and in many other tissue defects. Accordingly, these systems will help scientists to unravel the vital interactions and signaling cascades of these complex molecules in tissue repair.

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INVITED REVIEW ARTICLE

# Experimental tissue regeneration by DDS technology of bio-signaling molecules

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**KEYWORDS**
Drug delivery system (DDS);
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**Summary** The medical therapy of tissue regeneration achieved by biomaterial-based tissue engineering has been currently expected as the third option following reconstructive surgery and organ transplantation. The basic idea of this regenerative therapy is to assist the self-healing potentials of body to induce the natural regeneration and repairing of defective or injured tissue. To this end, it is practically important to create a local environment which enables cells to promote their proliferation and differentiation, resulting in the induction of cell-based tissue regeneration. Tissue engineering is a biomedical technology or methodology to build up this regeneration environment by making use of biomaterials. Drug delivery system (DDS) is a biomaterial technology to enhance the *in vivo* biological functions of bio-signaling molecules (growth factors and genes) for promoted tissue regeneration. This paper overviews the recent status of tissue regeneration therapy based on the DDS technology of bio-signaling molecules.


**Contents**



## 1. Tissue engineering necessary for tissue regenation therapy

Advanced surgical therapies currently available, such as reconstructive surgery and organ transplantation,

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undoubtedly have saved and improved many patient lives, but have still clinical limitations. For the former therapy, medical devices cannot completely substitute the biological functions even for a single tissue or organ and consequently cannot prevent progressive deterioration of injured tissue and organ, either. For the latter, the shortage of donor tissues or organs is a big problem. In addition, the permanent medication of immunosuppressive agents often causes side-effects, while virus infection is not completely ruled out. In this circumstance, a trial of novel therapy has been explored, in which treatment of diseases can be achieved by utilizing the natural-healing process of body itself. To realize this therapy of regenerative medicine, it is necessary to provide cells a local environment suitable to their natural proliferation and differentiation. Tissue engineering is a biomedical engineering technology and methodology to build up the environment for tissue regeneration. The basic concept of tissue engineering was originally introduced by Langer and Vacanti [1,2]. And several technologies have been reported in this field up to now [3–10]. The basic therapeutic idea to induce regeneration of defective or lost tissues by making use of the tissue engineering technique is applicable in a surgical or physical manner. For the surgical tissue engineering, biomaterials with or without cells and/or bio-signaling molecules combination are surgically applied to a body tissue defect to induce tissue regeneration for disease therapy. On the other hand, drugs are physically applied to the fibrotic tissue of chronic diseases to digest or loosen overproduced collagen matrices and consequently the fibrotic tissue is repaired by the regeneration induction based on the natural healing potential of the surrounding tissue, which is defined as physical tissue engineering of internal medicine.

## 2. Fundamental technology and methodology of tissue engineering

There are four fundamental technologies or methodologies of tissue engineering for regeneration therapy. The first technology is to prepare an artificial scaffold of cells for proliferation and differentiation. It is well known that the extracellular matrix (ECM) is not only a physical support of cells but also provides a natural environment for cell proliferation and differentiation or morphogenesis which contributes organogenesis and tissue repair [11]. And if there is a large tissue defect, it is unlikely that the defect will be naturally regenerated and repaired only by supplying cells to the defect area. Therefore, preparation of artificial ECM scaffold is very important to regulate the tissue regeneration. For example, one promising way is to artificially build an environment for cells suitable to assist cell attachment initially and the subsequent proliferation and differentiation. If the scaffold of artificial ECM is biologically compatible, it is highly expected that cells residing around the scaffold implanted infiltrate into it and proliferate and differentiate therein.

The second technology of tissue engineering is drug delivery system (DDS) to enhance the *in vivo* activity of bio-signaling molecules (Fig. 1). The DDS technology facilitates the molecule administration

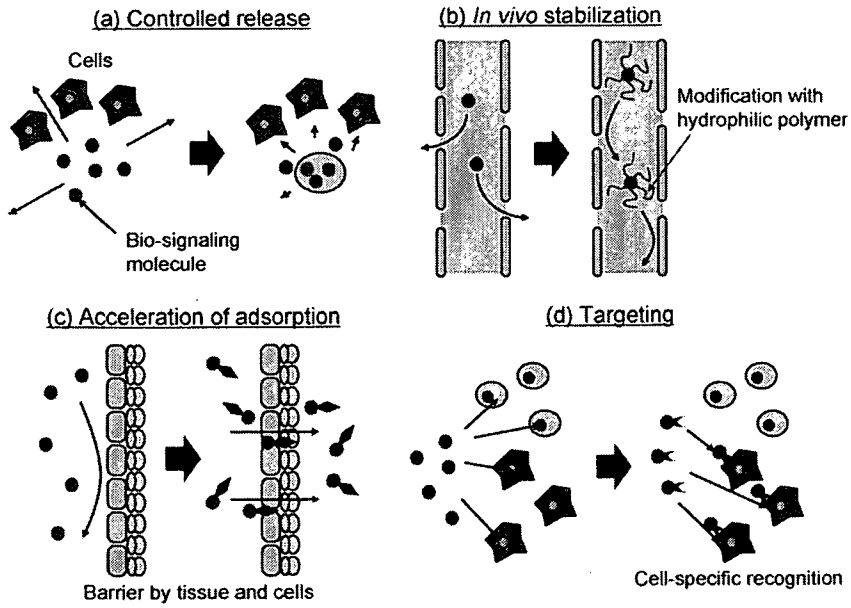


**Fig. 1** DDS technology with biomaterials for tissue engineering and regenerative medicine.

at the right place, the proper time and right concentration. When the tissue around the defect does not have any inherent ability for natural repairing, the tissue regeneration cannot be expected only by supplying the scaffold. Consequently, the scaffold should be combined with cells or/and bio-signaling molecules (growth factors and genes) which has a potential to accelerate tissue regeneration. However, the direct injection of growth factor in solution into the site to be regenerated is generally not effective. This is because the growth factor rapidly diffused from the injected site and is enzymatically digested or deactivated. DDS technology enables the growth factor to efficiently exert its biological function. Among the DDS technologies, the controlled release of growth factor at the site of action over an extended time period is achieved by incorporating the factor into an appropriate carrier. It is also highly possible that the growth factor is protected against its proteolysis, as far as it is, at least, incorporated in the release carrier, for prolonged retention of the activity *in vivo*. The release carrier should be degraded in the body since it is not needed after the growth factor release is completed. Other than the controlled release of drug, the objectives of DDS include the prolongation of drug half-life, the improvement of drug absorption and drug targeting. For example, it is a promising approach to promote tissue regeneration by targeting a growth factor with a prolonged half-life to the tissue site to be regenerated.

The third technology is the processing technique of cells for their isolation and proliferation. It is no doubt that cells with high proliferation and differentiation potentials, so-called precursor and stem cells, are important to induce tissue regeneration. However, one of the problems is the shortage of cells clinically available. Therefore, it is necessary to increase the number of stem cells with a high quality up to a level clinically applicable. For this purpose, *in vitro* cell isolation and culture techniques are highly required. The cell scaffold mentioned above can be utilized as the substrate for cell culture.

The fourth is for a physical barrier to protect cells transplanted and the area to be regenerated from immunological attack and fibroblast infiltration, respectively. When a body defect is produced, the defect space is usually occupied rapidly with the fibrous tissue accompanied by fibroblasts migration, because fibroblasts are present in the body ubiquitously and can proliferate rapidly. This is one of the typical wound healing processes to biologically maintain the living system. However, once this ingrowth of fibrous tissue into the space to be regenerated takes place, the regeneration and repairing of a target tissue at the space cannot be expected any more. To prevent this tissue ingrowth, a barrier membrane is highly required to keep a space for tissue regeneration. Also the immunoisolation membrane to protect cells transplanted from the biological attacks of humoral and cellular components is one of the barrier examples. Thus, by making use of cell scaffold, barrier and DDS technologies, biomedical technology or methodology to create an environment for the proliferation and differentiation of cells to induce tissue regeneration is tissue engineering.

## 3. Tissue regeneration based on DDS technologies of bio-signaling molecules

Tissue engineering can be classified into two categories in terms of the site where tissue regeneration is performed: *in vitro* and *in vivo* tissue engineering. *In vitro* tissue engineering involves tissue reconstruction and organ substitution. If a tissue can be reconstructed *in vitro* in factories or laboratories on a large scale, we can supply the tissue constructed to patients when it is needed. However, it is practically impossible to artificially arrange a biological environment for cell-based tissue regeneration under *in vitro* conditions only by using the present knowledge of biology and medicine or cell culture technologies currently available. Therefore, the *in vitro* tissue engineering approach has been unsuccessful other than a dermis-epidermis two-layers construct [12]. Another approach of *in vitro* tissue engineering is the functional substitution of organs with allo- or xenogeneic cells. Such engineered organs are called bioartificial hybrid organ. Research has been aimed at developing artificial liver and pancreas [13].

Distinct from the *in vitro* tissue engineering, the *in vivo* tissue engineering has an advantage for cell-induced tissue regeneration. Because it is likely that most biological components essential for tissue engineering are naturally supplied by the host. For example, only the application of a barrier membrane to keep a space for tissue regeneration enables the regeneration of periodontal tissues which has been clinically performed as guided tissue regeneration (GTR) [14]. Another example is the artificial dermis, which is prepared from the sponge scaffold of collagen, alginate or other materials. The sponges have a porous structure for cellular infiltration from the surrounding tissue and promote the regeneration of skin dermis following the implantation into the skin defect [15]. Although they cannot induce the regeneration of epidermis, they can serve many lives of patients with severe burns. Several experimental trials of *in vivo* tissue regeneration have been performed with the cell scaffold or the combination

with cells [16]. When the tissue around the defect site to be regenerated has an inherent activity toward tissue regeneration, it is likely that active and immature cells infiltrate into the scaffold implanted from the surrounding tissue, resulting in the formation of new tissue. However, additional treatments are required if the regeneration potential of tissue is poor, where the density of key cells and the concentration of bio-signaling molecules are low. One of the practical methods is to supply a growth factor to the site necessary for cell differentiation and proliferation in a controllable fashion. To enhance the *in vivo* efficacy of unstable growth factors for the induction of tissue regeneration, DDS technology is needed. We have succeeded in inducing the regeneration of various tissues and organs by the controlled release of various growth factors with the biological activities from biodegradable hydrogels (Table 1).

bFGF was originally characterized *in vitro* as a growth factor for fibroblasts and capillary endothelial cells and *in vivo* as a potent mitogen and chemoattractant for various cells. In addition, bFGF is reported to have a variety of biological activities [17] and be effective in enhancing wound healing through induction of angiogenesis as well as regeneration of various tissues, such as bone, cartilage and nerve. Human recombinant bFGF (Kaken Pharmaceutical Co. Ltd., Tokyo (product name: Fibrast® spray)) is on the market in Japan for the treatment of decubitus, a chronic ulcer of the skin. When a gelatin hydrogel incorporating bFGF was subcutaneously implanted into the mouse back subcutis, significant angiogenic effect was observed around the implanted site, in marked contrast to the injection of bFGF solution at higher doses [18]. The controlled release of growth factors other than bFGF has been achieved with the biodegradable hydrogel system and succeeded in the regeneration of various tissues (Table 1). The hydrogel system also permits the dual release of two factors to demonstrate a synergistic effect on angiogenesis and enhance the maturity of blood vessels regenerated [19].

There are two important objectives of angiogenesis in tissue engineering, the therapy of ischemic disease and prepare in advance angiogenesis for cell transplantation. As the former example, when injected into the ischemic site of myocardial infarction [20] or leg ischemia [21], gelatin microspheres incorporating bFGF induced therapeutically acceptable angiogenesis. This angiogenic therapy for leg ischemia has been started as a clinical study in different hospitals. The bFGF-induced angiogenic therapy has shown the good results.

It is no doubt that sufficient supply of nutrients and oxygen to the cells transplanted is indispensable for cell survival and the maintenance of biological functions. For successful cell transplantation, it is a practically promising to induce 'in advance' angiogenesis throughout the transplanted site, by using the release system of bFGF. This technology efficiently improved the biological functions of pancreatic islets [22], cardiomyocytes [23] and kidney cells [24]. We have succeeded in improving the cardiac functions of ischemic rat hearts by combination of cadiomyoblasts implantation with in advance angiogenesis induced by gelatin microspheres incorporating bFGF. The release system enabled bFGF, TGF-β1 and BMP-2 to enhance their activity of bone renegeration as well as bone regeneration induced by mesenchymal stem cells of bone marrow [25]. A hydrogel sheet incorporating bFGF was applied to the soft tissue around the sternum of diabetic rats of which sternum was cut and the bilateral arteries were ligated. As expected, bone regeneration at the cut line of sternum was achieved together with enhanced angiogenesis and the recovery of blood flow at the surrounding soft tissue [26]. This bFGF-induced simultaneous regeneration of bone and the surrounding blood vessels was also observed in a clinical study. De novo adipogenesis was succeeded by the preadipocytes isolated from human fat tissues, gelatin microspheres incorporating bFGF and a collagen sponge of cell scaffold [27]. Appropriate combination of all the three materials needed to induce this adipogenesis.

This system also permits the controlled release of plasmid DNA and small interference RNA (siRNA) to enhance the level of biological activities and prolong their time duration [28–31]. When intramuscularly injected into the ischemic leg of rats, the cationized gelatin microspheres incorporating a plasmid DNA of FGF-4-induced angiogenesis to a significantly higher extent than the plasmid DNA solution even at the dose 100 or 1000 times less than that of solution type [32]. The microspheres incorporating plasmid DNA was effective in genetically activating cells and consequently enhancing the efficacy of cell therapy. Cationized microspheres incorporating the plasmid DNA of adrenomedulin were prepared to allow them to internalize into endothelial progenitor cells. Intracellular controlled release of plasmid DNA enhanced the efficiently of gene transfection at the level higher than that of adenovirus transfection. The cells genetically engineered also functioned well to achieve higher therapeutic efficacy [33].

## 4. DDS technology for skin tissue regeneration

The therapeutic approach about skin tissue regeneration for severe burn or chronic ulcer has the longest history among other tissues and organs.

**Table 1** Regeneration induction of body tissues and organs based on the controlled release of bioactive growth factors from biodegradable hydrogels

| Materials | Growth factor | Animal | Effect | Objective | Reference |
|---|---|---|---|---|---|
| Acidic gelatin (pI 5.0) | bFGF | Mouse, rat and dog | Angiogenesis | Transplantation of Langerhans islands for diabetes therapy | [50,51] |
| | | Rat | Angiogenesis | Transplantation of hepatocytes for therapy of enzyme deficiency disease | [52] |
| | | Rat | Angiogenesis | Transplantation of renal epithelial cells | [24] |
| | | Rat and dog | Angiogenesis | Transplantation of cardiomyocytes | [23] |
| | | Rat and guinea pig | Angiogenesis | Promoted repairing of skin dermal layer | [40] |
| | | Rat and pig | Angiogenesis | Treatment of cardiac infarction | [20,53] |
| | | Rabbit | Angiogenesis | Treatment of lower limb ischemia | [21] |
| | | Rat, dog and monkey | Osteogenesis and angiogenesis | Repairing of sternum and connective tissue | [26,54,55] |
| | | Rat, rabbit and monkey | Osteogenesis | Repairing of skull and long bone | [56,57] |
| | | Mouse | Adipogenesis | Repairing of breast and soft tissue reconstruction | [27] |
| | | Mouse | Angiogenesis and activation of hair follicle tissue | Promotion of hair growth | [38,58] |
| | | Dog | Periodontium repair | Repairing of periodontium | [59] |
| | | Dog | Peripheral nerve repair | Nerve repairing | [60] |
| | | Dog | Osteogenesis | Repairing of mandiblular bone | [61] |
| | TGF-β1 | Rabbit and monkey | Osteogenesis | Repair of skull bone | [62-65] |
| | | Sheep | Chondrogenesis | Repairing of tracheal cartilages | [66] |
| | HGF | Mouse | Angiogenesis and activation of hair follicle tissue | Promotion of hair growth | [58] |
| | | Rat and pig | Angiogenesis and inhibition of apoptosis | Treatment of dilated cardiomyopathy | |
| | bFGF/ TGF-β1 | Rabbit | Osteogenesis | Repairing of skull bone | |
| | CTGF | Rabbit | Chondrogenesis | Repairing of articular cartilage | [67] |
| Basic gelatin (pI 9.0) | BMP-2 | Rat, dog and monkey | Osteogenesis | Repairing of skull and mandiblular bone | [68] |
| | | Dog | Chondrogenesis | Repairing of tracheal cartilages | [69] |
| Collagen | TGF-β1 | Rabbit | Osteogenesis | Repairing of skull bone | [70] |
| | | Rabbit | Osteogenesis | Promotion of engraftment of soft tissue grafts | [71] |
| | | Mouse | Angiogenesis and activation of hair follicle tissue | Promotion of hair growth | [39,58] |

bFGF, fibroblast growth factor; TGF-β1, transforming growth factor β1; HGF, hepatocyte growth factor; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; BMP-2, bone morphogenetic protein 2.

Several artificial skin grafts of biomaterials with or without cell seeding are commercialized in the world nowadays [12,34,35]. On the other hand, the DDS of angiogenic or other bio-signaling molecules or their combination with the graft is a promising and new approach to accelerate the *in vivo* skin regeneration [36–39].

Kawai et al. evaluated the incorporation effect of bFGF-impregnated gelatin microspheres into a collagen artificial dermis on the regeneration of dermis-like tissue in a full-thickness skin defect model of guinea pig [40], and on the wound healing for a pressure-induced decubitus ulcer model of genetically diabetic mice [41]. Combined release system

of bFGF in an artificial dermis in the skin defect model induced significantly infiltration of fibroblasts and the new formation of capillary blood vessels than the single application of bFGF solution on 5 days after implantation. Also in the site where necrotic tissues were resected after the pressure load over the femoral trochanter tertius of genetically diabetic mice, the release system of bFGF in implanted artificial dermis exhibited reduced infection and accelerated fibroblasts proliferation and capillary formation on 7 days after implantation. Generally, wound healing of diabetic mice is markedly delayed, and the mice have little tolerance for infection. It seemed that promoting neovascularization by the bFGF release system was significantly effective in suppressing the infection. By the

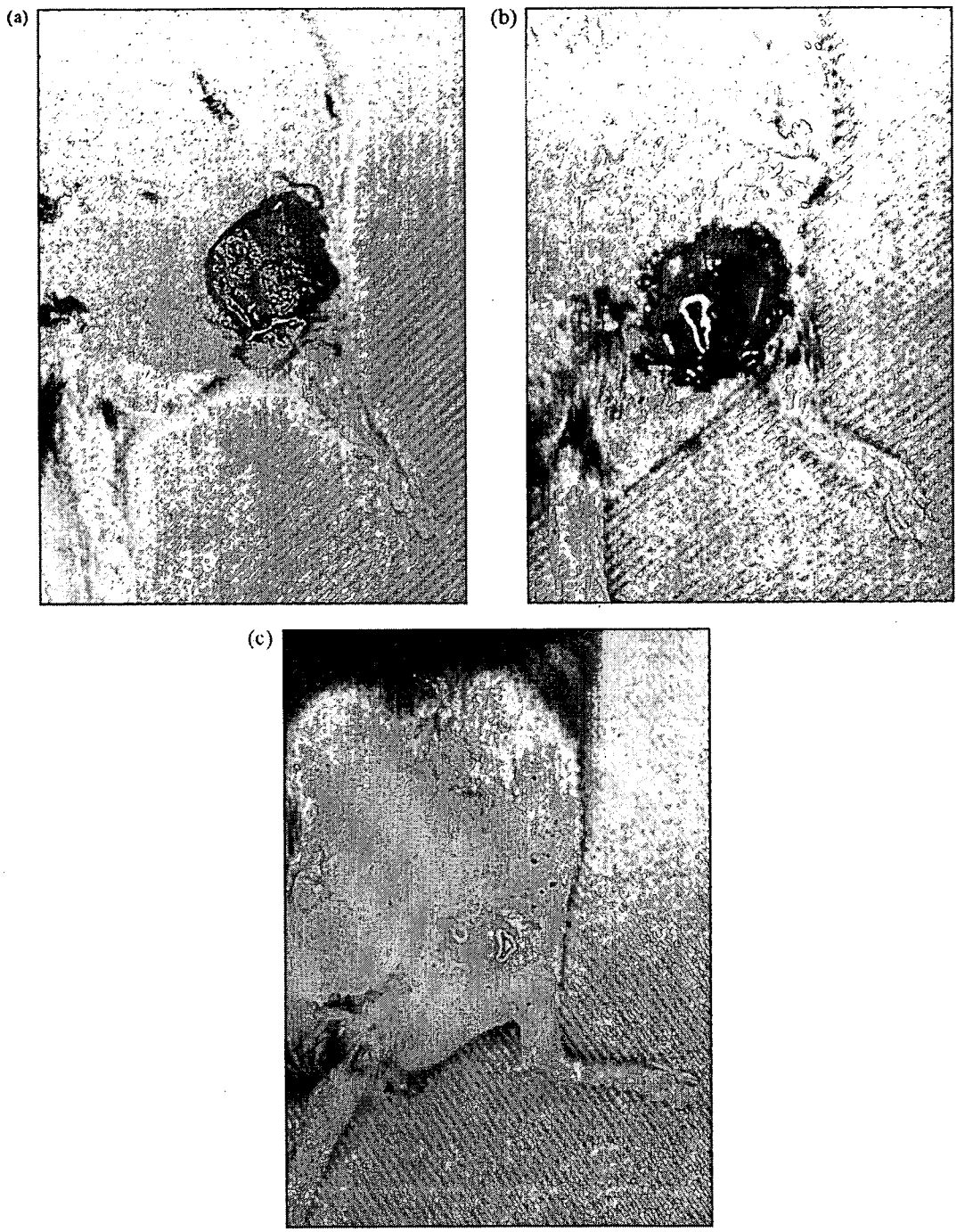


**Fig. 2** Macroscopic observations of artificial dermis-implanted site 7 days after implantation into a pressure-induced decubitus ulcer model of genetically diabetic mice (a) with bFGF-impregnated gelatin microspheres, (b) with solution form of bFGF and (c) without bFGF (Reprint from Ref. [41]).

combination of bFGF release system, the promoted degradation of collagen sponge by infection was suppressed and consequently tissue regeneration in the sponge remained was promoted (Fig. 2). In addition to bFGF, the controlled release of macrophage migration inhibitory factor (MIF) also induced accelerated wound healing in skin [42]. MIF-impregnated gelatin microspheres were administered around the full-thickness wound edge of normal mice and diabetic *db/db* mice. Significantly faster wound closure was observed in MIF-microsphere treated groups of both the animal models than the solution-injected groups. Additionally, the effect of MIF was evaluated by implanting the artificial dermis containing MIF-microsphere into a full-thickness skin wound model of MIF knockout mice. The result showed that enhanced procollagen $\alpha 1$ and VEGF were expressed into artificial dermis after implantation with MIF-microspheres and consequently higher CD31-positive cells and microvessel density were observed than those of the control group. MIF seemed to be a functional factor for neovascularization and granulation tissue development, and controlled release of MIF is of considerable promise in treatment to accelerate the rate of skin wound healing. Additionally, the controlled release system was adapted to anti-HGF IgG protein and the effect of HGF in cutaneous wound healing was evaluated. Neutralization of HGF by administration of anti-HGF antibody-incorporated gelatin microspheres around full-thickness skin wound retarded capillary formation, granulation tissue expansion, re-epithelialization and wound closure [43].

Furthermore, gelatin hydrogel microspheres containing bFGF were incorporated into cultured skin substrates (CSS) [44]. bFGF-impregnated gelatin microspheres were added to the preconfluent CSS before grafting, which were seeded $100 \times 10^3$ cells/$cm^2$ of fibroblasts combined with $100 \times 10^3$ cells/$cm^2$ of keratinocytes. Incorporation of bFGF significantly accelerated neovascularization and increased epidermal thickness, cellular components and thickness of the dermis at the grafted site of CSS in a full-thickness skin defect model of mice. And immunohistochemical staining of type IV collagen revealed that the skin construct 2 weeks after grafting has type IV collagen-positive layers between epidermal and dermal components at the site (Fig. 3). It seemed that the controlled release of bFGF was effective to promote the reconstruction of basement membrane into the CSS, as well as to accelerate epithelialization of the pre-confluent CSS after grafting. In the present day, CSS with both epidermal and dermal components have not been widely used clinically, partly because it takes several weeks to produce them. Decreasing the number of seeding cells may reduce the period required for production, but it still takes a long time before the cells become confluent and neovascularization is completed in CSS after grafting. Therefore, the incorporation of bFGF release system makes CSS a potential therapeutic approach for management of skin wounds.

## 5. Tissue engineering of internal medicine based on DDS technology to treat other medical conditions

At present, there is no successful therapy for chronic fibrosis, such as lung fibrosis, cirrhosis, dilated cardiomyopathy and chronic nephritis. In these diseases, the injured site is normally occupied with fibrous tissues of excessive collagen matrices. This tissue occupation physically impairs the natural tissue

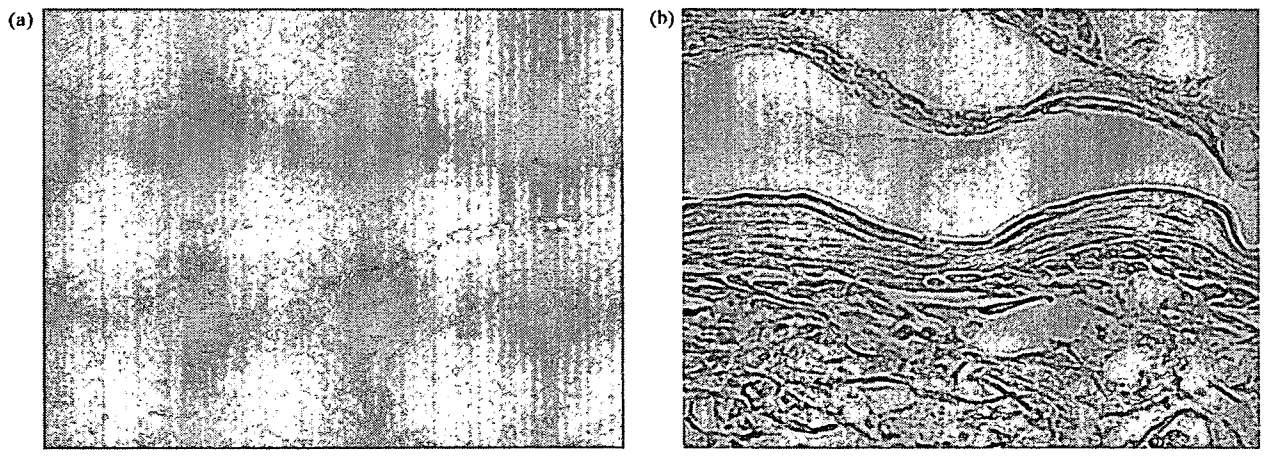


**Fig. 3** Immunohistochemical image of anti-human collagen type IV staining 2 weeks after implantation of cultured skin substrates (a) with or (b) without bFGF-impregnating gelatin microspheres into a full-thickness skin defect model of mice (magnification: ×400).

regeneration and repairing at the fibrotic site. If the fibrosis can be digested by any method to loosen or disappear, it is highly expected that the disease site is regenerated and repaired naturally based on the self-healing potential of the surrounding healthy tissue. It has been demonstrated that the injection of virus encoding a matrix metaloprotease (MMP) protein suppresses the tissue fibrosis to improve the disease symptoms [45]. The finding strongly suggests that when collagen in the fibrous tissue is enzymatically digested, fibrosis is repaired due to the body potential to induce tissue regeneration which is inherently equipped in the surrounding healthy tissue. This therapy for chronic fibrosis induced by autologous tissue regeneration is achieved by the drug treatment of internal medicine. Thus, it is a new direction of tissue engineering, defined as tissue engineering of internal medicine (Fig. 4). We have demonstrated that the controlled release of a MMP-1 plasmid DNA at the medulla of chronic renal sclerosis induced the histological regeneration of kidney structure, in contrast to the plasmid DNA solution [46]. Additionally, Kushibiki et al. reported that the controlled release of plasmid DNA encoding siRNA for transforming growth factor β (TGF-β) receptor suppressed progression of renal interstitial fibrosis in a mouse unilateral ureteral obstruction (UUO) model [30,47]. Selective digestion of TGF-βR mRNA by the siRNA expression significantly decreased the level of TGF-βR, the collagen content of mice kidney and the fibrotic area of renal cortex, in contrast to free plasmid DNA injection. We have demonstrated that the controlled release of siRNA itself with the cationized gelatin was effective for peritoneal sclerosis [48]. In the case of HGF protein, the liver fibrosis was histologically cured when gelatin microspheres incorporating hepatocyte growth factor (HGF) was administered by intraperitoneal injection into rats with liver cirrhosis [49].

## 6. Necessity of DDS technology in future tissue engineering to treat other medical conditions

Without using precursor and stem cells with high potential of proliferation and differentiation, recently, it has been possible to induce tissue regeneration only by using the controlled release system of biological active molecules. However, depending on the type of target tissue or organ and the site, it is necessary to make use of cells, scaffolds, growth factors and barrier membranes or their appropriate combinations. For the therapeutic approach of tissue engineering with growth factors, it is no doubt that the DDS technology or methodology will be indispensable in future. From the viewpoint of disease therapy based on the autologous repairing process of patients themselves, two approaches of tissue engineering in the surgical and internal medicine manners will be carried out in future.

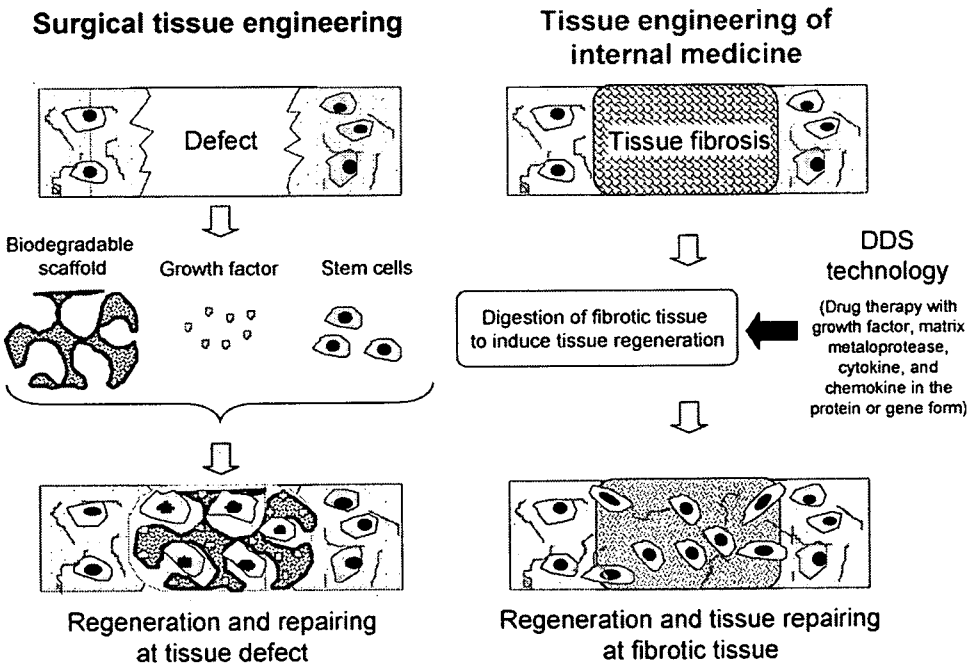


Fig. 4 The concept of surgical tissue engineering and tissue engineering of internal medicine to treat other medical conditions.

When the tissue damage is severe, such as infection and ischemia, the application of scaffold or barrier membrane alone does not always induce the tissue regeneration. In such a case, it is necessary to promote the process of tissue regeneration by any method. There are two choices for that currently available. One is to use cells of high proliferation and differentiation potentials, and the cell transplantation and the combination with the scaffold have been tried to demonstrate the therapeutic improvement. However, presently, it is scientifically impossible to completely manipulate the potential ability of cells for tissue regeneration. The survival rate of cells transplanted is very low and their functions therapeutically expected are not always exhibited. In addition, the *in vivo* regulation of cell differentiation is almost impossible. Taken together, the time may not be quite ripe for therapy by cell transplantation. Another approach is to make use of bio-signaling molecules. Since the molecules are generally unstable *in vivo*, it was practically difficult to expect the therapeutic function *in vivo*. However, the present situation has been changed by the recent development of biomedical technology and methodology to handle bioactive proteins and genes. By the combination of molecules with the appropriate DDS technology, it is possible to enhance the *in vivo* efficacy and induce the regeneration of tissue. From the viewpoint of technology, commercialization and law regulation, the DDS technology is more realistic to achieve the therapy of regenerative medicine.

If a key growth factor is supplied to the target site in a regulated time schedule and at the right concentration, the living body system will naturally direct toward the process of tissue repairing. Once the right direction is given, it is highly possible that the intact biological system of the body starts to physiologically function, resulting in natural achievement of tissue regeneration. There is no doubt that whenever growth factors and genes are used *in vivo*, their combination with DDS technology is essential. However, the present technology of controlled release does not always regulate accurately the amount and time period of growth factor release. Therefore, it is practically impossible to artificially control the process of cell differentiation only by the release technology of growth factors currently available, since the differentiation process is regulated by the complicated network of growth factor in the restricted time, site or concentration manner.

As tissue engineering is still in its infancy, it will take a long time to become well established; although a part of the research projects have already come close to the stage of clinical applications. In the future, drug delivery will be of increasing significance. We will be happy if this short review stimulates readers' interest in the idea and research field to assist understanding of release technology importance in tissue engineering and regenerative medicine.

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# Controlled release of platelet growth factors enhances bone regeneration at rabbit calvaria

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***Objective.*** Platelet-rich plasma (PRP) has been clinically employed to promote bone regeneration. However, few studies have investigated the enhancement of biological function of platelet growth factors after integration of PRP into biomaterials. In this study, the feasibility of gelatin hydrogels for controlled release of platelet growth factors and the consequent enhancement of PRP-induced bone regeneration were evaluated in rabbit calvarial defect.
***Study design.*** Gelatin hydrogels incorporating PRP, PRP-activated thrombin, or an empty gelatin hydrogel were applied to the defect, or the defect was left untreated. Bone regeneration was evaluated by microfocus computed tomography, peripheral quantitative computed tomography, and histological examinations.
***Results.*** Successful bone regeneration was observed at the bone defect applied with the gelatin hydrogel incorporating PRP, which is in marked contrast to other groups.
***Conclusion.*** The gelatin hydrogel is a promising material capable of controlled release of platelet growth factors to enhance bone regeneration. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;104:44-8)**

Recently, one of the predominant goals of oral and maxillofacial bone grafting has been to enhance bone formation and increase the rate of bone graft healing by use of platelet-rich plasma (PRP). PRP contains autologous platelet growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which accelerate bone regeneration and wound healing.[1] In 1998, Marx et al.[2] proposed the local application of PRP to enhance bone regeneration. Since this paper was published, many clinical or experimental investigations about bone regeneration with PRP have been reported.[3] Some studies have indicated a capability of PRP to promote bone regeneration.[4] On the contrary, some investigators have demonstrated no benefit or even a negative effect on bone regeneration with PRP. Aghaloo et al.[5] evaluated the effect of PRP on rabbit cranial bone healing, but no significant enhancement of bone formation was seen radiographically or histomorphometrically. Platelet-rich plasma has been combined with autologous bone or bone substitutes such as anorganic bone mineral and organic bone matrix.[6,7] It remains questionable whether or not these combinations enhance the biologic activity of PRP.[8] There are no clear scientific data to explain the role of biomaterials in PRP-induced augmentation of bone regeneration.[9] In addition, few studies have investigated the biologic function of PRP when combined with biomaterials, from the viewpoint of controlled release of growth factors.

Gelatin is a biodegradable material that has been extensively used for pharmaceutical and medical purposes and has proven to be biosafe through its long clinical application.[10] It is known that the isoelectric point (IEP) of gelatin can be changed by manipulating the conditions of preparation from collagen. We have prepared a biodegradable hydrogel from acidic gelatin that can ionically interact with alkaline growth factors. In this hydrogel system, the growth factor immobilized in the acidic gelatin hydrogel is released only when the hydrogel is degraded to generate water-soluble gelatin fragments.[11] Using the gelatin hydrogel, we have successfully achieved the controlled release of bioactive basic fibroblast growth factor,[12] TGF-β1,[13] PDGF,[14] and bone morphogenetic

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protein 2[15] with enhancement of biologic function.[16] It is highly expected that the gelatin hydrogel functions effectively as a carrier of platelet growth factors, because the controlled release of the growth factors has been experimentally confirmed.

The objective of the present study is to investigate the potential of the gelatin hydrogel as a biomaterial for controlled release of multiple growth factors present in PRP.

## MATERIAL AND METHODS

### Preparation of gelatin hydrogels

A gelatin with an IEP of 5.0 was supplied by Nitta Gelatin Co., Osaka, Japan. All chemicals were purchased from Wako Pure Chemical Industries Osaka, Japan and used without further purification.

Gelatin hydrogels were prepared by the glutaraldehyde cross-linking of gelatin as reported previously.[12] The water content of gelatin hydrogel (the weight ratio of water present in the hydrogel to the wet hydrogel) was 98 wt%. The resulting freeze-dried gelatin hydrogel was cut into a disk shape (5 mm in diameter and 3 mm height) and then sterilized with ethylene oxide gas.

### Preparation of PRP and gelatin hydrogels incorporating PRP

New Zealand white rabbits ranging from 3.0 to 3.5 kg of body weight (approximately 20 weeks old; Shimizu Laboratory Animal Supply, Kyoto, Japan) were used. All the animal experiments were approved by the Kyoto University Committee for Animal Experimentation. Briefly, 12 rabbits were anesthetized by the intramuscular injection of mixed 2 mL of ketamine hydrochloride (Ketalar, Sankyo Seiyaku, Tokyo, Japan) and 1 mL of xylazine (Seractarl Bayer, Tokyo, Japan) at a dose of 0.65 mL/kg body weight. Platelet-rich plasma was prepared according to the method reported.[5] The approximate volume of PRP obtained was 0.8 mL. The approximate density of platelets in the PRP prepared increased by 6.42-fold when compared to the original peripheral blood. The concentrated PRP (100 μL) was dropped onto the freeze-dried gelatin hydrogel disk, followed by incubation for 1 hour at 37°C for PRP impregnation to obtain the gelatin hydrogel incorporating PRP.

### Surgical procedure

All animal experiments were performed by the surgical procedure previously reported with slight modification.[5] An incision was made to the bony cranium while the periosteum was reflected. Bone defects of 5-mm diameter (4 defects per calvaria) were carefully created with a trephine bur, and 4 defects were randomly applied with a gelatin hydrogel incorporating PRP, or PRP (100 μL) activated with 10 IU of bovine thrombin in 10% calcium chloride solution (10 μL), or an empty gelatin hydrogel, or left untreated (Fig. 1). The wound was carefully closed with sutures, and a postoperative antibiotic (Fosmicin, Meiji Seika, Tokyo, Japan) was administered intramuscularly at a dose of 100 mg/kg per day for 3 days.

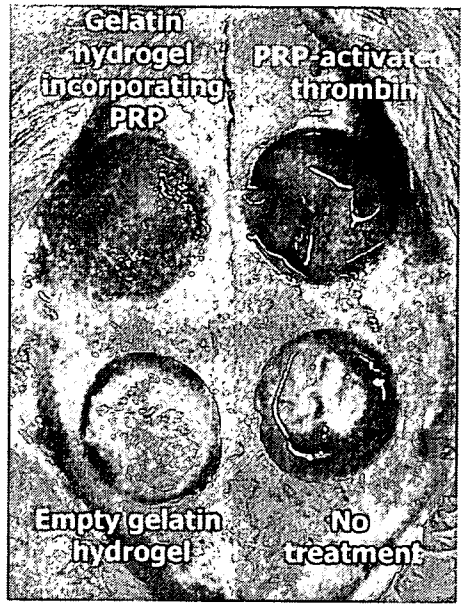


Fig. 1. A representative photograph of rabbit calvaria with surgical sites applied.

### Assessment of bone regeneration

Bone regeneration at the site of bone defects was assessed using microfocus computed tomography, peripheral quantitative computed tomography (pQCT), and histological examinations 8 weeks after application. Bone regeneration in the defects was evaluated using a microfocus x-ray CT system (SMX-130CT-SV3, Shimadzu Corp., Kyoto, Japan). Three-dimensional images were constructed with three-dimensional image visualization software (VG Studio MAX 1.2, Nihon Visual Science, Tokyo, Japan). For quantitative evaluation, the bone mineral density (BMD) of each bone defect was measured using pQCT (Stratec XCT Research SA+, Stratec Medizintechnik, Pforzheim, Germany). Briefly, prior to scanning, calibration of the pQCT was routinely performed with a hydroxyapatite phantom. The specimens were then placed in the holder, and the regenerated defect area was identified at the scout view window. Three slices, including the central slice at the defect and slices 0.2 mm to either side of the central slice, were scanned using a voxel size of 0.12 mm. All slices were analyzed for total volumetric BMD with the manufacturer-supplied software program (version 5.50). A density threshold of

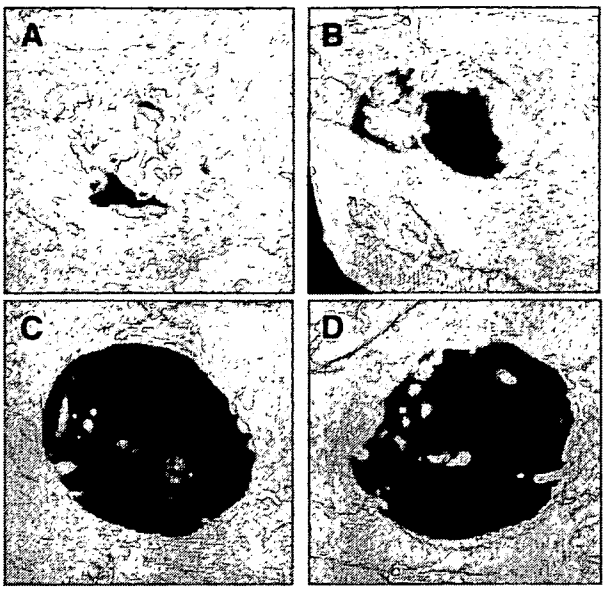


Fig. 2. Three-dimensional images of calvarial defects 8 weeks after treatment with a gelatin hydrogel incorporating platelet-rich plasma (PRP) (**A**), PRP-activated thrombin (**B**), an empty gelatin hydrogel (**C**), or without any treatment (**D**).

267 mg/cm$^3$ was used to differentiate bone from soft tissues. The mean BMD of the regenerated defect from the 3 slices per sample was calculated and compared.

Bone specimens were placed into 10% neutral phosphate-buffered solution of formaldehyde, decalcified with 10% formic acid, and processed for the paraffin embedding. The histological sections of 3-μm thickness were prepared and stained with hematoxylin-eosin for viewing by light microscopy (AX80T, Olympus Corp., Tokyo, Japan).

### Statistical analysis

All the data were statically analyzed using the Fisher least significant difference test for multiple comparisons, and statistical significance was accepted for $P$ values of less than .05. The experimental results were expressed as the mean ± the standard deviation of the mean.

## RESULTS

### Microfocus computed tomography evaluation

Fig. 2 shows the microfocus computed tomography image of bone defects 8 weeks after treatment with the gelatin hydrogel incorporating PRP or other agents. When the bone defect was treated with the gelatin hydrogel incorporating PRP (Fig. 2, *A*), complete bone regeneration at the defect was observed. On the other hand, incomplete bone regeneration was observed for the PRP-activated thrombin (Fig. 2, *B*). No bone regeneration was detected for the empty gelatin hydrogel and the untreated defect (Fig. 2, *C, D*).

### Histological evaluation

Fig. 3 shows the histological sections of calvarial defects 8 weeks after treatment. When treated with the gelatin hydrogel incorporating PRP, the bone defect was histologically closed by regenerated bone tissue (Fig. 3, *A*). Bone regeneration at the defect treated with PRP-activated thrombin was also observed, although the area of regenerated bone tissue was smaller (Fig. 3, *B*). No bone regeneration was detected at all in the defect treated with empty gelatin hydrogel and the untreated group, although an ingrowth of soft connective tissue into the defect was observed (Fig. 3, *C, D*).

### Quantitative evaluation with pQCT

Fig. 4 shows the BMD values of calvarial defects 8 weeks after treatment with the gelatin hydrogel incorporating PRP or other agents. The BMD value at the bone defect treated with the gelatin hydrogel incorporating PRP was significantly higher than that of bone defects treated with other agents.

## DISCUSSION

The present study used 5-mm calvaria defects to evaluate bone regeneration in adult rabbits (approximately 20 weeks old). There has been much debate in the literature regarding the appropriate size of the defects for bone regeneration in calvaria models, and defects that do not heal spontaneously have been termed critical size defects. Although the 5-mm defect used in this study is smaller than the critical size defect of 15 mm recommended for rabbits,[17] healing was still incomplete without any treatments (Fig. 2, *D*). Previous studies have also reported incomplete spontaneous healing for 5-mm calvaria defects in the rabbit model.[18] Meikle et al.[18] described that 5-mm defect size is clearly adequate for testing the performance of bone repair. Taken together, we believe that the 5-mm calvaria defect model of adult rabbits is an appropriate model to evaluate bone regeneration.

In living tissue, it is well known that various growth factors interact with extracellular matrix components, such as acidic polysaccharides, through various intermolecular interaction forces.[19] Furthermore, these physicochemical interactions serve to maintain and enhance growth factor biologic function in vivo. For instance, it is well recognized that basic fibroblast growth factor with an IEP of 9.6 forms a polyion complex with heparin and heparan sulfate in vivo, resulting in stabilization and regulation of biologic activity.[20,21] These natural physicochemical interactions form a starting point in the rational design of biomaterials for controlled release of growth

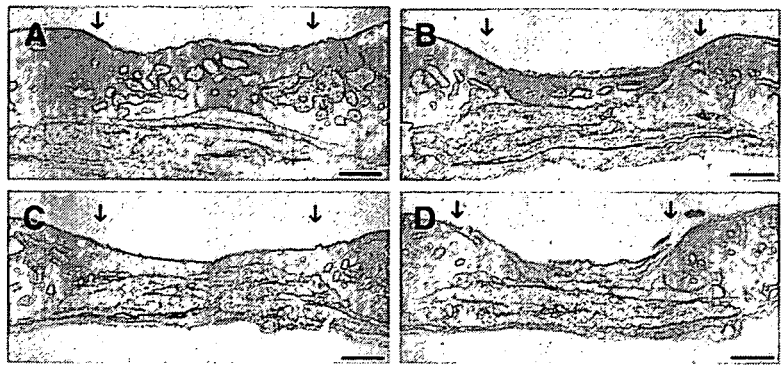


Fig. 3. Histological sections of calvarial defects 8 weeks after treatment with a gelatin hydrogel incorporating PRP (**A**), PRP-activated thrombin (**B**), an empty gelatin hydrogel (**C**) or without any treatment (**D**). *Arrows* indicate the margin site of defects prepared initially. The bar length is 1.0 mm.

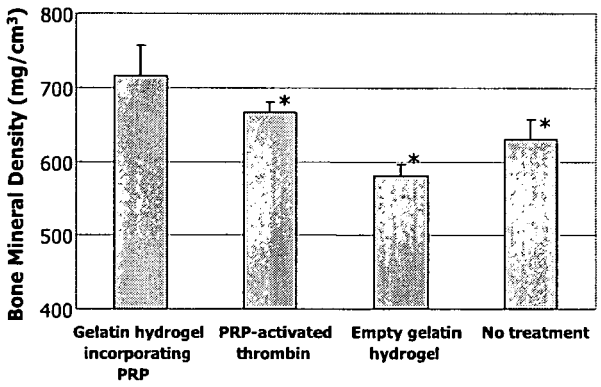


Fig. 4. The bone mineral density values at calvarial defects 8 weeks after treatment with gelatin hydrogels incorporating PRP, PRP-activated thrombin, empty gelatin hydrogels, or without any treatment. $*P < .05$; significance against the bone mineral density value at the bone defect treated with the gelatin hydrogels incorporating PRP.

factor. In this regard, a unique advantage of gelatin is its electrical nature, which can be readily changed by the manipulation processing method. For example, gelatin prepared with an alkaline process collagen has an IEP of 5.0 and is negatively charged at physiological pH, whereas an acidic process collagen gives a positively charged gelatin with an IEP of 9.0. This change can be used to interact electrostatically with growth factors, which are generally alkaline and therefore negatively charged.[22] Recently, recombinant growth factors have become commercially available and have been experimentally or clinically applied for tissue regeneration. Although there are some cases where direct application promotes tissue regeneration, the direct injection of growth factors in solution form is generally not effective. In solution form, growth factors tend to rapidly diffuse from the injection site and are also subject to enzymatic digestion or deactivation. Controlled-release drug delivery technology is therefore required to enable the growth factor to efficiently exert its biologic functions. We have successfully achieved the controlled release of TGF-β1 from acidic gelatin hydrogels with subsequent bone regeneration in rabbit and monkey skull defects. This regenerative effect was not observed for TGF-β1 in solution form. It was found that TGF-β1 was sorbed into the acidic gelatin hydrogel mainly due to the electrostatic interaction between the gelatin and TGF-β1 molecules.[23] Similarly, it was found that bioactive PDGF could also be released from acidic gelatin hydrogels.[14]

It has been recognized that platelets in PRP secrete various growth factors during the degranulation of α granules, and that the secreted factors exhibit various biologic activities.[4] It is well known that collagen and thrombin trigger platelet aggregation and the subsequent secretion of platelet growth factors.[24] We have demonstrated that gelatin, a derivative of collagen, can also trigger the platelet activation for growth factor secretion.[25]

Based on these findings, it is likely that PRP platelets are activated by exposure to gelatin molecules during the hydrogel impregnation process, resulting in the secretion of TGF-β1 and PDGF within the hydrogel. These growth factors will then be immobilized into the hydrogel through electrostatic interactions with gelatin molecules. During in vivo hydrogel degradation, the immobilized growth factors could then be released to the defect site.[22]

Biomaterials used to deliver osteoinductive growth factors to bone defect sites should not only promote biologic effects but also serve as scaffolds to assist bone regeneration.[13] When the rate of scaffold biodegradation is too fast compared with the rate of bone regeneration at the defect, the scaffold cannot physically prevent soft tissue infiltration, resulting in no bone regeneration. In this study, bone regeneration was ob-

served for PRP-activated thrombin, but the regenerated bone area was small (Fig 3, *B*). The clot prepared by PRP activated with thrombin could neither function as a release carrier of platelet growth factors nor as a scaffold for bone regeneration. In addition, because the clot degraded quickly, it did not efficiently prevent the infiltration of soft tissue into the bone defect and consequently suppressed regeneration of bone tissue. On the contrary, the gelatin hydrogel released platelet growth factors and effectively promoted their osteoinductive activity at the defect. The persistence of the hydrogel also functioned to physically prevent soft tissue infiltration. It appeared that hydrogel degradation was harmonized with the process of bone regeneration, resulting in effective defect closure by newly formed bone tissue (Fig. 3, *A*). The BMD value of defects treated with gelatin hydrogels incorporating PRP was significantly higher than that of other groups (Fig. 4). This finding indicates that a balance in the time profile between the release of growth factors and scaffold degradation is essential for bone regeneration induced by PRP. The gelatin hydrogel used in this study possessed a suitable in vivo degradability, which functioned not only as a space maker to prevent the growth of soft tissues into the bone defect, but also as a release carrier for platelet growth factors from PRP.

## CONCLUSION

For clinical application of platelet growth factors in PRP, one of the important technologies required is the controlled release of growth factors at the necessary site over an extended time period. This study indicates that the gelatin hydrogel is a promising biomaterial for the enhancement of bone regeneration by PRP platelet growth factors.

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# The Regenerative Effects of Platelet-Rich Plasma on Meniscal Cells *In Vitro* and Its *In Vivo* Application with Biodegradable Gelatin Hydrogel

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## ABSTRACT

**The objective of the study was to test the hypothesis that platelet-rich plasma (PRP) enhances meniscal tissue regeneration *in vitro* and *in vivo*. In the *in vitro* study, monolayer meniscal cell cultures were prepared, and 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay and 5-bromo-2′-deoxyuridine assay were performed to assess proliferative behavior in the presence of PRP. Alcian blue assay was performed to assess extracellular matrix (ECM) synthesis. To detect the fibrocartilage-related messenger ribonucleic acid (mRNA) expressions, real-time polymerase chain reaction was performed. In the *in vivo* study, 1.5-mm-diameter full-thickness defects were created in the avascular region of rabbit meniscus. Gelatin hydrogel (GH) was used as the drug delivery system for PRP growth factors. The defects were filled as follows: Group A, GH with PRP; Group B, GH with platelet-poor plasma; Group C, GH only. Each group was evaluated histologically at 4, 8, and 12 weeks after surgery. PRP stimulated deoxyribonucleic acid synthesis and ECM synthesis ($p < 0.05$). Meniscal cells cultured with PRP showed greater mRNA expression of biglycan and decorin ($p < 0.05$). Histological findings showed that remnants of gelatin hydrogels existed at 4 weeks, indicating that the hydrogels could control release for approximately 4 weeks. Histological scoring of the defect sites at 12 weeks revealed significantly better meniscal repair in animals that received PRP with GH than in the other two groups. These findings suggest that PRP enhances the healing of meniscal defects.**

## INTRODUCTION

THE MENISCI are wedge-shaped semi-lunar discs that play an important role in knee function.[1–8] Arthroscopic partial meniscectomy has been widely advocated for the treatment of meniscal injury, although biomechanical changes after meniscal injury were unavoidable.[9] Maintenance of the meniscus is important in the prevention of accelerated degeneration of the knee joint.

Because menisci tears located in the inner avascular part of the meniscus do not heal spontaneously, clinical repair techniques such as meniscal repair, rasping, and debridement[10,11] have been attempted to increase the healing potential of the meniscus.

Several growth factors, especially platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β), have been proven to be effective for meniscal tissue regeneration.[12–17] PDGF is considered to be one of most

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potent factors for mitogenic response of meniscal cells in monolayer and explant culture.[12–14] However, the cost of almost all genetically engineered growth factors is prohibitively high, and only some growth factors, such as basic fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor-1 (IGF-1) are available for clinical use.[18]

When the clinical application of growth factors was considered, platelet-rich plasma (PRP) provided one of the most attractive sources for growth factors. PRP could be easily prepared from a patient's own peripheral blood using a number of simple centrifugation steps and contained rich growth factors, such as PDGF and TGF-β. As an autologous source of growth factors, PRP has been investigated and proven to be useful for bone regeneration.[19–30] Thus, if PRP growth factors were also effective for meniscal tissue, PRP could be an attractive clinical source for meniscal tissue regeneration.

For the *in vivo* application of PRP, we used acidic gelatin hydrogels as a source of delivery for PRP growth factors. Gelatin is a biodegradable material that has been extensively used for pharmaceutical and medical purposes and has been proven to be biosafe throughout its long history of clinical applications. It was found that gelatin could control the release of PRP growth factors, such as TGF-β and PDGF, for an average of approximately 2 weeks in an *in vivo* environment.[31,32] Hokugo *et al.* previously reported that gelatin could effectively release growth factors containing PRP.[31]

Thus the purpose of this study was to investigate the effectiveness of PRP for the promotion of meniscal tissue regeneration. In our *in vitro* study, we first showed that PRP promoted meniscal cell proliferation and extracellular matrix (ECM) synthesis. We also examined the regenerative effects of PRP on meniscal tissues with rabbit meniscal defect *in vivo*.

## MATERIALS AND METHODS

### *Collection and preparation of PRP (rabbit)*

Blood samples ($n = 4$ in each group) were obtained from Japanese white rabbits (Kitayama Labs, Nagano, Japan), weighing $2.8 \pm 3.2$ kg. The Animal Research Committee of Kobe University Graduate school of Medicine approved all procedures. PRP was prepared using 2 centrifugation techniques, as previously reported.[29,30] Briefly, rabbits were anesthetized using intravenous injection of 2.0 to 2.4 mL Pentobarbital sodium solution at a dose of 30 mg/kg body weight. Sixteen mL of whole blood was drawn from each subject into tubes containing 4 mL of acid citrate dextrose-A solution as an anticoagulant and equally separated into 2 sterile tubes. An aliquot was removed from each tube to determine the platelet count. The tubes were then spun in a laboratory centrifugation apparatus (6800; Kubota, Tokyo, Japan) at 4°C for 15 min at 800 rpm, and all plasma was transferred to 2 new sterile tubes to be further centrifuged at 4°C for 10 min at 2000 rpm. The supernatant plasma was discarded and the remaining approximately 0.8 mL of plasma and precipitated platelet was designated PRP. Platelet-poor plasma (PPP) was collected using a further centrifugation step (at 4°C for 10 min at 3000 rpm) in the remaining red cell fraction after the first centrifugation. Platelet counts were also performed for samples of PRP and PPP. Samples were thawed and stored at −80°C until used.

### *Enzyme-linked immunosorbent assay analysis of growth factors in PRP and PPP*

To measure the concentration of growth factors secreted from PRP and PPP, enzyme-linked immunosorbent assay (ELISA) was performed ($n = 7$ in each group). Briefly, the PRP and PPP samples were thawed and centrifuged at 4°C for 10 min at 3000 rpm to remove platelet membranes, and then the supernatants were used. Commercial human PDGF, TGF-β1, and vascular endothelial growth factor (VEGF) ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions. All samples for TGF-β1 analysis were acid activated with 1 N hydrochloric acid.

### In vitro *study*

*Tissue and culture preparation.* In this study, meniscal tissue was prepared from skeletally mature female Japanese white rabbits (Kitayama Labs), weighing $2.8 \pm 3.2$ kg. The rabbits were euthanized with an overdose of pentobarbital sodium solution injected intravenously. Soon after sacrifice, the knee joint was opened, and the bilateral meniscus were harvested and carefully dissected free from the adherent synovium and the capsule. Only the inner two-thirds, avascular zone of the meniscus was used. The pieces were diced manually into smaller pieces and digested using 0.2% collagenase (Sigma, St. Louis, MO) for 1 h at 37°C, followed by a subsequent digestion in 0.05% trypsin for 30 min at 37°C to release the cells from the ECM. The digests were filtered to remove any undigested material using cell strainers with a pore size of 45 μm and then centrifuged and washed 2 times with phosphate-buffered saline (PBS). The pellets of the cells were resuspended and cultured in 75-$cm^2$ culture flasks containing Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS, penicillin G (100 U/mL), and streptomycin (100 μg/mL). The cultures were maintained at 37°C in a humidified 5% carbon dioxide ($CO_2$) atmosphere. The cells reached confluence at an average of 3 weeks.

*Cell viability: 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay.* Meniscal cell viability was assayed using the 3-(4, 5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay technique (CellTilter 96 Aqueous; Promega, Madison, WI) ($n = 5$