Fundamental study of controlled-release effect of bioactive substances using gelatin scaffold for regenerative therapy

（再生医療におけるゼラチン足場材料を用いた
生理活性物質の徐放効果の基礎的研究）

The United Graduate School of Veterinary Science,
Yamaguchi University

Ryohei TAKAOKA

2010

Contents

General Introduction

Recently, regenerative medicine has received much attention. Regenerative medicine, which is term often used interchangeably with tissue engineering, merges the fields of life sciences and engineering, and aims to orchestrate body regeneration by specifically controlling the biological environment [1]. Many studies about regenerative medicine are based on the tissue engineering. It is difficult to completely distinguish between regenerative medicine and tissue engineering. Regeneration in the adult often represents a recapitulation of developmental processes, and strives to maintain and/or restore tissue integrity and functionality. Inspiration for regenerative medicine strategies commonly derives from our increasing understanding of how cells and biological systems decipher cues, and aims to replicate biological concepts and instructions expressed during embryonic development, including signal transduction pathways, transcription factor instructions and protein regulation. Tissue engineering is composed mainly of studies about scaffold, which cells attach. In addition, these scaffold sometime can include and release some growth factors [2,3], such as bone morphogenetic protein (BMP) -2 [4], transforming growth factor (TGF) -β [5], vascular endothelial growth factor (VEGF) [6], and basic fibroblast growth factor (bFGF) [7]. Growth factors play a crucial role in information transfer between cells and their microenvironment in tissue engineering and regeneration. They initiate their actions by binding to specific receptors on the surface of target cells and the chemical identity, concentration, duration, and context of these growth factors contain information that dictates cell fate. Hence, the importance of exogenous delivery of these molecules in tissue engineering

is unsurprising, considering their importance for tissue regeneration. However, the short half-lives of growth factors, their relatively large sizes, slow tissue penetration, and their potential toxicity at high systemic levels, suggest that conventional routes of administration are unlikely to be effective [8].

Naturally occurring materials such as silk, keratin, collagen, gelatin, fibrinogen, elastin, chitosan, hyaluronic acid, starch, carrageenan, cellulose and alginate have also gained wide attention as drug carriers. Gelatin is a denatured collagen and commercially available as a biodegradable polymer. It has been extensively utilized for pharmaceutical and medical purposes, and its bio-safety has been proven through the long clinical applications [9]. Other advantages of gelatin are the easiness of chemical modification and the commercial availability of samples with different physicochemical properties. It has been experimentally demonstrated from some researchers are promoted by the surface coating of gelatin [10-12].

One of the problems with the achievement of regeneration within an organism is a nutrient-supply from the vessels. Cells need to continuous supply of a nutrient and oxygen. The deficiency in nutrient-supply to cells will induce the apoptosis of cell.

Many papers have already reported about the neoangiogenesis. One of effective plans to induce neoangiogenesis is to use growth factors. Until now, vascular endothelial growth factor (VEGF) [13], platelet-derived growth factor (PDGF) [14] and basic fibroblast growth factor (bFGF) [15] is often used for growth factor to induce the neoangiogenesis. For giving the cells an environment suitable to their survival and functional achievement, biomaterials play a key

role in creating the environment for cells. The scaffold to promote the proliferation and differentiation is prepared from biomaterials, while the biomaterial is used as the delivery carrier of bio-signaling molecules as the cell nutrients to biologically activate cell. Combination with biomaterials enables an angiogenic factor to efficiently induce in vivo angiogenesis, which gives nutrients and oxygen to the transplanted cells. Biomaterials need to assist the approach of cell transplantation and enhance the therapeutic efficacy.

However, as far as we know, the biomaterials with ability of angiogenesis are not usually available in clinical field. The objective of chapter 1 was to combine biomaterial with a gelatin hydrogel incorporating growth factor aiming at the efficient induction of angiogenesis around the biomaterial.

Bone defects that are generated by tumor resection, trauma, and congenital abnormality have been clinically treated by the implantation of bioceramics or autogenous and allogenous bone grafts. Although autografting is a popular procedure for reconstructive surgery, it has several disadvantages, such as the shortage of donor supply, the persistence of pain, the nerve damage, fracture and cosmetic disability at the donor site. On the other hand, there are no donor site problems for allografting, while allografting has some clinical risks including disease transmission and immunological reaction [16]. As one trial to overcome the problems, bone tissue engineering has been attracted much attention as a new therapeutic technology which induces bone regeneration by making use of osteoinductive growth factors, osteogenic cells, and scaffolds or their combination [17].

Osteoinductive growth factors, such as BMP, TGF-β, and bFGF, have been investigated to

induce bone regeneration, have already been applied clinically for bone regeneration at the bone defect, because of their high osteoinduction activity [18]. However, the using of growth factors for bone regeneration, and the other purposes, has a problem in terms of cost. The cost of growth factors is usually expensive to use for medical products.

Lactoferrin (LF) is an iron-binding glycoprotein of transferring family. It is present in breast milk, especially colostrum (6-8 mg/ml) and in the secondary granules of neutrophils. It has been reported that LF has the bioactivity of the proliferation and differentiation of osteoblasts [19,20] and can inhibit the cell apotosis [21]. On the other hand, LF increases the calcification of extracellular matrix by human osteoblast-like cells [22], which experimentally a great potential of LF for bone regeneration.

Various materials have been investigated as the material for sustained release of growth factors [23]. Among them, gelatin is being used for the material of various growth factors release, such as basic fibroblast growth factor (bFGF) [24], bone morphogenetic protein 2 (BMP-2) [25], transform growth factor (TGF) β-1 [5] and vascular endothelial growth factor (VEGF) [6]. These growth factors are certainly hopefulness as the factor of bone regeneration. However, one of the biggest issues for using these growth factors is their cost. These growth factors are usually too expensive for using in usual clinical field.

In this study, we take notice LF, which we could get cheaper than above growth factors, for the factor of bone regeneration. Stallmann et al. reported that the continuous release of human LF from calcium phosphate bone substitutes [26], was effective in enhancing bone regeneration. However, few papers reported that the sustain release of LF. In addition, the

paper investigated effect of LF in terms of in vitro and vivo is very few.

The objective of chapter 2 was to evaluate the potential of LF to induce bone regeneration, when LF is applied in a sustained release fashion. A biodegradable gelatin hydrogel was prepared to allow LF to release in a sustained manner. After applied to the bone defect of rat skulls and rabbit ulnas, the bone regeneration by the hydrogel incorporating LF was assessed. In addition, we examined the in vitro proliferation of cells by addition of LF in a repeated fashion and compared with that of cells cultured LF added one time. The scaffold of sponge shape has been used for cell scaffold from the viewpoint of good nutrients and oxygen supply to cells and superior cell infiltration. However, generally a porous structure weakens the mechanical strength of scaffold. Therefore, the compression modulus of sponge scaffold is not enough strong for cell scaffold application. Three-dimensional biodegradable materials with a porous structure, such as glycolide-lactide copolymer non-woven fabrics, collagen sponges, and calcium phosphate ceramics, have been used [27], since it is preferable that the scaffolds for bone regeneration basically function as the substrate for the attachment and proliferation of osteogenic cells, Among them, hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) of bioactive ceramics have been extensively investigated as the cell scaffold for bone tissue engineering [27-37] because it is well recognized that they are compatible to natural bone tissue. However, since HAp is not practically degraded under the physical condition, it remains inside the bone tissue regenerated. Therefore, as one trial to improve the poor degradability in vivo, HAp has been attempted to mix with organic materials of collagen and glycolide-lactide copolymer. The combination was effective in manipulating the degradation and mechanical properties for the HAp scaffolds [38-43]. β-TCP is advantageous from the

viewpoint of materal biodegradability, though brittle compared with HAp. We examined the characteristics of gelatin sponges incorporating β-TCP prepared by different conditions.

As described above, gelatin is biodegradable and has been extensively used for food, pharmaceutical, and medical purposes and its biosafety has been proven through their long practical applications [9]. The objective of this study was to evaluate and follow after the convenience of gelatin scaffold for tissue engineering, by not only in vitro but also in vivo experiments.

Chapter 1

Vascularization around poly(tetrafluoroethylene) mesh with coating of gelatin hydrogel incorporating basic fibroblast growth factor

INTRODUCTION

All over the world, approximately 1 million surgeries are being performed per year [44]. The biomaterials routinely used most frequently is the repairing membrane of abdominal wall defects. The representative biomaterials for this purpose are polypropylene (PL) and poly(tetrafluoroethylene) (PTFE) meshes [45]. The role of mesh is to achieve the mechanical and physiological reinforcements of abdominal walls through the induction of fibrosis. The mesh usage often leads to clinical outcomes better than the surgical procedure performed without the mesh, including the lower recurrence rate and less postoperative pain [46-48]. The process of acute wound healing composes of complex, but well-orchestrated molecular and cellular events which initiate from hemostasis and inflammation and subsequently angiogenesis and fibroplasia to form a firm fibrous tissue that can resist the distractive force of abdominal walls [49].

It has been widely recognized that growth factors greatly contribute to tissue regeneration at different stages of cell proliferation and differentiation [50]. However, successful tissue regeneration induced by the growth factor has not been always achieved. One of the reasons is that the in vivo half-life period of growth factor is too short to expect the biological activities. It is necessary for their enhanced in vivo activity to develop the drug delivery system (DDS). We have explored biodegradable hydrogels of gelatin for growth factor release and succeeded in the growth factor-induced regeneration and repairing of tissues [7,51]. Among the hydrogel could release basic fibroblast growth factor (bFGF) to induce angiogenesis [52,53]. When a biomaterial is implanted into the tissue, the ischemic conditions around the biomaterial implanted often allow to delay the wound healing, which is one of the clinically large problems

to be resolved.

A poly(tetrafluoroethylene) (PTFE) mesh (Motif mesh®; Proxy Biomedical Ltd., Galway, Ireland) (MM) was developed for hernia repairing [54]. This mesh has a specific pore which gives the PTFE material homogeneous tensile strength [55]. It is demonstrated that the MM functions well as the patch material of soft tissue defects [54,55]. However, for the application to the ischemic tissue, there are some rooms in the material property to be improved.

The objective of this study was to combine the MM with a gelatin hydrogel incorporating bFGF aiming at the efficient induction of angiogenesis around the MM. The gelatin hydrogel was coated onto the surface of MM for the controlled release of bFGF. Following the subcutaneous implantation of MM coated with the hydrogel incorporating bFGF, the angiogenesis around the implanted site was evaluated. We performed the surface treatment of MM with corona discharge to facilitate the hydrogel coating. The profile of bFGF release from the hydrogel coated was investigated to compare with that of the MM without hydrogel coating.

MATERIALS AND METHODS

Materials

Gelatin sample with an isoelectic point (IEP) of 5.0, prepared through an alkaline process of bovine bone, was kindly supplied from Nitta Gelatin Co., Osaka, Japan. An aqueous solution of bFGF (IEP = 9.6, 10 mg/ml) was obtained by Kaken Pharma ceutical Co., Tokyo, Japan. The poly(tetrafluoroethylene) mesh (MotifMesh®; MM) was kindly supplied by Proxy Biomedical Ltd., Galway, Ireland.

Corona discharge treatment of MM and water contact angle measurement

The surface of MM was treated by corona discharge at 100 W for 30 sec on corona discharge device (TK-1; Iwatsu electric Co., Ltd., Tokyo, Japan.)

To evaluate change in the surface property of MM, the water contact angle was measured with a goniometer (contact angle meter CA-X, Kyowa Inter face Science, Saitama, Japan) according to the method previously reported [56]. Briefly, a water drop (9 μl) was dropped onto the surface of MM at room temperature and the water contact angle was measured independently 15 times for each MM to obtain the average value.

Coating of gelatin hydrogel onto the MM surface and surface observation

The coating of biodegradable hydrogel onto the MM was performed by the surface coating of gelatin solution, followed by the glutaraldehyde cross-linking of gelatin. Briefly, the MM was immersed into the mixed solution of gelatin (5 wt%) and glutaraldehyde (0.05 wt%), followed by leaving at 4 ℃ for 15 min to allow gelatin to cross-link. This procedure was repeated three times. Next, the MM coated with the hydrogel cross-linked was placed in 100 mmole/l glycine aqueous solution to chemically block the residual aldehyde groups of glutarardehyde, and then washed three times with double-distilled water (DDW). The MM coated with gelatin hydrogel prepared was freeze-dried and sterilized with ethylene oxide gas. When calculated as the weight percentage of water present in the hydrogel to the hydrogel in wet, the water content of gelatin hydrogel prepared was 95 %.

The surface of MM with or without the coating of gelatin hydrogel was observed on a

scanning electron microscope (SEM, S-2380N; Hitachi Ltd., Tokyo, Japan) at 2.5 or 3.0 KV of an accelerated voltage. The MM sample was coated with gold on an ion sputterer (E-1010; Hitachi Ltd., Tokyo, Japan) at 50 mtorr and 5 mA for 30 s to view.

Bonding strength measurement between the gelatin hydrogel and MM surface

Two MM samples (18 × 9 mm) with or without corona discharge were coated with the mixed solution of gelatin and glutaraldehyde, and immediately after that superposed in the half area to allow to crosslink gelatin for hydrogel formation. The sample was reacted with the glycine solution, washed, and freeze-dried. After swelling in phosphate-buffered saline solution (PBS, pH 7.4), the bonding strength between the two MM samples superposed was measured on an autograph machine (AGS-5D; Shimadzu Co., Kyoto, Japan) at 5 mm/min and room temperature to evaluate the hydrogel adhesion to the MM surface. The experiment was performed for three samples independently.

Evaluation of bFGF release from gelatin hydrogel coated on MM surface

bFGF was radioiodinated according to the method of Greenwood et al. [57]. To impregnate bFGF into the gelatin hydrogel coated on the MM, 20 μl of aqueous solution containing ^{125}I-labeled growth factor was dropped onto a freeze-dried gelatin hydrogel, and then left 37 ℃ for 1 hr to obtain the hydrogel incorporating ^{125}I-labeled bFGF. Since the volume of growth factor solution of growth factor was much smaller than that theoretically impregnated into the hydrogel, 100 % of growth factor added could be entirely incorporated into the hydrogel. The MM coated with gelatin hydrogel incorporating ^{125}I-labeled bFGF was

implanted into the back subcutis of 6-week-old female ddy mice (Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) at the central position 15 mm away from the tail root. As a control, 20 μl of aqueous solution of ^{125}I-labeled bFGF at the same dose was subcutaneously injected into the mouse back. Each experimental group was composed of four mice.

At different time intervals, the mice were sacrificed, and the back skin (3 × 5 cm^2) around the hydrogel implanted or injected site of growth factor was cut out and the corresponding facia was thoroughly wiped off with a filter paper to absorb the remaining ^{125}I-labeled bFGF. The radioactivity of remaining gelatin hydrogels, the skin piece, and the filter paper was measured on the gamma counter (ARC-301B, Aloka Co., Ltd., Japan). The percentage of remaining radioactivity was expressed as the radioactivity ratio of test samples to the original hydrogel or aqueous solution containing ^{125}I-labeled bFGF.

Quantitative and histological evaluation of angiogenesis

Gelatin hydrogel-coated MM samples incorporating bFGF were carefully implanted into the back subcutis of mice 15 mm apart from the tail root at the body center. The bFGF doses were 0, 1, 30, and 100 μg / mouse and four to eight mice were used for every experimental group. The mice were sacrificed at 7 and 21 days to evaluate angiogenesis in the mouse back subcutis on the basis of the weight of tissue hemoglobin [58,59]. Briefly, a fixed area of subcutaneous tissue around the implanted site of MM (2×2 cm^2) was cut off using a blade and immersed in 17 mM Tris-HCl buffer solution (pH 7.6) containing 0.75 wt% ammonium chloride for 24 hr at 4 ℃ to extract hemoglobin from the tissue. The extracted hemoglobin was quantitated using a hemoglobin assay kit (Wako Pure Chemicals Co., Ltd., Kyoto, Japan) based on a calibration

curve which had been prepared using standard hemoglobin solutions.

The histological section of tissue around the implanted site of MM with or without the gelatin hydrogel coating and bFGF incorporation was viewed after the staining with hematoxylin and eosin.

Statistical analysis

All the data were statistically analyzed by Fisher's LSD test for multiple comparisons and statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the mean ± the standard derivation of the mean.

RESULTS

Characterization of MM surface and gelatin hydrogel coated

Figure 1 shows the water contact angle of MM surface with or without corona discharged. The water contact angle of MM surface was significantly decreased by the corona discharge.

Figure 2 shows the SEM photographs of MM samples with or without the coating of gelatin hydrogel. From the SEM observation, it is apparent that the gelatin hydrogel was coated on the surface of MM at 50 μm thickness.

Figure 3 shows the bonding strength between the two MM samples superposed. The bonding strength between the two gelatin hydrogel-coated MM with or without corona treatment were similar although it tended to be larger for the corona-discharged sample.

bFGF absorption for MM samples with or without hydrogel coating

Figure 4 shows the remaining radioactivity of ^{125}I-radiolabeled bFGF on the MM surface with or without corona discharge and gelatin hydrogel coating. For the MM samples with hydrogel coating, the amount of ^{125}I-radiolabeled bFGF absorbed was significantly high compared with that of samples without hydrogel coating. The amount was similar to that of gelatin hydrogel itself. However, no difference in the amount of bFGF absorbed was observed between the hydrogel-coated MM samples corona discharged and non-discharged.

Time profiles of bFGF release from gelatin hydrogel coated on the MM surface

Figure 5 shows the in vivo time profiles of bFGF release from gelatin hydrogel coated on the MM surface. The radioactivity of ^{125}I-labeled bFGF incorporated in the gelatin hydrogel coated on the MM decreased more slowly than that of ^{125}I-labeled bFGF on the hydrogel-free non-coated MM with or without corona discharge.

Assessment of angiogenesis

Figure 6 shows the hemoglobin amount of tissue 7 days after subcutaneous implantation of different MM samples coated with or without bFGF incorporation.

When corona discharged MM samples with gelatin hydrogel incorporating bFGF were implanted into the back subcutis of mice, the amount of hemoglobin was significantly high compared with that of other groups. On the other hand, the hemoglobin increase was not induced by the injection of bFGF solution. No significant difference in the amount of hemoglobin was observed among the hydrogel-free MM samples discharged and non-discharged.

Figure 7 shows the effect of bFGF dose on the angiogenesis induced by MM samples coated with bFGF-incorporated gelatin hydrogel at 7 or 21 days after subcutaneous implantation. At the bFGF dose of 100 μg, significantly high amount of hemoglobin was observed for the MM samples coated with the hydrogel, irrespective of the corona discharge.

Histological observation

Figure 8 shows the histological section of tissue 7 days after subcutaneous implantation of different MM samples. Histological observation appearance greatly dependent on the MM samples implanted. The gelatin hydrogel incorporating bFGF coated on the surface of MM promoted the migration of cells and induced vascularizarion around the MM implanted, significant angiogesis was induced at bFGF dose of 30 and 100 μg. However, the vascularization was not induced on the original and corona discharged MM. The corona discharge treatment did not affect the migration of cells and vascularizaion.

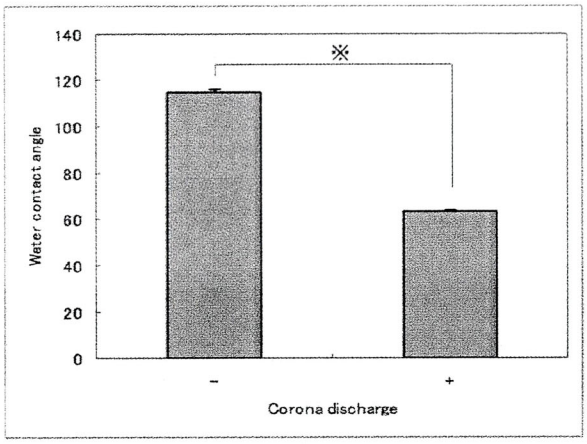


Figure 1. Change in the water contact angle of MM surface with (+) or without corona discharge (−).

※, $p<0.05$; significant between the two groups indicated.

Figure 2. Scanning electron micrographs of MM with or without coating of gelatin hydrogel: (A) the original MM or (B) and (C) gelatin-coated MM ((B) surface and (C) cross-section views)

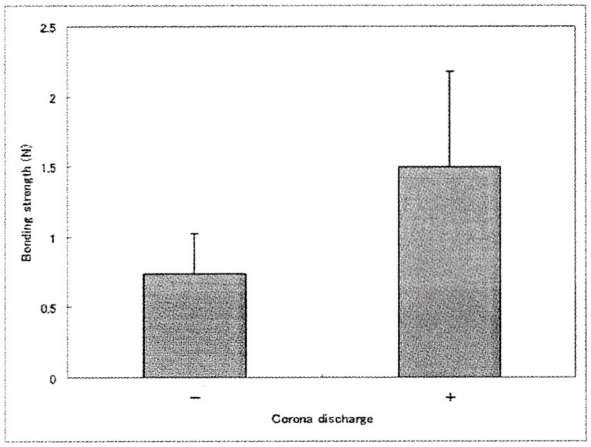


Figure 3. Bonding strength between the gelatin hydrogel coated and the surface of MM with (+) or without corona discharge (−).

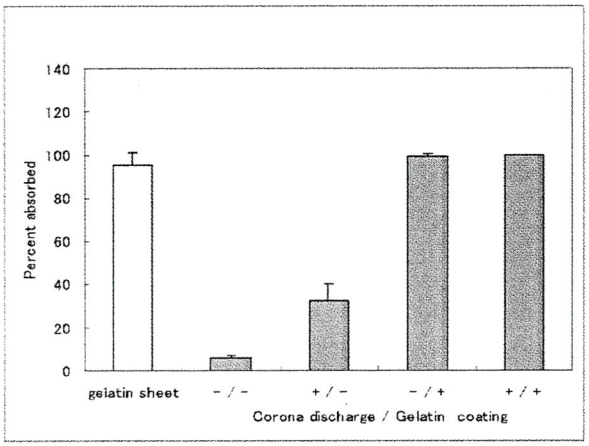


Figure 4. bFGF absorption onto the MM surface with (+) or without corona discharge (−). The MM was not coated (−) or coated with gelatin hydrogel(+). The percent absorbed is indicated as the amount of bFGF added initially of 100%.

※, $p<0.05$; significant between the two groups indicated.

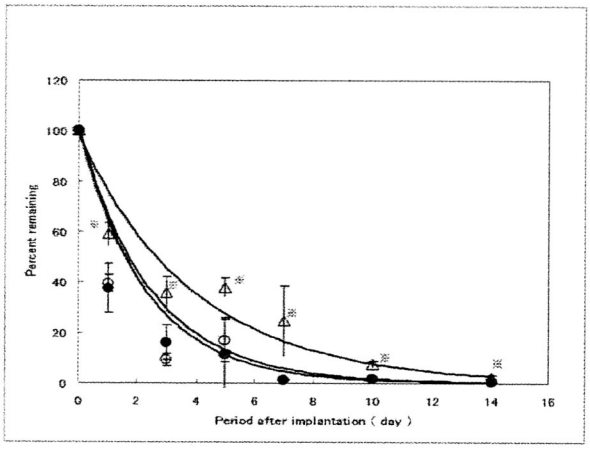


Figure 5. Time profiles of radioactivity remaining after subcutaneous implantation of different MM samples incorporating with ^{125}I-labeled bFGF. The MM was corona discharged and absorbed with ^{125}I-labeled bFGF solution (○) while it without corona discharge was absorbed (●). The MM was corona discharged and coated with the gelatin hydrogel, followed by the incorporation of ^{125}I-labeled bFGF solution (△).

※, indicates significance at $p < 0.05$ against the value of non corona discharged mesh on the coreesponding day.

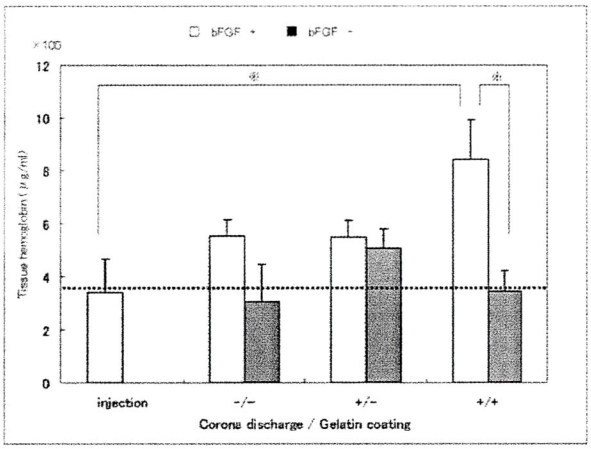


Figure 6. Hemoglobin amount of tissue 7 days after subcutaneous implantation of different MM samples with (□) or without bFGF incorporation (■). The MM was corona discharged and absorbed with bFGF solution (+/−) while it was absorbed without corona discharge (—/—). The MM was corona discharged and coated with gelatin hydrogel, followed by the incorporation of bFGF solution (+/+). As a control, bFGF solution was injected subcutaneous (injection). The bFGF dose is 100 μg / mouse. The dotted line indicates the natural amount of tissue hemoglobin.

※, $p<0.05$; significant between the two groups indicated.

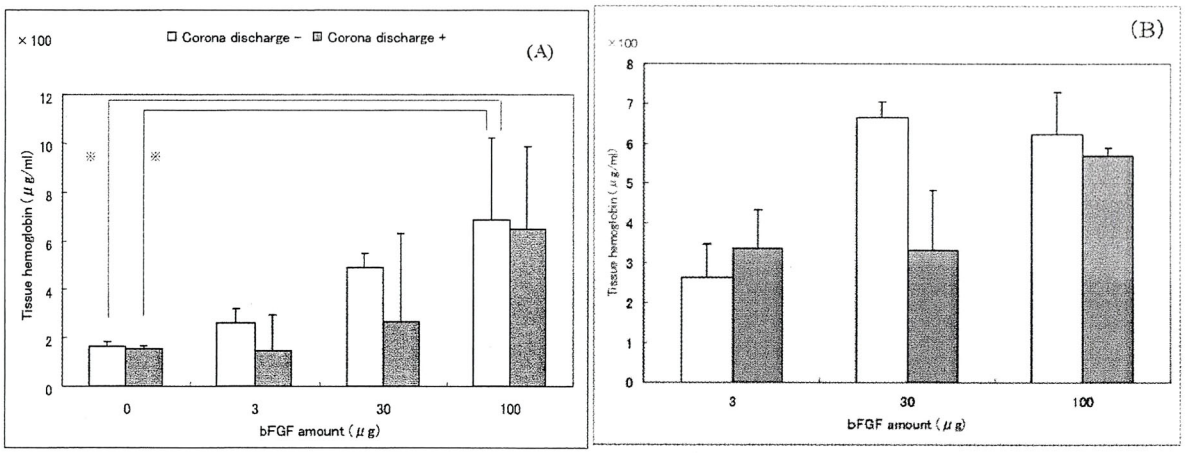


Figure 7. (A) Hemoglobin amount of tissue 7 days after subcutaneous implantation of gelatin hydrogel-coated MM samples with (■) or without corona discharge (□) incorporating 0, 3, 30, and 100 μg of bFGF.

※, $p<0.05$; significant between the two groups indicated.

Figure 7. (B) Hemoglobin amount of tissue 21 days after subcutaneous implantation of gelatin hydrogel-coated MM samples with (■) or without corona discharge (□) incorporating 3, 30, and 100 μg of bFGF.

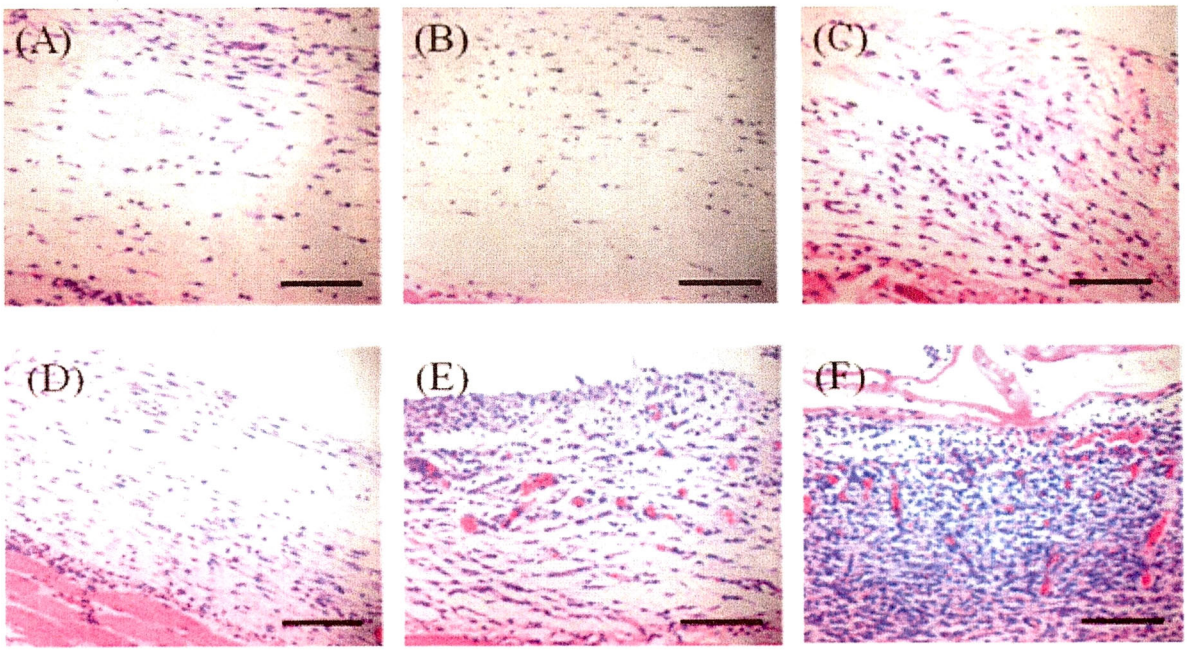


Figure 8. Histological sections of tissue 7 days after subcutaneous implantation of different MM samples incorporating bFGF: (A) the original MM, (B) the MM corona discharged, (C) the gelatin hydrogel-coated MM corona discharged, and gelatin hydrogel-coated MM corona discharged incorporating 1 (D), 30 (E) or 100 μg (F).

The bar length is 100 μm.

DISCUSSION

The objective of the present study was to provide the MM with the nature for the controlled release of bFGF to induce vascularization. The MM surface is so hydrophobic that cannot be readily coated with gelatin hydrogel of hydrophilicity. Therefore, the surface was treated by corona discharge to convert into hydrophilic. The decrease in the water contact angle of MM surface discharged implies the hydrophilic conversion of surface by the corona discharge. Higher bonding strength, though no significant difference, may be explained in terms of high miscibility between the gelatin hydrogel and the MM surface. It is possible that corona discharge makes the MM surface hydrophilic, resulting in the enhanced interaction between gelatin molecules and MM surface.

In this study, bFGF was selected as a growth factor to induce in vivo angiogenesis. It is reported that the production of bFGF in the drainage of wound fluid from patients promoted incisional hernia repairing [60]. From the implantation experiment, there was no significant difference in the amount of tissue hemoglobin between the corona-treated and non-corona treated MM. The amount of bFGF absorbed was significantly enhanced by the coating of gelatin hydrogel (Figure 4). Similar bFGF absorption was observed on the surface of MM with or without corona discharge treatment. The gelatin hydrogel would be coated strongly to function as the carrier of bFGF release. As expected, angiogenesis was significantly enhanced by the hydrogel coating at a bFGF of 100 μg.

As apparent from Figure 8, significantly higher number of blood vessels was observed for

the MM sample coated with with the hydrogel incorporating bFGF, in remarked contrast to other groups. The infiltration of inflammatory cells was sometimes observed. It has been demonstrated reported that inflammation was often important to recover from hernia [61]. The inflammatory fluid contains some growth factors and cytokines [60,62-66]. These factors may contribute to accelerate the natural repairing of hernia.

It has been demonstrated that bFGF release can be controlled by the biodegradation of hydrogel itself [58,67,68]. It is highly possible that the bFGF molecule, once ionically complexed with the acidic gelatin, is not released from the gelatin unless the hydrogel is degraded to generate water-soluble gelatin fragments. The initial bFGF release from the gelatin hydrogel can be explained in terms of bFGF diffusion. Because the present condition of bFGF impregnation was not sufficient to complete poly ion complexation between the bFGF and gelatin molecules, the uncomplexed bFGF would be diffused out initially [58]. The bFGF of 100 μg was enough to induce angiogenesis which is experimentally conformed by significant increase of hemoglobin amount. increase of hemoglobin amount. In case the mesh is used at an ischemic site, bFGF-induced angiogenesis must assist to repair hernia. Blood vessel can not only accelerate the wound healing, also enhance the recruitment of key cells to the wound site for tissue regeneration.

Chapter 2

Bone regeneration by lactoferrin released from gelatin hydrogel

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein of transferring family. It is present in breast milk, especially colostrum (6-8 mg/ml) and in the secondary granules of neutrophils. It has been reported that LF has several biological activities, such as the enhancement of cell proliferation and differentiation [19,69], and endothelial cells adhesion [70], anti-tumor [71], and the modulation of inflammatory responses [72]. In addition, it modifies the proliferation and differentiation of osteoblasts [19,20] and can inhibit the cell apotosis [21]. On the other hand, LF increases the calcification of extracellular matrix by human osteoblast-like cells [22], which experimentally a great potential of LF for bone regeneration.

It is widely recognized that growth factors play an important role in tissue regeneration at different stages of cell proliferation and differentiation [50]. However, successful tissue regeneration by the growth factor has not been always achieved. One of the reasons is that the in vivo half-life period of growth factor is too short to expect the biological activities. It is necessary for their enhanced in vivo activity to develop the drug delivery system (DDS) of growth factor. For example, it has been reported that the sustained release of bone morphogenetic protein-2 (BMP-2) enhances the activity to induce bone formation whereas the solution was not effective in vivo [73,74].

Various materials have been investigated as the material for sustained release of growth factors [23]. Among them, gelatin is being used for the material of various growth factors release, such as basic fibroblast growth factor (bFGF) [24], bone morphogenetic protein 2 (BMP-2) [25],

transform growth factor (TGF) β-1 [5] and vascular endothelial growth factor (VEGF) [6]. Gelatin has been widely used for pharmaceutical and medical applications to demonstrate the biocompatible and biodegradable natures on the basis of the long-term clinical trials. Stallmann et al. reported that the continuous release of human LF from calcium phosphate bone substitutes, was effective in enhancing bone regeneration [26].

The objective of this study was to evaluate the potential of LF to induce bone regeneration when LF is applied in a sustained release fashion. A biodegradable gelatin hydrogel was prepared to allow LF to release in a sustained manner. After applied to the bone defect of rat skulls, the bone regeneration by the hydrogel incorporating LF was assessed. We examined the in vitro proliferation of cells by addition of LF in a repeated fashion and compared with that of cells cultured LF added one time.

MATERIALS AND METHODS

Materials

A gelatin sample with an isoelectric point of 5.0 was kindly supplied by Nitta Gelatin (Osaka, Japan). Bovine LF (WAKO Pure Chemical Industries, Osaka, Japan, Lot No ALR2632.), glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification.

Preparation of gelatin hydrogels

Gelatin hydrogels were prepared by the chemical cross-linking of gelatin with GA. Briefly, 4.29 wt% aqueous solution of gelatin (70 ml) was mixed at 5,000 rpm at 37 °C for 3 min with a

homogenizer (ED-12, Nihonseiki Co., Tokyo, Japan). After the addition of 2.17 wt% GA aqueous solution (30 ml), the mixed solution was agitated for 15 second by the homogenizer. The resulting solution was cast into a polypropylene dish of 138×138 cm^2 and 5 mm depth, followed by leaving at 4 °C for 12 hr for gelatin cross-linking. Then, the cross-linked gelatin hydrogels were placed into 100 mM of aqueous glycine solution at 37 °C for 1 hr to block the residual aldehyde groups of GA. Following complete washing with double distilled water (DDW), the hydrogels were freeze-dried and cut into the disc in 8 mm diameter and 2 mm thickness for the following implantation experiments into the bone defect model in rats, or 5 × 5 × 20 mm into the bone defect model in rabbits.

Sustained release test of LF from gelatin hydrogels

LF was radioiodinated according to the method of Greenwood et al.[57]. Then, 20 μl of aqueous solution containing ^{125}I-labeled LF was dropped onto a freeze-dried gelatin (2 mg), and then left 37 ℃ for 1 hr to obtain the gelatin sponge incorporating ^{125}I-labeled LF. Since the volume of LF solution added was much smaller than that theoretically impregnated into the sponge, 100 % of LF added could be completely incorporated into the hydrogel. The gelatin hydrogel incorporating ^{125}I-labeled LF was implanted into the back subcutis of 6-week-old female DDY mice (Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) at the central position 15 mm away from the tail root. As a control, 20 μl of aqueous solution of ^{125}I-labeled LF at the same dose was subcutaneously injected into the mouse back. Each experimental group was composed of three mice. At different time intervals, the mice were sacrificed, and the back skin (3 × 5 cm^2) around the sample implanted or injected site of LF was cut out and

the corresponding facia was thoroughly wiped off with a filter paper to absorb the ^{125}I-labeled LF remaining. The radioactivity of remaining gelatin hydrogel, the skin piece, and the filter paper was measured on the gamma counter (ARC-301B, Aloka Co., Ltd., Japan). The percentage of remaining radioactivity was expressed as the radioactivity ratio of test samples to the original hydrogel or aqueous solution containing ^{125}I-labeled LF.

In addition above, we investigated the repercussions of different administration routes in terms of amount of LF. Injected LF as solution or implanted LF with hydrogels was estimated from remaining radioactivity one day after the application.

Cell culture experiment with LF

MC3T3-E1 cells (mouse derived osteoblasts) were seeded into each well of 24-well multi dish culture plate (Corning Inc., Corning, NY) at a cell density of 2×10^4 cells / well and cultured in 500 μ l of culture MEM medium with 10 wt% fetal bovine serum (FBS) for one day (day 0). After the incubation for one day, each well was washed twice with the phosphate-buffered saline solution (PBS, pH 7.4) twice. Then, the cells were cultured in FBS-free medium for 3 days. Fresh FBS-free medium (500 μ l/well) was changed every day, LF was added into the medium in one-time or repeated time fashion. For the group of one-time, single addition, the LF solution was added to each well at total concentrations from 0.15 to 1500 μ g/ml on day 1. On day 2 and 3, 500 μ l of PBS was added to each well. For the group of repeated addition, the LF solution was added 3 times to each well at the total concentrations from 0.05 to 500 μ g/ml every day for 3 days. As a control group, MC3T3-E1 cells were incubated in the medium containing 10 wt% FBS for 3 days, while the medium was changed every day by the PBS

addition at same amount of other groups.

MTT assay method

The cells were washed twice with PBS. The culture medium (500 μl) was added into each well. The cell proliferation was evaluated with MTT assay kit (Nacalai tesque Inc., Kyoto, Japan). Briefly, to each well, 50 μl of solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; WST-8) was added followed by incubation for futher 2 hr. The absorbance of cell supernatants was measured on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The number ratio of cell proliferated was expressed as 1.0 for the group where cells were cultured in the medium containing 10 wt% FBS.

DNA assay method

The number of proliferated cells was measured by a DNA assay method [57]. The cells were washed twice with PBS, underwent the conventional freeze-thaw process, and finally incubated in 1 ml of aqueous solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate for 1 hr to completely dissolve cells. The cell lysate (100 μl) was mixed with 100 μl of 1 μl/ml Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trihydrochloride dimethyl sulfoxide solution, Nacalai Tesque, Inc., Kyoto, Japan) containing 9.0 mg/ml NaCl and 4.4 mg/ml sodium citrate. The fluorescence intensity of mixed solution was measured on a fluorescence spectrophotometer (Spectra Max Gemini Em, Molecular Device Japan Co., Osaka, Japan) at exciting and emission

wavelengths of 355 and 460 nm. The cell number was determined by the calibration curve prepared from the fluorescent measurement for the known number of cells.

Bone regeneration assay of gelatin hydrogels incorporating LF

Rat model

LF solution at concentrations of 0, 1, 10, and 100 mg/ml (300 μ l), was dropped onto the disc of gelatin hydrogel freeze-dried, followed by leaving at 4 ℃ for overnight in dark to obtain the gelatin sponge incorporating 0, 0.3, 3, and 30 mg of LF. Fisher 344 rats (14-week-old, Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) were used divided into 4 groups (3 rats / group). Under anesthesia with a pentobarbital (40 mg/kg), a flap was raised and a bone defect of 8-mm diameter was created with a bone trephine bur. Then, the hydrogels incorporating LF were applied to the defect, and then the flap was repositioned and sutured. The rats were sacrificed 8 weeks after the hydrogel application. The calvaria were dissected out and examined with soft X-ray radiography (Hitex-100, Hitachi, Tokyo, Japan) at 20 kVP and 2.0 mA for 200 sec. X-ray radiographs around the bone defect (8 × 8 mm square) were mechanically changed to the binary images on a soft ware (Photoshop, Adobe Systems Incorporated, San Jose, CA, USA) at the region of interest of 8 × 8 mm square. Then, 3 binary images for each experimental group were analyzed to calculate the average percentage of ossified area ± the standard error.

Rabbit model

The implantation was preformed by a surgical procedure previously reported[75], with a slight modification. The site to be operated, either the left or right front limb of rabbits (New Zealand white rabbit, Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) was shaved, prepped, and draped for aseptic surgery in the supine position. A 2 cm-long superomedial incision was made and the tissue overlying the diaphysis of the radius was dissected. 2 cm segmental defect was prepared in the radius with a surgical oscillating saw supplemented by copious sterile saline water irrigation. Gelatin hydrogel incorporating LF (0.3, 3.0, 30 mg) and empty gelatin hydrogel were applied to defects, and some defects were left without any application. Fixation of the ostectomized bone was unnecessary because of the fibro-osseous union between the ulna and radius located distal and proximal to the surgical site. The soft tissue was approximated with interrupted 4-0 Vicryl (Ethicon, Someville, NJ) and the skin was closed with 3-0 silk sutures. A post operative antibiotic (fosfomycin) ([Fosmicin]; Meiji Seika, Tokyo, Japan) was administered intramuscularly at a dose of 100 mg/kg per day for 3 days. The radius-ulna complex containing the defect was then taken out and fixed in 10 w% formaldehyde solution in PBS for assessment of bone regeneration. Bone regeneration at the site of bone defect was assessed by soft X-ray 12 weeks after application. Soft X-ray photographs of bone specimens were taken with above soft X-ray system at 20 kVP and 2.0 mA for 240 s.

Preparation of gelatin sponges with β-TCP

Gelatin sponges incorporating β-TCP were prepared by chemical cross-linking of gelatin with glutaraldehyde in the presence of β-TCP granules (50 wt%). Briefly, 4.29 wt% aqueous

solution of gelatin at β-TCP (70 ml) was mixed at various rpm for different time at 37 ℃ (table 1) by using a homogenizer (ED-12, NIhonseiki Co., Tokyo, Japan). After addition of 2.17 wt% of glutaraldehyde aqueous solution (30ml), the mixed solution was further mixed 15 s with the homogenizer. The resulting solution was cast into a polypropylene dish of 138 × 138 cm^2 and 5mm depth, followed by leaving at 4 ℃ for 12 h for gelatin cross-linking. Then, the cross-linked gelatin hydrogels with β-TCP were placed into 100 mM of aqueous glycine solution at 37 ℃ for 1 h to block the residual aldehyde groups of glutaraldehyde. Following complete washing with double distilled water (DDW), the hydrogels were freezed at various conditions (table 1) and dried. Then, the freeze-dried gelatin hydrogels with β-TCP were cut into cubes of 5 × 5 × 5 mm^3.

Table 1. Prepared condition of gelatin sponges incorporating β-TCP

	Mixed revolution (rpm)	Cooling condition (℃)	Mixed time (min)
1	1,500, 5,000, 9,000	-80	3
2	5,000	-30, -80, LN	3
3	5,000	-80	1,3,30s

LN=liquid nitrogen

The resulting sponges were sputter-coated with gold / palladium and viewed both on a scanning electron microscope (SEM, S-2380N, HITACHI, Japan). The average pore size and porosity of sponges were measured by the methods reported previously [76,77].

Culture of cell under the gelatin sponge with or without β-TCP

MC3T3-E1 cells were seeded into each well of 96-well multi dish culture plate (Corning Inc., Corning, NY) at a cell density of 1×10^5 cells / well and cultured in 200 μl of culture MEM medium with 10 wt% fetal bovine serum (FBS) for 6 h. After the incubation for one day, each well was washed twice with the phosphate-buffered saline solution (PBS, pH 7.4) twice. Then, gelatin sponges with or without β-TCP sponge were placed onto each well. A fresh medium was changed every 3 days for cell proliferation without touch gelatin sponge.

Measurement of cell number and observation of gelatin sponge with β-TCP

The cultured sponges on the well plate were washed twice with PBS. The number of cells in washed sponges was measured by above DNA assay method.

The cultured sponges on the well plate were washed twice with PBS, and fixed into 0.2 wt% glutaraldehyde solution for 1 h at 4 ℃. The fixed sponges were washed twice with PBS, and reserved in 70 % ethanol solution until observation. The reserved sponges were freezed and dried before SEM observation. The resulting sponges were sputter-coated with gold / palladium and viewed above SEM system.

Statistic analysis

Statistical analysis was performed by the Tukey-Kramer method, and p value less than 0.05 was considered significant.

RESULTS

Release profile of LF from gelatin hydrogels incorporating LF

Figure 9a and 9b show the remaining ratio and amount of applied LF at the site of implantation after one day after the application. The applying efficiency of LF with hydrogel was dearly better than administration as solution.

Figure 9c shows the release profile of LF from gelatin hydrogels incorporating with ^{125}I-labeled LF after subcutaneous implantation. The remaining radioactivity of ^{125}I-labeled LF solution injected subcutaneously was 2.96 and 0.82 % on Day 1 and 3, respectively. On the contrary, the remaining radioactivity of ^{125}I-labeled LF in the hydrogel incorporated form was 10.14 and 5.26 % on 1 and 3 days. The remaining radioactivity of LF for a long time period was observed by the sustained release with the hydrogel.

In vitro cells proliferation

Figure 10 shows the mitochondrial metabolism activity of cells proliferated 1 and 3 days in the presence of LF in the one-time or repeated additional fashion. The mitochondrial metabolism activity of cells was measured by MTT assay method. The activity of cells on 3 days was lower than 1 day, because of culture without FBS. On 1 day, with an increase of LF concentration, the cell activity tended to promote. On 3 days, although cell activity of all groups was decreased, with an increase of LF concentration, the cell activity tended to keep the level of 1 day.

Figure 11 shows the mitochondrial metabolism activity of cells proliferated on 3 days in the presence of LF in the one-time or repeated additional fashion. With an increase of LF concentration, the cell activity was promoted. At higher concentrations of LF, the activity of

cells proliferated was significantly higher for the repeated additional group than that of single additional group.

Figure 12 shows the cell number cultured on 1 and 3 days in the presence of LF in the one-time or repeated additional fashion. The number of cells was measured by DNA assay method. With an increase of LF concentration, the cell number was increased, especially repeated additional group. In single additional group, there were no different in cell number between on 1 and 3 days. However, in repeated additional group, the number of cell cultured on 3 days with high dose of LF was increased compared with on 1 day, in the face of a culture without FBS.

Figure 13 shows the cell number on 3 days. After MC3T3-E1 cells was cultured under 10 % FBS media for 1 day, the cell number of initial attachment was 18,996 cells (dot-line). With the culture under 10 % FBS media for further 3 days, the cell number increased to 13 times its number (control). On the other hand, the cell proliferation was prevented by the culture of serum-free media. However the supply of 150 or 1500 μg/ml LF (both single and continuous) induced to significantly increase of cell number compared with initial attachment number.

In vivo bone regeneration by gelatin hydrogels incorporating LF

Figure 14 shows the soft X-ray radiography pictures of rat calvarial defect after implantation of gelatin hydrogels incorporating various amounts of LF. Implantation of LF-free gelatin hydrogels did not induce bone regeneration. On the other hands, bone regeneration at the defect site was observed by the implantation of gelatin hydrogels incorporating 30 mg of LF.

Figure 15 shows the soft X-ray radiography pictures of rabbit ulna defect after implantation

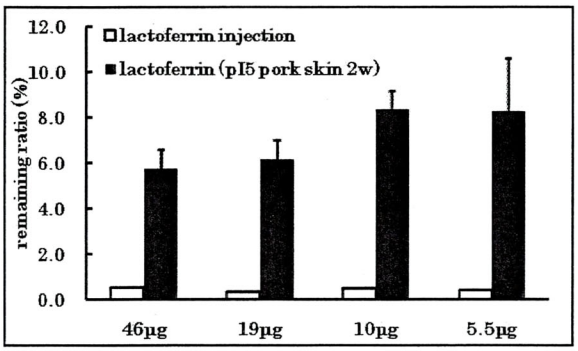
of gelatin hydrogels incorporating various amounts of LF. Sham operation and implantation of hydrogel incorporating 0.3 mg LF did not induce bone regeneration. On the other hands, bone regeneration at the defect site was observed by the implantation of gelatin hydrogels incorporating 3.0 and 30 mg of LF.

Pore size of gelatin sponge with β-TCP

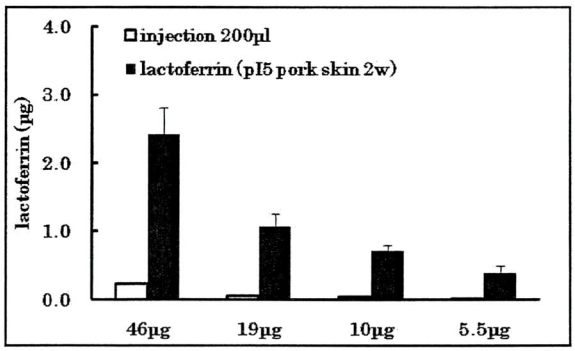
Figure 16 a, b, and c show mean pore size of gelatin sponges with β-TCP prepared by various conditions. Although mixed time did not affect their pore size, lower temperature was inclined to reduce the pore size.

During cell culture, the cells under the sponges infiltrated the sponges and produced extracellular matrix (ECM). Figure 17 shows the number of cells in the gelatin sponges with or without β-TCP cultured 7 and 14 days in vitro. In both sponges, the number of cells infiltrated into sponges at 14 days were almost twice compared with its of 7 days. The number of cells in the gelatin sponge with β-TCP was significantly larger than it in the sponge without β-TCP at 14 days. Figure 18 shows the surface, observed by SEM, of sponges infiltrated cells. Although, unfortunately, we did not determine quantity of ECM in the sponges, it seemed that the amount of ECM around sponge with β-TCP was more than it around sponge without β-TCP.

A



B



C

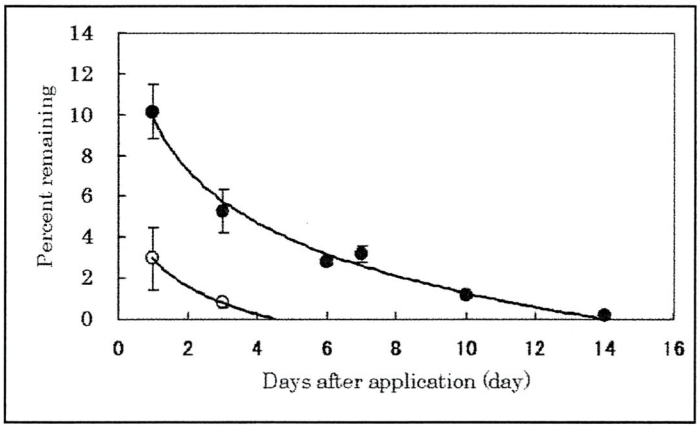


Figure 9.

a; Remaining ratio of LF, when infected as solution or implanted with gelatin hydrogels.

b; Remaining amount of LF, when injected as solution or implanted with gelatin hydrogels.

c; Time profiles of radioactivity remaining after the subcutaneous injection of ^{125}I-labeled LF solution (○) and implantation of gelatin hydrogel incorporating with ^{125}I-labeled LF (●).

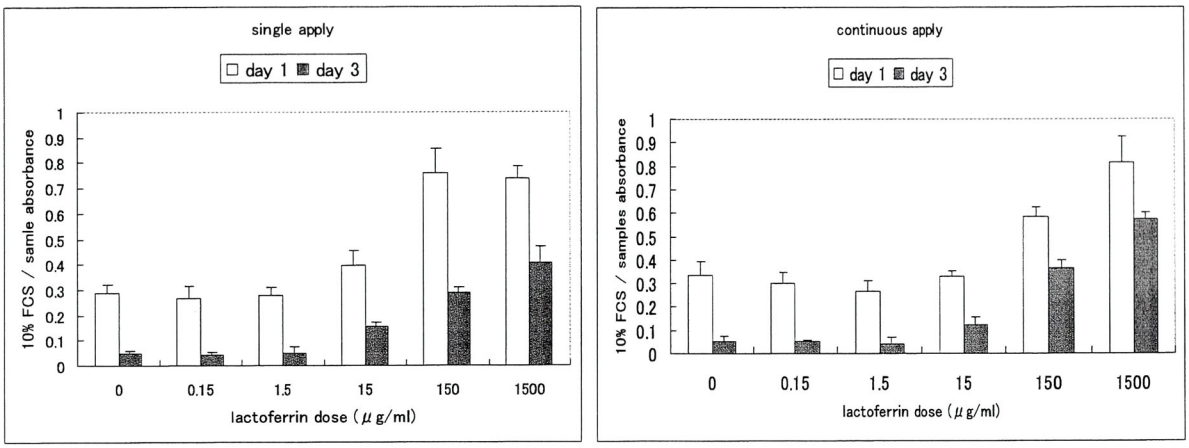


Figure 10. Time profile of mitochondrial metabolism activity of MC3T3-E1 cells in the presence of LF in the single (left) and 3-times addition of LF (right), evaluated by MTT assay on 1 and 3 days.

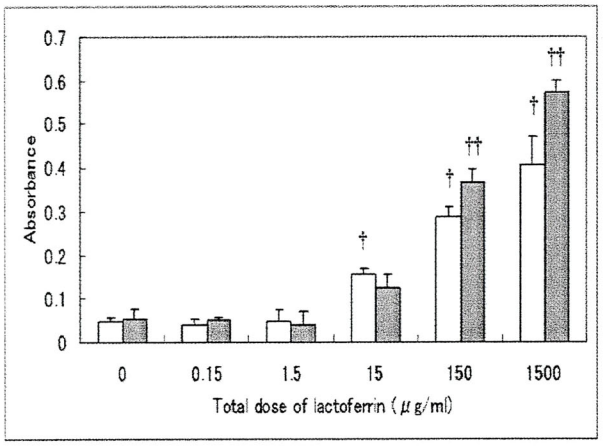


Figure 11. The mitochondrial metabolism activity of MC3T3-E1 cells in the presence of LF in the single (□) and 3-times addition of LF (■), evaluated by MTT assay on 3 days.

†, $p<0.05$; significant against the activity of cells cultured in the LF-free medium

††, $p<0.05$; significant against the activity of cells cultured on the single addition of LF into medium

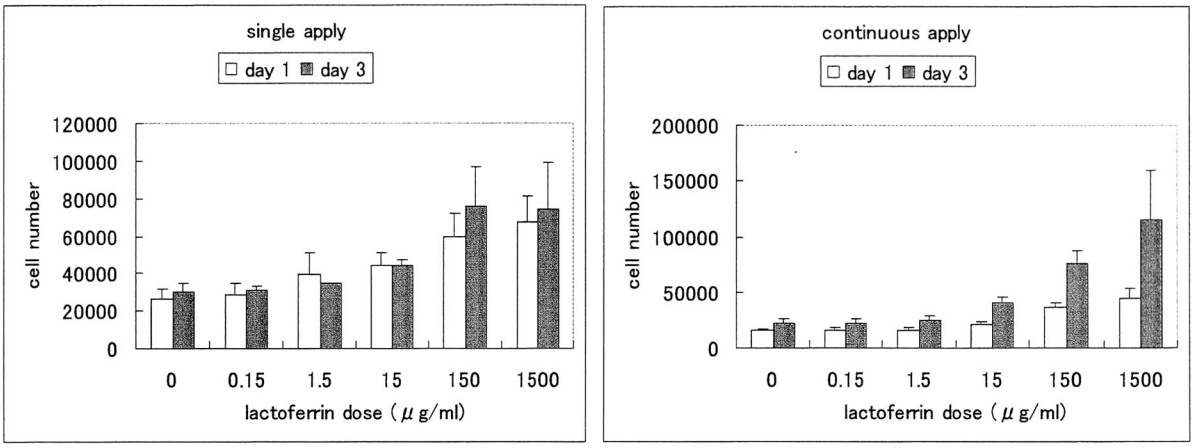


Figure 12. Time profile of MC3T3-E1 cells proliferation in the presence of LF in the single (left) and 3-times addition of LF (right), evaluated by DNA assay on 1 and 3 days.

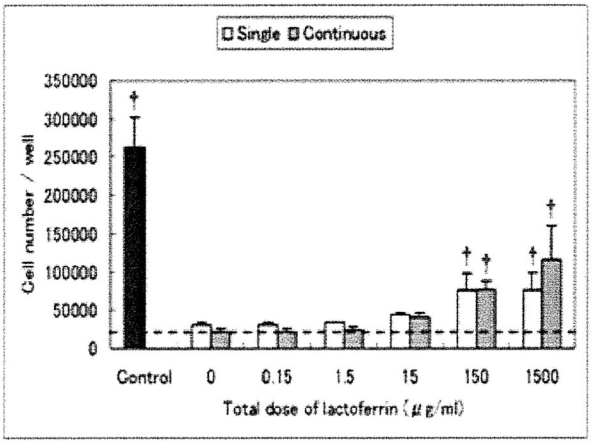


Figure 13. Figure 13 shows the effect of LF supply on the proliferation of MC3T3-E1 cells evaluated by DNA assay. MC3T3-E1 cells were incubated under 10 % fetal bovine serum (FBS) media. After MC3T3-E1 cells was cultured to induce initial cell attachment under 10 % FBS media for 1 day, then were incubated under a serum-free condition, except the control, with single or continuous supply of LF for 3 days. The dot line means the cell number of initial attachment.

†, $p<0.05$; significant against the number of initial attached cells cultured in the 10 % FBS on 1 day.

Amount of LF incorporated (mg)

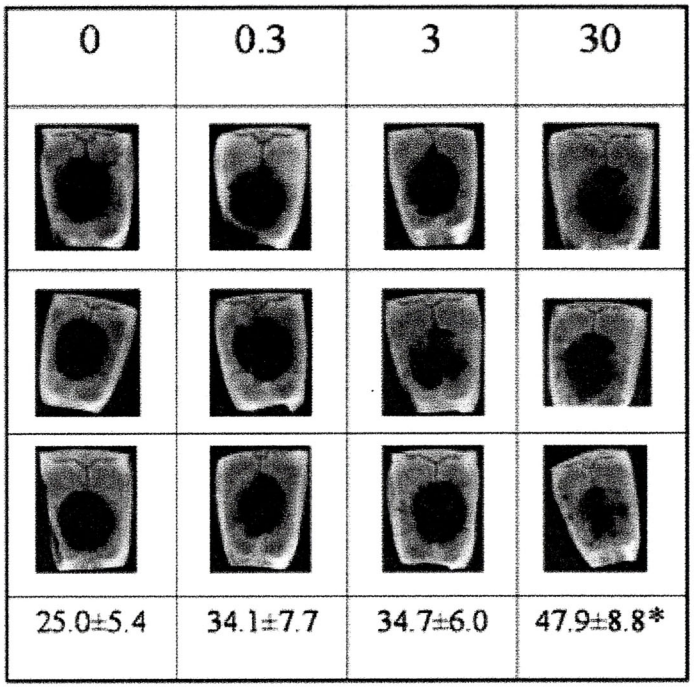


Figure 14. Soft X-ray radiographies of rat calvarial defect 8 weeks after implantation of gelatin hydrogels incorporating 0, 0.3, 3, and 30 mg LF. The lower stand value indicates the average percentage of area ossified in 8 × 8 mm square around bone defect. No statistical significant was observed between LF 0 and 0.3 or 3 mg group.

*, $p<0.05$; significant against the percentage of ossified area in square around defect implanted with gelatin hydrogels incorporating 0 mg of LF.

Amount of LF incorporated (mg)

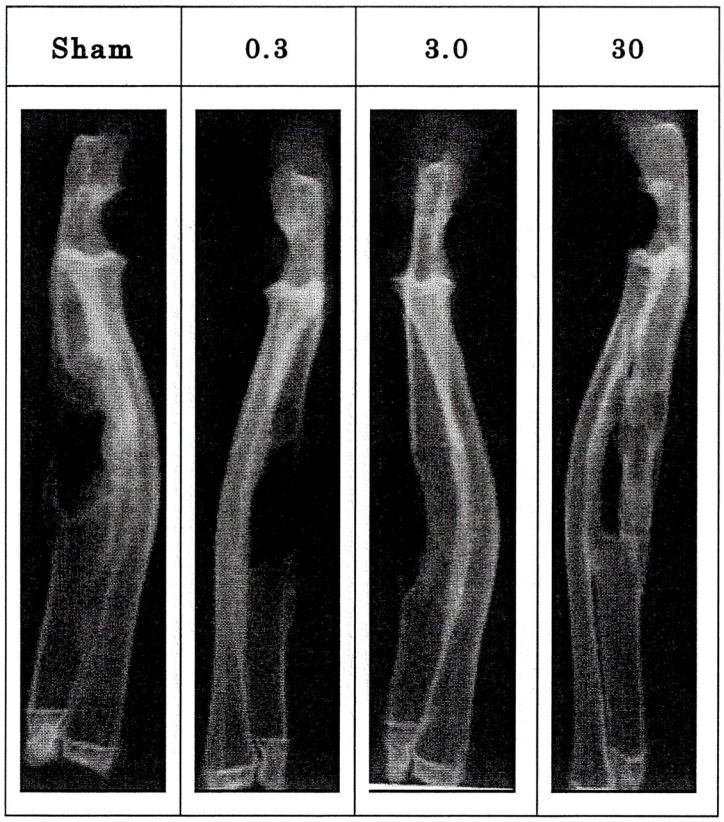


Figure 15. Soft X-ray radiographies of rabbit ulna defect 12 weeks after implantation of gelatin hydrogels incorporating 0.3, 3, and 30 mg LF and sham operation.

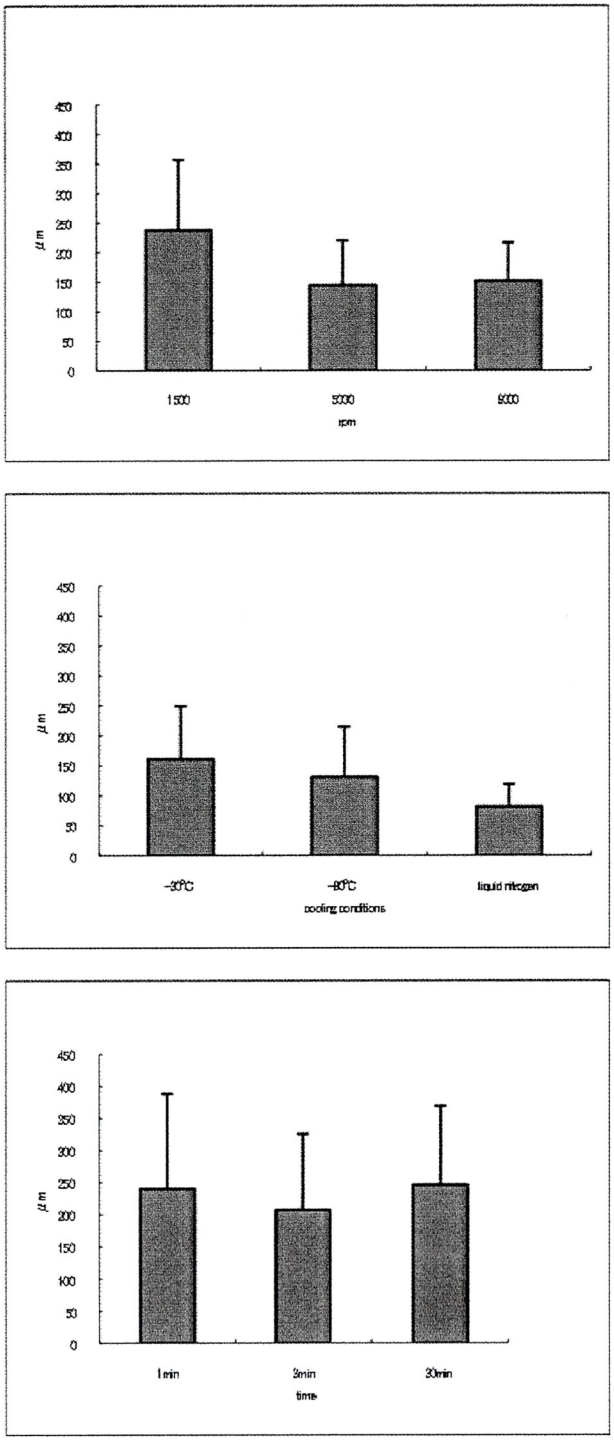


Figure 16. These figures show the pore size of gelatin sponges with β-TCP prepared by various conditions. The pore size of sponges was measured by observation of SEM pictures.

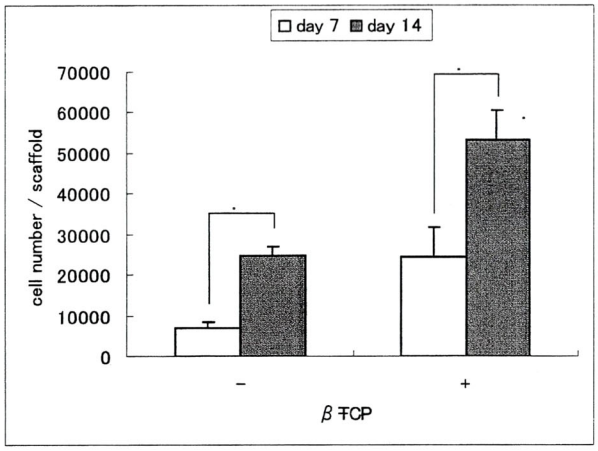


Figure 17. This figure shows the number of cells in the gelatin sponges with or without β-TCP cultured on the well plate seeded MC3T3-E1 cells for 7 and 14 days.

†, $p<0.05$; significant different between these groups

††, $p<0.05$; significant against the number of cells in the β-TCP (-) gelatin sponge.

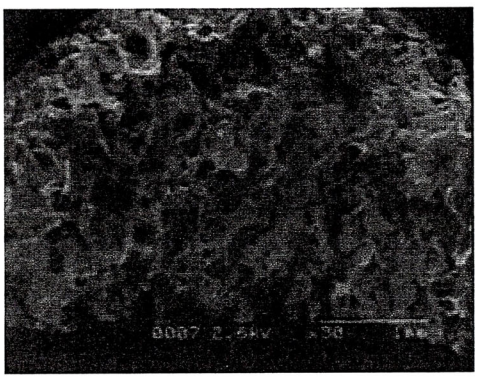
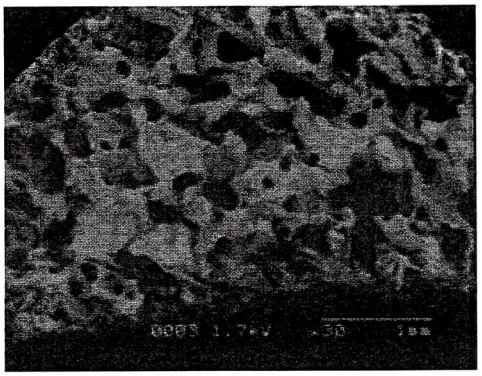
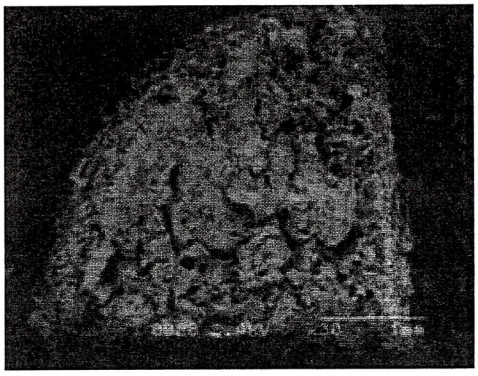
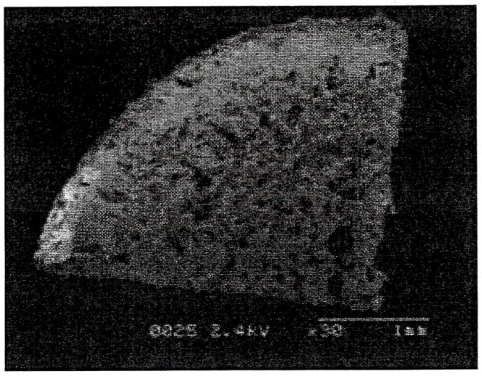
β-TCP	Day 7	Day 14
−		
+		

Figure 18. This gifure shows SEM pictures observed the surface of gelatin sponge with or without β-TCP cultured on the well plate seeded MC3T3-E1 cells for 7 and 14 days. Cells and ECM attached surface on the scaffolds. It seemed the amount of ECM around sponge with β-TCP was more than it around sponge without β-TCP.

DISCUSSIONS

The present study demonstrates that the sustained release with the gelatin hydrogel enabled LF to enhance the potential of bone regeneration. Stallman et al. reported that the continuous-release of human LF from calcium phosphate bone substitutes was effective in enhancing bone regeneration [26]. The release technology is necessary to enhance LF-induced bone regeneration.

It is well recognized in the polymer science that a positively or negatively charged polyelectrolyte ionically interacts with the oppositely charged one. Thus, it is highly possible that the LF molecule of a positive charge is not released from the acidic gelatin hydrogel (negative charge) unless the hydrogel is degraded to generate water-soluble fragments. On the other hand, LF will be diffused out of the basic gelatin hydrogel of positive charge since there is no electrostatic interaction between the LF and gelatin molecules. The initial release from the acidic gelatin hydrogel can be explained in terms of this LF diffusion. Because the present LF impregnation condition was not sufficient to complete poly-ion complexation between the LF and the acidic gelatin molecules, the uncomplexed LF would be diffused out initially. There have been reported on many carriers for the controlled release of proteins[4,6,73,78]. The profile of LF release from other carriers is under investigation at present to compare that of gelatin hydrogel.

We investigated the effect of LF additional fashion on the proliferation of cells. Repeated addition of LF was more effective in enhancing the cell proliferation than the single addition. The enhanced extent became high as the LF concentration increased. Cornish et al.

investigated that the effect of bovine LF on the proliferation of human osteoblasts to demonstrate the promoted proliferation of primary or cell line cultures of human or rat osteoblast-like cells in the dose-dependent manner (1-100 μg/ml). It is reported that in the healthy body, the serum level of LF ranges from 2 μg/ml to 7 μg/ml [79]. On the other hand, Lorget et al. have reported that bovine LF inhibited the in vitro bone resorption (200 μg/ml) in the in vitro culture of rabbit bone cells. Therefore, the concentration of LF to affect the cell behavior in vitro should be examined considering the type of cells. In this study, we did not check the time course of LF concentration in culture medium and around bone defect. These points should be checked in order to confirm the effective concentration of LF is released from the hydrogel.

It is apparent in Figure 2 that the repeated addition of LF effectively enhanced the in vitro proliferation of cells. This is just an in vitro experiment, but this clearly indicates that a continuous supply of LF to the cell culture system was effective in the enhancement of cells proliferation. Bone regeneration could be detected in a rat skull bone regeneration model 8 weeks and a rabbit ulna bone regeneration model 12 weeks after implantation. On the other hand, ^{125}I-labelled LF was not detected in the tissue around implanted hydrogel on 14 days after implantation. This time difference is often observed in the case of protein-induced bone regeneration. The protein is locally released for 14 days to enhance the number of key cells and activate their activity. Then, the cells initiate to work on the regeneration of bone tissues. It is possible that it needs some time period to achieve the cell-based bone regeneration. It is well known that LF interacts with the receptors of cells to exert their biological functions.

There is the cell surface receptor of LF for various types of cells, such as T cells [80], monocytes [81], liver cells [82], intestinal cells [83], platelets [84], and kidney, thyroid or parathyroid glands [85]. However, we did not check which cells were key cell affected by LF.

There are two important advantages to use gelatin hydrogels over the LF solution upon applying to the defect site. One of the advantages is the sustained release of LF which can be achieved with the gelatin hydrogel. The other is that the gelatin hydrogel could maintain the LF concentration at the bone defect site. The LF molecule of positive charge, once ionically complexed with the acidic gelatin of negative charge, is not released from the acidic gelatin hydrogel unless the hydrogel is degraded to generate water-soluble fragments. As one control experiment, the repeated addition of LF was effective in the enhancement of cell proliferation and activity in vitro. This indicates that continuous release enables LF to induce the biological activity. Taken together, we can say with certainly that the sustained release of LF promotes LF-based the ability of bone regeneration.

Although we should add the number of trials, especially in rabbit model, in vivo experiments (Figure 14, 15) revealed that the gelatin hydrogel functioned well to increase in the number of key cells, resulting in cell-based bone regeneration. Some papers demonstrate that bone regeneration was promoted by growth factors, especially bone morphogenetic proteins (BMPs) [4,25,86] and basic fibroblast growth factor (bFGF) [7]. We think that comparing with other growth factors, an important advantage to use LF is the low cost compared with other growth factors. Smith et al. reported that bone regeneration in a rabbit calvarial was induced by 288 μ

g/defect of BMP-2 with a collagen sponge [86]. Yamamoto et al. succeed in the dose reduction of BMP-2 dose (17 μg/defect) induce bone regeneration by using the gelatin hydrogel of release carrier [25]. Tabata et al. demonstrate bone regeneration in a rat calvarial defect by gelatin hydrogel incorporating 100 μg of bFGF [7]. Although it is reported that the dual release of growth factors could reduce their dose requested to induce bone regeneration [87]. However, from the view point of cost, it is practically difficult to say that the growth factors are available for therapy. In this study, we use a 30 mg of LF for bone regeneration in the rat calvarial defect. Mountziaris PM et al reported on the modulation of the inflammatory response for enhanced bone tissue regeneration [88]. An anti-inflammatory property is useful for bone regeneration since inflammation is mainly associated with a loss of bone mass. LF is reported to have an anti-inflammatory activity[72]. On the contrary, this property is not observed for BMPs and bFGF. It is highly conceivable that LF has an advantage over other growth factors in terms of anti-inflammation.

This study experimentally confirmed that the combination of LF and gelatin hydrogel was useful to induce bone regeneration. However, it is difficult to make a direct comparison in the bone regeneration between this and other papers because there are some differences in the animal species, the defect model, and the speed and degree of bone regeneration. In spite of this, we believe that the cost-benefit performance of LF is very practical compared with other growth factors which are widely used for the bone regeneration.

In previous experiment, we already checked the compression resistance of gelatin and gelatin incorporating β-TCP sponges in the freeze-dried state [74]. The compression modulus

of gelatin sponge was 0.27±0.01 MPa. On the contrary, its of gelatin sponges incorporating, 25, 50, 75 and 90 wt%, β-TCP were respectively 0.52±0.14, 1.13±0.13, 2.60±0.32 and 4.97 ±0.73. These results indicate that gelatin scaffold will be reinforced by the addition of microsphere. In this study, we prepared some gelatin sponges incorporating β-TCP with various conditions and evaluated their pore size. Although the mixed time did not affect the pore size of sponges, cooling temperature and mixed revolution affected. Cooling temperature affects the size of ice crystal, when freezed gelatin solution. The size of ice crystal is influenced by the cooling temperature. Because of the pore due to melting of ice crystal, we could change the pore size of gelatin scaffolds by cooling temperature. As it has been reported that the optimal pore size for attachment, differentiation and growth of osteoblasts and vascularization is approximately 300-400 μm [89], there must be the optimal pore size which have a significant influence on cell signal expression and differentiation. In addition, the change of pore size of scaffold means the change of superficial area of it. The superficial area of scaffold affects the degradation rate of it and the release profiles of growth factors. We should investigate the optimal design parameters, including porosity, pore size, interconnectivity, and mechanical properties, for cells. Furthermore, we must develop the process of manufacture to prepare the scaffold with optimal parameters. Biodegradable hydrogels containing microsphere subunits such as β-TCP provide new types of gelatin scaffold with stable and controlled degradability, which be able to incorporate growth factors. We aspect that it will be easy the characteristics of this gelatin scaffold with β-TCP to be modified to optimal specifications for tissue regeneration.

General Conclusion

In chapter 1, we tried to produce the biomaterial with angiogenesis ability using gelatin and bFGF. Although Motif Mesh has hydrophobic nature, corona discharge induced the reduced hydrophobicity (Figure 1). The gelatin could attach strongly the surface of MM by this artifice (Figure 3). The strongly attached gelatin was not easily diffused after the implantation. Significantly higher number of blood vessels was observed for the MM coated with the gelatin hydrogel incorporating bFGF, in remarked contrast to other groups (Figure 8). To use the gelatin and bFGF induced angiogenesis around the material, which even had hydrophobic nature. These results suggest that these artifices can be applied to the other biomaterials. Combination with biomaterials enables an angiogenic factor to efficiently induce in vivo angiogenesis.

In chapter 2, we tried to prepare the gelatin sponge which has an ability of sustain release of LF. In addition, we checked the bone regeneration by this gelatin sponge incorporating LF in rat and rabbit defect models. Injected LF back of mice was diffuse in a day. On the other hand, LF incorporated in gelatin sponge was sustained release during 12 days. In addition, the repeated addition of LF was effective in the enhancement of cell proliferation compared with the single addition in vitro. These results indicate that the bioactivity of sustain released LF is higher than it of injection in vivo. This assumption was supported by some results of in vivo experiment. The drug in liquid form is difficult just to keep at the defect site without diffusion. It is easy to imagine that to use of scaffold, which could incorporate liquid, has an advantage in terms of preventing diffusion. Although it needs some additional studies to control release

profile of LF from gelatin scaffold, we aspect that complex of gelatin scaffold and LF have good prospects as biomaterial for bone regeneration.

Furthermore, we investigated the characteristic of gelatin sponge incorporating β-TCP in chapter 2. The freezing temperature at the stage of fabrication had an influence on the pore size of gelatin sponge with β-TCP. On the contrary, the mixed time and revolution did not affect the pore size of it. In previous study, we already checked that the gelatin sponge with the addition of β-TCP was reinforced as compared with gelatin sponge without β-TCP. In this study, we checked that this reinforced gelatin sponge had a property to easy infiltration of cells (Figure 17 and 18). These results indicated that local environment, as enables cells to infiltrate and promote their proliferation and differentiation, was created by reinforced gelatin sponge incorporating β-TCP.

Tissue engineering is a newly emerging biomedical technology and methodology to assist and accelerate the regeneration based on the natural healing potentials of patients themselves. Biomaterial technology plays an important role in the creation of a local environment, which enhances and regulates cell proliferation and differentiation. To combine gelatin scaffold with bFGF and LF can induce the angiogenesis and bone regeneration. Furthermore, gelatin scaffold can be modified to suitable shape, hardness, and fabric for tissue regeneration. We aspect that gelatin scaffold will play an important role in tissue engineering field.

Abstract

The medical meshes have been used for the treatment of hernia. Mesh herniorrhaphy is the surgical intervention that has decreased recurrent incisional hernia formation. However, the incidence of recurrent incisional hernias remains unacceptably high even after mesh repair. For successful mesh herniorrhaphy, it is important to induce angiogenesis and fibroplasia around the mesh implanted site. The purpose of this study is to combine a mesh with a gelatin hydrogel for basic fibroblast growth factor (bFGF) release and evaluate the biological activity in vivo. The MotifMesh® (MM) of poly(tetrafluoroethylene) was treated with corona discharge to facilitate the coating of gelatin hydrogel. When implanted into the back subcutis of mice, the gelatin hydrogel-coated MM incorporating bFGF showed significant angiogenesis around the implanted site, in contrast to the MM without gelatin hydrogel or bFGF incorporation. It is concluded that coating of hydrogel incorporating bFGF is a promising technology to give the mesh an angiogenic property.

The objective of chapter 2 is to evaluate the potential of lactoferrin (LF), an iron-binding glycoprotein, to induce bone regeneration. A biodegradable hydrogel of gelatin was prepared to allow the LF to release in vivo in a sustained fashion. When subcutaneously implanted into the back of mice, the gelatin hydrogel incorporating LF showed a longer time period of LF retention at the site implanted than that of LF solution infection. An in vitro culture experiment of 3T3E1 cells (mouse derived osteoblasts) revealed that the cells were proliferated by the repeated addition of LF to a significantly great extent compared with the

single addition of LF at the same dose. Following the implantation of gelatin hydrogels incorporating LF into a skull bone defect of rats, significantly stronger bone regeneration at the defect was observed than LF-free or -low. It is concluded that the sustained release with the gelatin hydrogels enables LF to enhance the in vivo activity of bone regeneration. Furthermore, we investigated the influences of manufacture condition toward pore size in gelatin sponge incorporating β-TCP. Although mixed time did not affect their pore size, lower temperature was inclined to reduce the pore size. In addition, the reinforced gelatin sponges with β-TCP showed higher cell infiltration compared with gelatin sponges. We aspect that gelatin scaffold will play an important role in tissue engineering field, because gelatin scaffold can include growth factors and be modified to suitable shape, hardness, and fabric for tissue regeneration.

Acknowledgements

I would like to thank my doctoral course supervisor Professor Dr. Yoshiaki Hikasa (Tottori University) for his guidance and support throughout my graduate student years. He is a talented and brilliant scientist and a great mentor, from whom I have learned a great deal. His enthusiasm and passion for science is contagious and the freedom that he gives his students to explore ne ideas is remarkable. I am grateful to Professor Dr. Takashi Takeuchi (Tottori University) and Professor Dr. Yamamoto (Yamaguchi University) for their guidance as assistant supervisors during my study.

Special thanks to Professor Dr. Yasuhiko Tabata (Institute for Frontier Medical Sciences, Kyoto University) for his kind and useful advices during my study. He is well known around the world as the researcher of tissue engineering. He gave me, who was newcomer about this field, many precise and significant advices during my study.

I feel especially grateful to all students of Institute for Frontier Medical Sciences, Kyoto University including Tomoaki Takamoto, Yu Kimura, Jo Junichiro, Inoue Sachiko, Kentaro Hayashi, and Kazunari Asano for their indispensable technical assistance and acquaintance during my experiments. It is no doubt that my research work could not have been completed without their cooperation. And I feel grateful to my family for their cooperation. At the last, during my study, a lot of animals, mice, rats and rabbits, were sacrificed for some experiments. I should pray for the victims of their experiments.

References

1. Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface. Aug 18.

2. Tabata Y. Biomaterial technology for tissue engineering applications. J R Soc Interface. 2009 Jun 6;6 Suppl 3:S311-24.

3. Ikada Y, Tabata Y. Protein release from gelatin matrices. Adv Drug Deliv Rev. 1998 May 4;31(3):287-301.

4. Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev. 2003 Nov 28;55(12):1613-29.

5. Fan H, Hu Y, Qin L, Li X, Wu H, Lv R. Porous gelatin-chondroitin-hyaluronate tri-copolymer scaffold containing microspheres loaded with TGF-beta1 induces differentiation of mesenchymal stem cells in vivo for enhancing cartilage repair. J Biomed Mater Res A. 2006 Jun 15;77(4):785-94.

6. Patel ZS, Ueda H, Yamamoto M, Tabata Y, Mikos AG. In vitro and in vivo release of vascular endothelial growth factor from gelatin microparticles and biodegradable composite scaffolds. Pharm Res. 2008 Oct;25(10):2370-8.

7. Tabata Y, Yamada K, Miyamoto S, et al. Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels. Biomaterials. 1998 Apr-May;19(7-9):807-15.

8. Tayalia P, Mooney DJ. Controlled growth factor delivery for tissue engineering. Adv Mater.

2009 Sep 4;21(32-33):3269-85.

9. Zekorn D. Intravascular retention, dispersal, excretion and break-down of gelatin plasma substitutes. Bibl Haematol. 1969;33:131-40.

10. Bordenave L, Caix J, Basse-Cathalinat B, et al. Experimental evaluation of a gelatin-coated polyester graft used as an arterial substitute. Biomaterials. 1989 May;10(4):235-42.

11. Warocquier-Clerout R, Lee YS, Penhoat J, Sigot-Luizard MF. Comparative behaviour of L-929 fibroblastic and human endothelial cells onto crosslinked protein substrates. Cytotechnology. 1990 May;3(3):259-69.

12. Ma Z, Gao C, Gong Y, Ji J, Shen J. Immobilization of natural macromolecules on poly-L-lactic acid membrane surface in order to improve its cytocompatibility. J Biomed Mater Res. 2002;63(6):838-47.

13. Rajappa M, Saxena P, Kaur J. Ocular angiogenesis: mechanisms and recent advances in therapy. Adv Clin Chem.50:103-21.

14. Hellberg C, Ostman A, Heldin CH. PDGF and vessel maturation. Recent Results Cancer Res.180:103-14.

15. Chang JH, Han KY, Azar DT. Wound healing fibroblasts modulate corneal angiogenic privilege: interplay of basic fibroblast growth factor and matrix metalloproteinases in corneal angiogenesis. Jpn J Ophthalmol. May;54(3):199-205.

16. Langer R, Vacanti JP. Tissue engineering. Science. 1993 May 14;260(5110):920-6.

17. Petite H, Viateau V, Bensaid W, et al. Tissue-engineered bone regeneration. Nat Biotechnol. 2000 Sep;18(9):959-63.

18. Gittens SA, Uludag H. Growth factor delivery for bone tissue engineering. J Drug Target. 2001;9(6):407-29.

19. Cornish J, Callon KE, Naot D, et al. Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo. Endocrinology. 2004 Sep;145(9):4366-74.

20. Naot D, Grey A, Reid IR, Cornish J. Lactoferrin--a novel bone growth factor. Clin Med Res. 2005 May;3(2):93-101.

21. Grey A, Zhu Q, Watson M, Callon K, Cornish J. Lactoferrin potently inhibits osteoblast apoptosis, via an LRP1-independent pathway. Mol Cell Endocrinol. 2006 Jun 7;251(1-2):96-102.

22. Takayama Y, Mizumachi K. Effect of bovine lactoferrin on extracellular matrix calcification by human osteoblast-like cells. Biosci Biotechnol Biochem. 2008 Jan;72(1):226-30.

23. Devescovi V, Leonardi E, Ciapetti G, Cenni E. Growth factors in bone repair. Chir Organi Mov. 2008 Nov 29.

24. Layman H, Spiga MG, Brooks T, Pham S, Webster KA, Andreopoulos FM. The effect of the controlled release of basic fibroblast growth factor from ionic gelatin-based hydrogels on angiogenesis in a murine critical limb ischemic model. Biomaterials. 2007 Jun;28(16):2646-54.

25. Yamamoto M, Takahashi Y, Tabata Y. Enhanced bone regeneration at a segmental bone defect by controlled release of bone morphogenetic protein-2 from a biodegradable hydrogel. Tissue Eng. 2006 May;12(5):1305-11.

26. Stallmann HP, Faber C, Slotema ET, et al. Continuous-release or burst-release of the

antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes. J Antimicrob Chemother. 2003 Nov;52(5):853-5.

27. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater. 1991 Fall;2(3):187-208.

28. Yang Z, Yuan H, Tong W, Zou P, Chen W, Zhang X. Osteogenesis in extraskeletally implanted porous calcium phosphate ceramics: variability among different kinds of animals. Biomaterials. 1996 Nov;17(22):2131-7.

29. Knabe C, Driessens FC, Planell JA, et al. Evaluation of calcium phosphates and experimental calcium phosphate bone cements using osteogenic cultures. J Biomed Mater Res. 2000 Dec 5;52(3):498-508.

30. Yuan H, Li Y, de Bruijn JD, de Groot K, Zhang X. Tissue responses of calcium phosphate cement: a study in dogs. Biomaterials. 2000 Jun;21(12):1283-90.

31. Mankani MH, Kuznetsov SA, Fowler B, Kingman A, Robey PG. In vivo bone formation by human bone marrow stromal cells: effect of carrier particle size and shape. Biotechnol Bioeng. 2001 Jan 5;72(1):96-107.

32. Erbe EM, Marx JG, Clineff TD, Bellincampi LD. Potential of an ultraporous beta-tricalcium phosphate synthetic cancellous bone void filler and bone marrow aspirate composite graft. Eur Spine J. 2001 Oct;10 Suppl 2:S141-6.

33. Livingston T, Ducheyne P, Garino J. In vivo evaluation of a bioactive scaffold for bone tissue engineering. J Biomed Mater Res. 2002 Oct;62(1):1-13.

34. Boo JS, Yamada Y, Okazaki Y, et al. Tissue-engineered bone using mesenchymal stem cells and a biodegradable scaffold. J Craniofac Surg. 2002 Mar;13(2):231-9; discussion

40-3.

35. Kasten P, Luginbuhl R, van Griensven M, et al. Comparison of human bone marrow stromal cells seeded on calcium-deficient hydroxyapatite, beta-tricalcium phosphate and demineralized bone matrix. Biomaterials. 2003 Jul;24(15):2593-603.

36. Shimaoka H, Dohi Y, Ohgushi H, et al. Recombinant growth/differentiation factor-5 (GDF-5) stimulates osteogenic differentiation of marrow mesenchymal stem cells in porous hydroxyapatite ceramic. J Biomed Mater Res A. 2004 Jan 1;68(1):168-76.

37. Ruhe PQ, Kroese-Deutman HC, Wolke JG, Spauwen PH, Jansen JA. Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants in cranial defects in rabbits. Biomaterials. 2004 May;25(11):2123-32.

38. Ignatius AA, Ohnmacht M, Claes LE, Kreidler J, Palm F. A composite polymer/tricalcium phosphate membrane for guided bone regeneration in maxillofacial surgery. J Biomed Mater Res. 2001;58(5):564-9.

39. Laurencin CT, Attawia MA, Lu LQ, et al. Poly(lactide-co-glycolide)/hydroxyapatite delivery of BMP-2-producing cells: a regional gene therapy approach to bone regeneration. Biomaterials. 2001 Jun;22(11):1271-7.

40. Du C, Cui FZ, Zhu XD, de Groot K. Three-dimensional nano-HAp/collagen matrix loading with osteogenic cells in organ culture. J Biomed Mater Res. 1999 Mar 15;44(4):407-15.

41. Itoh S, Kikuchi M, Koyama Y, Takakuda K, Shinomiya K, Tanaka J. Development of an artificial vertebral body using a novel biomaterial, hydroxyapatite/collagen composite. Biomaterials. 2002 Oct;23(19):3919-26.

42. Rodrigues CV, Serricella P, Linhares AB, et al. Characterization of a bovine

collagen-hydroxyapatite composite scaffold for bone tissue engineering. Biomaterials. 2003 Dec;24(27):4987-97.

43. Lickorish D, Ramshaw JA, Werkmeister JA, Glattauer V, Howlett CR. Collagen-hydroxyapatite composite prepared by biomimetic process. J Biomed Mater Res A. 2004 Jan 1;68(1):19-27.

44. Schumpelick V, Klinge U, Welty G, Klosterhalfen B. [Meshes within the abdominal wall]. Chirurg. 1999 Aug;70(8):876-87.

45. Bellon JM, Bujan J, Contreras L, Hernando A. Integration of biomaterials implanted into abdominal wall: process of scar formation and macrophage response. Biomaterials. 1995 Mar;16(5):381-7.

46. Vrijland WW, van den Tol MP, Luijendijk RW, et al. Randomized clinical trial of non-mesh versus mesh repair of primary inguinal hernia. Br J Surg. 2002 Mar;89(3):293-7.

47. Melis P, van der Drift DG, Sybrandy R, Go PM. High recurrence rate 12 years after primary inguinal hernia repair. Eur J Surg. 2000 Apr;166(4):313-4.

48. McGillicuddy JE. Prospective randomized comparison of the Shouldice and Lichtenstein hernia repair procedures. Arch Surg. 1998 Sep;133(9):974-8.

49. Dubay DA, Wang X, Kuhn MA, Robson MC, Franz MG. The prevention of incisional hernia formation using a delayed-release polymer of basic fibroblast growth factor. Ann Surg. 2004 Jul;240(1):179-86.

50. Stocum DL. New tissues from old. Science. 1997 Apr 4;276(5309):15.

51. Taipale J, Keski-Oja J. Growth factors in the extracellular matrix. Faseb J. 1997 Jan;11(1):51-9.

52. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. Structural characterization and biological functions of fibroblast growth factor. Endocr Rev. 1987 May;8(2):95-114.

53. Rifkin DB, Moscatelli D. Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol. 1989 Jul;109(1):1-6.

54. Voskerician G, Gingras PH, Anderson JM. Macroporous condensed poly(tetrafluoroethylene). I. In vivo inflammatory response and healing characteristics. J Biomed Mater Res A. 2006 Feb;76(2):234-42.

55. Voskerician G, Rodriguez A, Gingras PH. Macroporous condensed poly(tetra fluoro-ethylene). II. In vivo effect on adhesion formation and tissue integration. J Biomed Mater Res A. 2007 Aug;82(2):426-35.

56. Hori Y, Inoue S, Hirano Y, Tabata Y. Effect of culture substrates and fibroblast growth factor addition on the proliferation and differentiation of rat bone marrow stromal cells. Tissue Eng. 2004 Jul-Aug;10(7-8):995-1005.

57. Greenwood FC, Hunter WM, Glover JS. The Preparation of I-131-Labelled Human Growth Hormone of High Specific Radioactivity. Biochem J. 1963 Oct;89:114-23.

58. Tabata Y, Ikada Y. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials. 1999 Nov;20(22):2169-75.

59. Tabata Y, Miyao M, Ozeki M, Ikada Y. Controlled release of vascular endothelial growth factor by use of collagen hydrogels. J Biomater Sci Polym Ed. 2000;11(9):915-30.

60. Di Vita G, Patti R, D'Agostino P, et al. Modifications in the production of cytokines and growth factors in drainage fluids following mesh implantation after incisional hernia

repair. Am J Surg. 2006 Jun;191(6):785-90.

61. Di Vita G, Milano S, Frazzetta M, et al. Tension-free hernia repair is associated with an increase in inflammatory response markers against the mesh. Am J Surg. 2000 Sep;180(3):203-7.

62. Holzheimer RG, Steinmetz W. Local and systemic concentrations of pro- and anti-inflammatory cytokines in human wounds. Eur J Med Res. 2000 Aug 18;5(8):347-55.

63. Chow LW, Loo WT, Yuen KY, Cheng C. The study of cytokine dynamics at the operation site after mastectomy. Wound Repair Regen. 2003 Sep-Oct;11(5):326-30.

64. Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol. 1998 Jun;152(6):1445-52.

65. Nissen NN, Polverini PJ, Gamelli RL, DiPietro LA. Basic fibroblast growth factor mediates angiogenic activity in early surgical wounds. Surgery. 1996 Apr;119(4):457-65.

66. Vogt PM, Lehnhardt M, Wagner D, Jansen V, Krieg M, Steinau HU. Determination of endogenous growth factors in human wound fluid: temporal presence and profiles of secretion. Plast Reconstr Surg. 1998 Jul;102(1):117-23.

67. Yamamoto M, Ikada Y, Tabata Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. J Biomater Sci Polym Ed. 2001;12(1):77-88.

68. Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. Tissue Eng. 1999 Apr;5(2):127-38.

69. Bi BY, Lefebvre AM, Dus D, Spik G, Mazurier J. Effect of lactoferrin on proliferation and differentiation of the Jurkat human lymphoblastic T cell line. Arch Immunol Ther Exp

(Warsz). 1997;45(4):315-20.

70. Baveye S, Elass E, Fernig DG, Blanquart C, Mazurier J, Legrand D. Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopolysaccharide complex. Infect Immun. 2000 Dec;68(12):6519-25.

71. Artym J. [Antitumor and chemopreventive activity of lactoferrin]. Postepy Hig Med Dosw (Online). 2006;60:352-69.

72. Conneely OM. Antiinflammatory activities of lactoferrin. J Am Coll Nutr. 2001 Oct;20(5 Suppl):389S-95S; discussion 96S-97S.

73. Igai H, Chang SS, Gotoh M, et al. Tracheal cartilage regeneration and new bone formation by slow release of bone morphogenetic protein (BMP)-2. Asaio J. 2008 Jan-Feb;54(1):104-8.

74. Takahashi Y, Yamamoto M, Tabata Y. Enhanced osteoinduction by controlled release of bone morphogenetic protein-2 from biodegradable sponge composed of gelatin and beta-tricalcium phosphate. Biomaterials. 2005 Aug;26(23):4856-65.

75. Hollinger JO, Schmitt JM, Buck DC, et al. Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. J Biomed Mater Res. 1998 Winter;43(4):356-64.

76. Chen G, Ushida T, Tateishi T. A biodegradable hybrid sponge nested with collagen microsponges. J Biomed Mater Res. 2000 Aug;51(2):273-9.

77. Matsuda K, Suzuki S, Isshiki N, Yoshioka K, Okada T, Ikada Y. Influence of glycosaminoglycans on the collagen sponge component of a bilayer artificial skin. Biomaterials. 1990 Jul;11(5):351-5.

78. Hokugo A, Sawada Y, Hokugo R, et al. Controlled release of platelet growth factors enhances bone regeneration at rabbit calvaria. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007 Jul;104(1):44-8.

79. Caccavo D, Sebastiani GD, Di Monaco C, et al. Increased levels of lactoferrin in synovial fluid but not in serum from patients with rheumatoid arthritis. Int J Clin Lab Res. 1999;29(1):30-5.

80. Bi BY, Leveugle B, Liu JL, et al. Immunolocalization of the lactotransferrin receptor on the human T lymphoblastic cell line Jurkat. Eur J Cell Biol. 1994 Oct;65(1):164-71.

81. Eda S, Beppu M, Yokoyama N, Kikugawa K. Novel lectin-like proteins on the surface of human monocytic leukemia cell line THP-1 cells that recognize oxidized cells. Arch Biochem Biophys. 2001 Jan 1;385(1):186-93.

82. Bennatt DJ, McAbee DD. Identification and isolation of a 45-kDa calcium-dependent lactoferrin receptor from rat hepatocytes. Biochemistry. 1997 Jul 8;36(27):8359-66.

83. Suzuki YA, Shin K, Lonnerdal B. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. Biochemistry. 2001 Dec 25;40(51):15771-9.

84. Leveugle B, Mazurier J, Legrand D, Mazurier C, Montreuil J, Spik G. Lactotransferrin binding to its platelet receptor inhibits platelet aggregation. Eur J Biochem. 1993 May 1;213(3):1205-11.

85. May P, Herz J, Bock HH. Molecular mechanisms of lipoprotein receptor signalling. Cell Mol Life Sci. 2005 Oct;62(19-20):2325-38.

86. Smith DM, Afifi AM, Cooper GM, Mooney MP, Marra KG, Losee JE. BMP-2-based repair of large-scale calvarial defects in an experimental model: regenerative surgery in

cranioplasty. J Craniofac Surg. 2008 Sep;19(5):1315-22.

87. Gomez G, Korkiakoski S, Gonzalez MM, et al. Effect of FGF and polylactide scaffolds on calvarial bone healing with growth factor on biodegradable polymer scaffolds. J Craniofac Surg. 2006 Sep;17(5):935-42.

88. Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue Eng Part B Rev. 2008 Jun;14(2):179-86.

89. Tsuruga E, Takita H, Itoh H, Wakisaka Y, Kuboki Y. Pore size of porous hydroxyapatite as the cell-substratum controls BMP-induced osteogenesis. J Biochem. 1997 Feb;121(2):317-24.