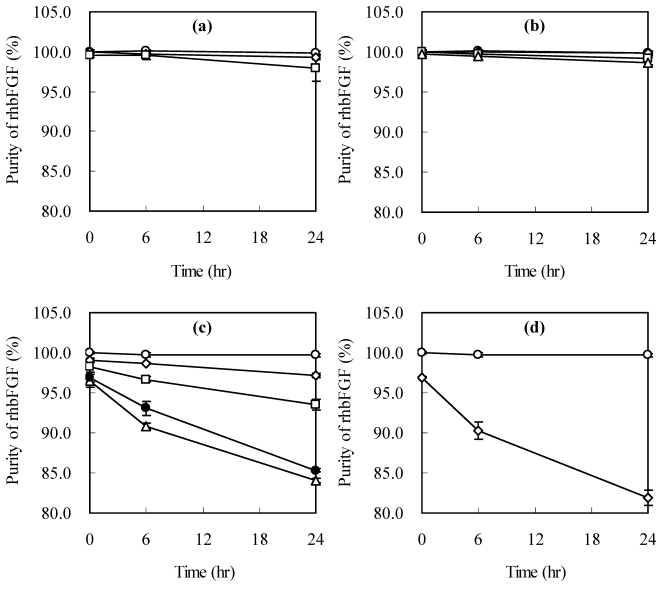

Fig. 5. Purity of rhbFGF Remaining in Formulations Stored for 24 h at 25 °C

(a) Purity of rhbFGF in (○) water (control), (◇) 3% HPC(H) and (□) 6% HPC(M). (b) Purity of rhbFGF in (○) water (control), (◇) 1.25% HEC(HHX), (□) 2% HEC(HX) and (△) 2.4% HEC(M). (c) Purity of rhbFGF in (○) water (control), (◇) 2.3% MC(SM8000), (□) 4% MC(SM1500), (△) 6% MC(SM100) and (●) 9% MC(SM25). (d) Purity of rhbFGF in (○) water and (◇) 10% CMCNa. Each point is the mean±S.D. of 3 experiments.

the sample was turbid. This was thought to be a denaturing of rhbFGF due to the acidic property of the CVP formulation, whose pH value was about 2 (not shown in this report). So, a re-test was conducted with neutralized CVP formulation. However, when CVP solution was mixed with lyophilized formulation including rhbFGF, the viscosity of CVP formulation was decreased rapidly. This phenomenon is likely to be caused by citric acid essential as a pH regulator included in the lyophilized formulation. In general, the viscosity of electrolyte-type polymer gels, such as CVP, is known to be decreased rapidly when salts are coexistent.[19] Therefore, CVP was considered to be unsuitable for the formulation of periodontal disease. In the case of sodium alginate formulation, the rhbFGF peak was not detected in the HPLC measurement. This was thought to be because rhbFGF was covered with an egg-box type cross-linking of sodium alginate gel formed by divalent metal ion in water, and sodium alginate is known to become a gel in the slight presence of divalent metal ion.[15] Or, it was considered that the positive charged part of rhbFGF might be strongly trapped in the structure of sodium alginate as well as metal ion. Sodium alginate was considered to be unsuitable because rhbFGF could not be released from the gel formulation after administration. The time-dependent purity decrease of rhbFGF was observed as 90% or less at 24-h measurement with 10% CMCNa, 4% MC(SM1500), 6% MC(SM100), and 9% MC(SM25) formulations. Each of these formulations had a relatively higher concentration than other cellulose derivatives and a concentration-dependent purity decrease was observed with the MC solution. On the other hand, PAA-Na solution was not measured because it became a gel and was judged to be unsuitable for HPLC measurement.

Thickeners with multiple grades, such as MC, HPC and HEC, showed a concentration-dependent purity decrease. In addition, thickeners with more than 6% concentration tend to affect the stability of rhbFGF. This suggested that although cellulose derivatives showed neutral pH and did not have strong ionic dissociable groups, the increment of the thickener per definite quantity of rhbFGF was unfavorable for the stability of rhbFGF.

When rhbFGF is applied to periodontal disease, the formulation should be prepared just before use. In clinical practice, such usage is basic, but sometimes the formulation is prepared the day before use. Thus, formulation stability for 24 h after preparation is important. Based on the stability of rhbFGF in thickener solutions, water-soluble cellulose derivatives, such as HPC, were considered to be useful. The cellulose derivatives selected in this work had no strong ionic dissociable groups that would form an ionic complex with bFGF and their solution had a neutral pH. These properties were thought to contribute to the stability of rhbFGF in thickener solutions.

As a result, HPC and HEC were found to be suitable thickeners for rhbFGF formulations applied to periodontal disease. It was also found that the concentration of thickeners should be low because higher thickener concentrations might affect the stability of rhbFGF.

Conclusion

To design a suitable periodontal drug formulation using rhbFGF, it is necessary to maintain rhbFGF stably and to administer it homogenously to complex and small volume disease sites without dripping.

In this work, suitable thickeners for rhbFGF formulations for periodontal disease were screened from the viewpoints of legality, formulation properties such as viscosity *etc.*, and inertness to rhbFGF. As a result, it was revealed how to select thickeners suitable for the formulation of periodontal disease. It was also found that there is an optimum concentration range for thickener solutions despite the thickener type and grade; they must be prepared at 2—3% with optimum viscosity (about 1×10^4mPa·s). In addition, several water-soluble cellulose derivatives, HPC and HEC in low concentration, were found to be inert to bFGF because of their structure, without strong ionic dissociable groups, and neutral pH. Thus, these cellulose derivatives were suggested to have required properties for a dental drug formulation including bFGF.

These results obtained from this study gave novel and useful findings for a formulation design of periodontal disease, such as the desired structure and the optimal concentration of thickeners.

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