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Nanomaterials of Drug Delivery Systems for Tissue Regeneration

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Summary

A new therapeutic trial aimed at assisting tissue regeneration at a body defect in size too large for self-repair has recently begun. The objective is to substitute the biological functions of damaged and injured organs by taking advantage of cells. For successful tissue regeneration, it is absolutely indispensable not only to have cells of high proliferation and differentiation potential, but also to create an environment suitable for inducing regeneration. Such creation can be artificially achieved only by providing various biomaterials to promote cell proliferation and differentiation, such as cell scaffold and growth factors. Growth factors are often required to promote tissue regeneration because they can induce angiogenesis, which promotes a sufficient supply of oxygen and nutrients to effectively maintain the biological functions of cells transplanted for organ substitution. However, because of their poor in vivo stability, the biological effects of growth factors cannot always be expected unless these drug delivery systems (DDSs) are contrived. In this chapter, several research approaches to tissue regeneration are reviewed to emphasize the significance of biomaterials and DDS technologies in regenerative medicine.

Key Words: Tissue engineering; tissue regeneration; regenerative medicine; biomaterials; drug delivery systems; growth factor release.

1. Introduction

1.1. Technologies Necessary for Tissue Regeneration

When a body tissue or organ is severely injured, largely lost, or malfunctioning, it is clinically treated using either reconstructive surgery or organ transplantation. Although there is no doubt that these procedures have saved and improved the lives of countless patients, these therapies at present are facing several clinical challenges. One promising alternative to such complications is to allow patients to induce regeneration of their body tissues and organs by

From: *Methods in Molecular Biology, vol. 300:*
Protein Nanotechnology, Protocols, Instrumentation, and Applications
Edited by: T. Vo-Dinh © Humana Press Inc., Totowa, NJ

making use of their self-healing potential. A new biomedical field focused at bringing about this new therapeutic approach is tissue engineering. Tissue engineering is one of the biomedical technologies aimed at assisting clinical applications of basic research, which results in "regenerative" medicine and "regenerative" medical therapy for patients, including regeneration of natural tissues as well as creation of biological substitutes for defective or lost tissues and organs, starting at the cellular level ***(1)***.

To successfully achieve tissue regeneration, it is undoubtedly necessary not only to increase the number of cells constituting the tissue, but also to reconstruct a structure to support the proliferation and differentiation of the cells' so-called extracellular matrix (ECM). In addition, growth factors are often required to promote tissue regeneration, depending on the type of tissue. In summary, the components necessary for tissue engineering include cells, the scaffolds for cell proliferation and differentiation, and growth factors.

Recently, it has been well recognized that the ECM not only physically supports cells, but also plays an important role in both cell proliferation and differentiation (or morphogenesis), which results in tissue regeneration and organogenesis. It is unlikely that a large defect of tissue will be naturally regenerated and repaired by merely supplying cells to the defect. Nonetheless, there are a few cases in which successful tissue regeneration is achieved by simply adding healthy cells to the appropriate site. For example, one practical approach is to provide an environment suitable for induction of tissue regeneration at the defect site by first building a scaffold as an artificial ECM that temporarily promotes cell attachment and the subsequent cell proliferation and differentiation. It is highly expected that self-derived cells residing around the scaffold or the cells preseeded in the scaffold will proliferate and differentiate on the foundation of the provided scaffold if the artificial ECM is compatible with the cells. Once the regeneration process of a new tissue is initiated, the cells constituting tissue eventually produce the natural and appropriate ECM. However, any remaining cell scaffolds physically hinders tissue regeneration during the process. Thus, for successful tissue regeneration, a period of scaffold degradation at the defect should be optimized and controlled. For cases in which the tissue to be repaired has a high activity toward regeneration, a new tissue will be formed in the biodegradable scaffold matrix by active, immatured cells infiltrated by the surrounding healthy tissue. However, additional means are required if the regeneration potential of tissue is very low, because of, e.g., the poor infiltration of cells and low concentration of growth factors responsible for new tissue generation. The simplest method to offset these conditions is to supply growth factors to the site of regeneration for cell differentiation and proliferation.

However, the direct injection of growth factors in solution form into sites to be regenerated is often not effective, because injected growth factors are rapidly diffused out and excreted from the site. One promising way to enhance growth factor efficiency in vivo is to use drug delivery systems (DDSs). For example, the controlled release of growth factor at the site of action over an extended time period is readily possible by incorporating the factors into appropriate carriers. It is likely that a growth factor is protected against proteolysis, as long as it is held within the carrier, hence enabling prolonged retention of the activity in vivo. After the release of the growth factor, the carrier should be degraded in the body because it is no longer needed. Thus, for tissue regeneration or organ substitution, it is of prime importance to create an environment suitable for induction of tissue regeneration by making use of the biodegradable scaffold and DDS technologies. Even if the basic biology of and medicine for cells are greatly advanced, "regenerative" medical therapy for patients will never be realized by the research results alone unless this regeneration environment is provided appropriately.

1.2. Overview of Tissue Engineering

Tissue engineering is classified into two categories in terms of the site where tissue engineering is performed: in vitro and in vivo (**Fig. 1**). In vitro tissue engineering involves tissue reconstruction and organ substitution, otherwise known as bioartificial organs. **Table 1** provides tissues and organs undergoing tissue engineering. The targeted tissues and organs have been extensively investigated based on combinations of cells, scaffolds, and growth factors ***(1)***.

If tissues can be reconstructed in vitro in factories or laboratories on a large scale, the tissue constructs can be supplied to patients when they are needed. This approach would be very attractive for commercialization once the feasibility is established. However, it is quite difficult to completely reconstruct the event in vitro using the cell culture technologies currently available. Another approach to in vitro tissue engineering is the substitution of organ functions by the use of allogenic or xenogenic cells. Such engineered organs have been called bioartificial organs because they are composed of heterogeneic cells and man-made membranes or porous constructs for immunoisolation to protect the cells from host attack and maintain cell function. This approach has been performed for the liver, pancreas, and kidney ***(2,3)***.

Distinct from in vitro tissue engineering, in vivo tissue engineering has the advantage of using the native environment for induction of tissue regeneration. Most of the materials necessary for tissue regeneration are automatically supplied by the host living body. Therefore, almost all the approaches to tissue engineering have been currently performed in vivo with or without biodegradable scaffolds. This approach is more realistic and clinically acceptable if it

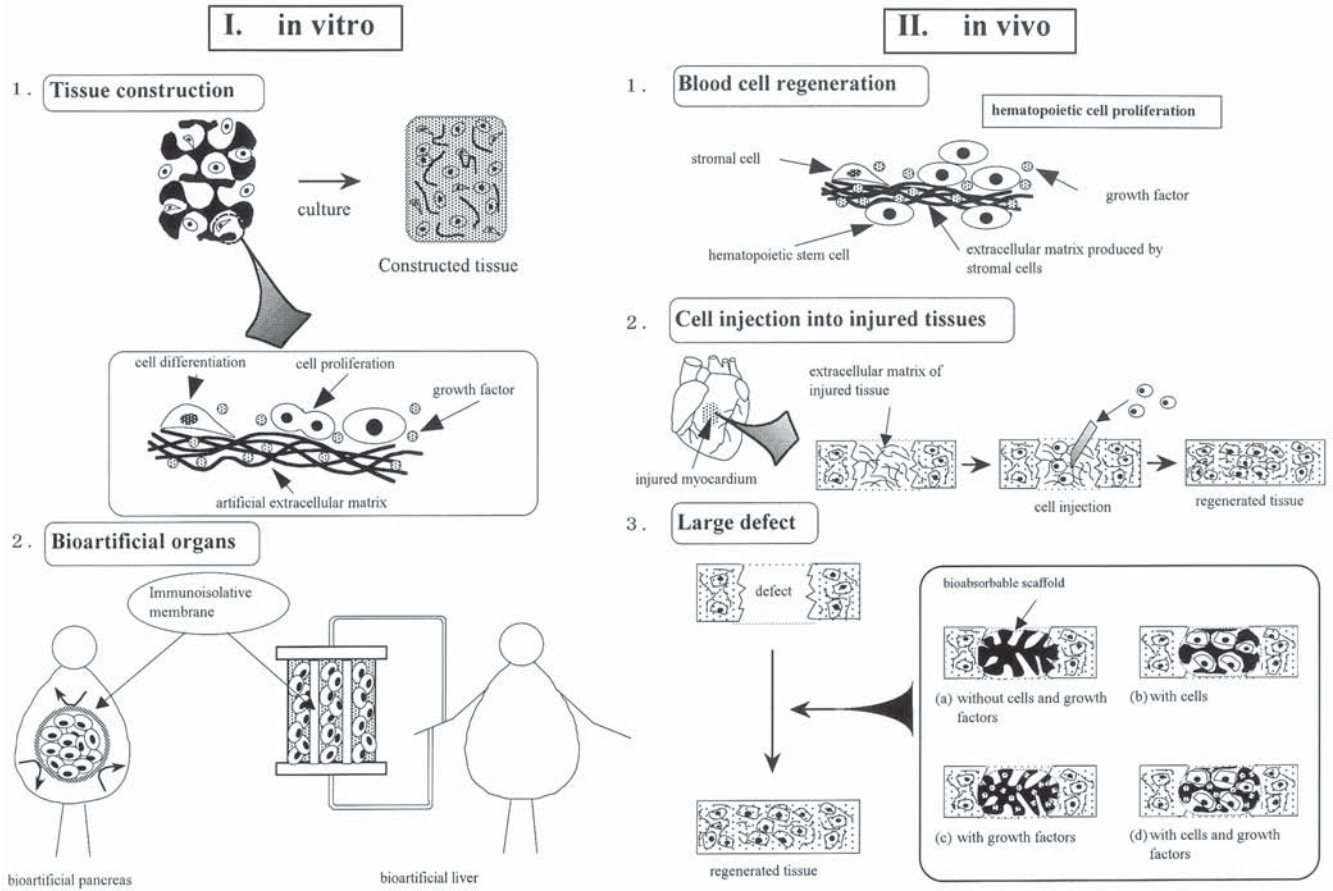

Fig. 1. Basic principle of tissue engineering.

Table 1
Tissues and Organs Being Regenerated or Reconstructed by Tissue Engineering

		Tissue regeneration			Organ substitution
Fabricated site	Cell	Scaffold	Scaffold + growth factor	Barrier membrane	Barrier membrane
In vitro	Necessary	Skin (epidermis + dermis), articular cartilage, bone, artery, myocardium	—	—	Liver, pancreas, kidney
In vivo	Not necessary	Dermis, dura mater, esophagus, trachea	Bone (skull, jaw, long), hair, arteriole, smooth muscle, bladder, periodontal tissue	Peripheral nerve, periodontal tissue, alveolar bone	—
In vivo	Necessary	Skin, cornea, retina, artery, cartilage (fibrous and hyaline), bone (skull, jaw, long), myocardium, trachea, esophagus, small intestine, stomach, smooth muscle, bladder, ureter, central nerve	Mamma, fat, hair, myocardium, liver, kidney	Peripheral nerve	Liver, pancreas, chromaffin cells (angiogenesis)

works well. If the healthy ECM is still available in the body, no artificial scaffold is needed. In addition to bone marrow transplantation, eye-related stem cells are being used for regeneration of defective cornea and retina ***(4)***, and the transplantation of myocardial cells has been experimentally tried for the treatment of myocardial infarction ***(5)***. For the regeneration of a large defect, it is absolutely necessary to use a biodegradable scaffold. The scaffold is implanted with or without cell seeding. For example, sponge form collagen is the most popular material ***(6)***, because a collagen scaffold is compatible with cells and is degraded in the body, hence preventing a physical hindrance to new tissue construction. In vivo tissue engineering using a collagen sponge or a biodegradable polymer sheet with no cell seeding has succeeded in inducing regeneration of the skin dermis ***(7)***, trachea ***(8)***, esophagus ***(9)***, and dura mater ***(10)***.

There are many body tissues that cannot be regenerated unless the scaffold used is seeded with the cells specifically needed for tissue regeneration. Regeneration of epidermis and cartilage necessitates seeding of the scaffold with keratinocytes and chondrocytes, respectively. Cells isolated from blood vessels and small intestines have been combined with biodegradable scaffolds to achieve in vivo regeneration of the respective organs ***(11,12)***. Bone marrow cells have also been widely used to this end; bone marrow cells contain mesenchymal stem cells (MSCs) that can differentiate into the osteocytic lineage ***(13,14)***. It is possible to seed more than one type of cell for regeneration of tissue composed of several subtissues. For example, phalanges and small fingers could be reconstructed by using three different scaffolds combined with periosteum, chondrocytes, and tenocytes for reconstruction of bone, cartilage, and tendon (ligament), respectively ***(15)***.

Sometimes successful tissue regeneration cannot be achieved by merely combining cells and their scaffolds. In such cases, one practical, possible way to promote tissue generation is to use suitable growth factors as well. The type of growth factor depends not only on the target tissue under investigation but also the site where the tissue is expected to generate. Besides the single use of growth factor, sometimes a combination of multiple growth factors with the scaffold preseeded with cells is required to accelerate tissue regeneration.

When a body defect is incurred, the defect space is soon filled with fibrous tissue produced by fibroblasts, which are ubiquitously present in the body and can rapidly proliferate. Once this ingrowth of fibrous connective tissue takes place, further repair or regeneration of other tissues is effectively terminated. To prevent tissue ingrowth, additional biomaterials, known as barrier membranes, are needed. The objective is to make space for tissue regeneration and prevent the undesirable tissue ingrowth, thereby permitting repair of the defect by natural tissue. Some successful examples include guided channels for lost peripheral nerve fibers ***(16)*** and guided regeneration of lost periodontal tissues

and alveolar bone ***(17)***. Barrier membranes should be prepared from biodegradable materials, because they are no longer needed after completion of tissue regeneration. This chapter presents an overview of several research trials on tissue regeneration based on the use of DDS growth factors with or without cells and/or the scaffolds.

2. Materials

An aqueous solution of human recombinant basic fibroblast growth factor (bFGF) with a p*I* of 9.6 (10 mg/mL) was kindly supplied by Kaken (Tokyo, Japan). A gelatin sample with a p*I* of 5.0 (Nitta Gelatin, Osaka, Japan) was extracted from bovine bone (type I collagen) by an alkaline process. $Na^{125}I$ aqueous solution in 0.1 *N* NaOH (NEZ033, 740 MBq/mL) and *N*-succinimidyl-3-(4-hydroxy-3,5-di^{125}I iodophenyl) propionate (^{125}I-Bolton-Hunter reagent, NEX-120H, 147 MBq/mL in anhydrous benzene) were purchased from NEN, DuPont (Wilmington, DE). Glutaraldehyde, glycine, and other chemicals were purchased from Wako (Osaka, Japan) and used without further purification.

3. Methods

3.1. Preparation of Gelatin Hydrogel Incorporating Growth Factor

Gelatin hydrogel was prepared by glutaraldehyde crosslinking of a gelatin aqueous solution. Briefly, after mixing 40 μL of aqueous glutaraldehyde solution (25 wt%) with 40 mL of aqueous gelatin solution (5 wt%) preheated at 40°C, the mixed aqueous solution was cast into a polypropylene tray (18×18 cm^2); the mixture was left for 12 h at 4°C to allow chemical crosslinking of the gelatin. The resulting hydrogel sheet was then punched out to obtain gelatin hydrogel disks (6 mm in diameter, 3 mm thick), and the disks were placed in 50 mL of 100 m*M* glycine aqueous solution, followed by agitation at 37°C for 1 h to block residual aldehyde groups of unreacted glutaraldehyde. The crosslinked hydrogel disks were twice washed with double-distilled water (ddH_2O), freeze-dried, and sterilized with ethylene oxide gas. The water content of gelatin hydrogel (the weight ratio of water present in the hydrogel to the wet hydrogel) was 96.0 wt%, as calculated from the hydrogel weight before and after swelling in phosphate-buffered saline solution (pH 7.4) for 24 h at 37°C.

The original bFGF solution was diluted with ddH_2O to adjust the solution concentration. The aqueous bFGF solution (20 μL) was dropped onto a freeze-dried gelatin hydrogel disk for impregnation of bFGF into the disk. The bFGF solution was completely sorbed into the hydrogel disk at 25°C for 2 h, because the solution volume was less than that theoretically required for the equilibrated swelling of hydrogels. Similarly, empty gelatin hydrogels without bFGF were prepared by adding ddH_2O as the solution.

3.2. Characterization of Gelatin Hydrogel Incorporating Growth Factor

In vivo degradation of gelatin hydrogels was evaluated in terms of the loss of radioactivity of implanted ^{125}I-labeled gelatin hydrogels. Gelatin hydrogels were radioiodinated using ^{125}I-Bolton-Hunter reagent. Briefly, 100 μL of ^{125}I-Bolton-Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene evaporation was completed. Then, 1 mL of 0.1 *M* sodium borate-buffered solution (pH 8.5) was added to the dried reagent to prepare an aqueous solution of ^{125}I-Bolton-Hunter reagent. The aqueous solution was impregnated into freeze-dried disks of gelatin hydrogels at a volume of 20 μL/disk. The resulting swollen hydrogels were kept at 4°C for 3 h to introduce ^{125}I residues into the amino groups of gelatin. The radioiodinated gelatin hydrogels were placed in ddH_2O, which was exchanged periodically at 4°C for 4 d to exclude noncoupled, free ^{125}I-labeled reagent from ^{125}I-labeled gelatin hydrogels. When measured periodically, the radioactivity of the ddH_2O returned to a background level after 3 d of rinsing. The resulting swollen hydrogels were freeze-dried. In vivo degradation of gelatin hydrogels was evaluated in terms of the loss of radioactivity of implanted ^{125}I-labeled gelatin hydrogels. Various types of ^{125}I-labeled gelatin hydrogels were implanted into the back subcutis of ddY mice (three mice per group, 6 to 7 wk old). At 1, 3, 5, 7, 10, 14, and 21 d after hydrogel implantation, the radioactivities of explanted hydrogels were measured on a gamma counter. Next, the mouse back skin around the hydrogel site was cut into a 3 × 5 cm strip, and the corresponding facia site was thoroughly wiped off with filter paper and measured to evaluate the remaining radioactivity of tissue around the implanted hydrogel. The ratio of total radioactivity measured to the radioactivity of the initially implanted hydrogel was expressed as the percentage of remaining activity for hydrogel degradation.

An aqueous solution of ^{125}I-labeled bFGF was sorbed into freeze-dried gelatin hydrogel disks to prepare gelatin hydrogel incorporating ^{125}I-labeled bFGF. ^{125}I-labeled bFGF was prepared according to the chloramines T method reported previously ***(18)***. Various types of gelatin hydrogels incorporating ^{125}I-labeled bFGF were implanted into the backs of mice. An aqueous solution of ^{125}I-labeled bFGF was subcutaneously injected into the backs of mice. At different time intervals, areas of mouse skin containing the implanted hydrogels or directly injected ^{125}I-labeled bFGF were thoroughly wiped off with filter paper in a way similar to that just described. The radioactivities of the residual gelatin hydrogels and the skin strip plus filter paper were measured on a gamma counter, and their radioactivity ratios to the bFGF initially used were expressed as the percentage of remaining activity for in vivo bFGF release.

Recently, much research has been devoted to tissue regeneration through combinations of growth factors with various carrier materials (**Table 2**).

Table 2
Experimental Trials for Tissue Regeneration by Combination of Growth Factor With Carrier[a]

Growth factor	Carrier	Animal	Tissue regenerated
BMP	PLA	Dog	Long bone
	Collagen sponge	Rat	Long bone
		Dog, monkey	Periodontal ligament and cementum
	β-TCP	Rabbit	Long bone
	Porous HA	Rabbit	Skull bone
rhBMP-2	Porous PLA	Dog	Spinal bone
		Rat	Skull bone
	PLA microsphere	Rabbit	Skull bone
	Collagen sponge	Dog	Periodontium
	Gelatin	Rabbit	Skull bone
	PLA-coating gelatin sponge	Dog, monkey	Long bone, jaw bone, skull bone
	Porous HA	Monkey	Skull bone
	PLA-PEG copolymer	Rat	Long bone
rhBMP-7	Collagen	Dog	Spinal bone
		Dog	Long bone
EGF	Agarose	Hamster	Angiogenesis
	PVA	Rat	Dermis
aFGF	PVA	Mouse	Angiogenesis
	Alginate	Mouse	Angiogenesis
bFGF	Alginate	Mouse	Angiogenesis
	Agarose/heparin	Mouse, pig	Angiogenesis
	Amylopectin	Mouse	Angiogenesis
	Gelatin	Mouse	Angiogenesis, dermis, adipogenesis
		Rabbit, monkey	Skull bone
		Dog	Nerve
	Fibrin gel	Mouse	Angiogenesis
	Collagen minipellet	Rabbit	Long bone
	Collagen	Mouse	Cartilage
	Poly(ethylene-co-vinyl acetate)	Rat	Nerve
NGF	Collagen minipellet	Rabbit	Nerve
	PLGA	Rat	Nerve
TGF-β1	PEG	Rat	Dermis
	Gelatin	Rabbit	Skull bone
	Plaster of Paris, PLGA	Rat	Skull bone
	TCP	Dog	Long bone
	Porous HA	Dog	Long bone
	Collagen	Baboon	Skull bone
		Mouse	Dermis
PDGF-BB	Porous HA	Rabbit	Long bone
	Collagen	Rat	Dermis
	Chitosan	Rat	Periodontal bone
VEGF	Collagen	Mouse	Angiogenesis
	Alginate	Mouse	Angiogenesis
HGF	Gelatin	Mouse	Angiogenesis
IGF-1	PLGA-PEG	Rat	Adipogenesis
IGF-1/bFGF	PLGA-PEG	Rat	Adipogenesis
PDGF/IGF-1	Titanium implant	Dog	Jaw bone

[a]aFGF, acid fibroblast growth factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; EGF, epidermal growth factor; HA, hydroxyapatite; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; PDGF-BB, platelet-derived growth factor-BB; PEG, poly(ethylene glycol); PLA, polylactide; PLGA, glycolide-lactide copolymer; PVA, poly(vinyl alcohol); rhBMP, recombinant human bone morphogenetic protein; TCP, tricalcium phosphate; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

All results claim the necessity of combining the growth factors with carriers to induce in vivo tissue regeneration. In addition to proteinaceous growth factor, the gene encoding growth factor has recently been applied to promote tissue regeneration ***(19)***. If the corresponding gene is transfected into the cells existing in the site of regeneration, it is highly possible that the cells can secrete the growth factor for a certain time period, resulting in promoted tissue regeneration. The angiogenetic therapy of ischemic diseases ***(20)*** and bone tissue regeneration ***(21)*** have been attempted by using the corresponding growth factor genes.

4. Notes

1. *Characteristics of gelatin hydrogel for controlled release of growth factor.* One of the largest problems in protein release technology is the loss of biological activity of proteins released from protein-carrier formulations. It has been demonstrated that this loss of activity results mainly from denaturation and deactivation of proteins during preparation of carrier formulations. Therefore, a method to prepare protein release carriers with inert biomaterials should be exploited to minimize protein denaturation. From this viewpoint, a polymer hydrogel may be a preferable candidate for use as a protein release carrier because of its biocompatibility and its high inertness toward protein drugs.

   We have created a release system for growth factors that mimics the native mode of growth factor delivery in the living body. **Figure 2** shows a conceptual illustration for the controlled release of growth factor from a biodegradable polymer hydrogel based on physicochemical interaction forces between the growth factor and polymer molecules. For example, a hydrogel is prepared from a biodegradable polymer with negative charges. The growth factor with a positively charged site is electrostatically attracted to the polymer chain and thereby is physically immobilized in the hydrogel carrier. If an environmental change, such as increased ionic strength, occurs, the immobilized growth factor is released from the factor-carrier formulation. Even if such an environmental change does not take place, degradation of the carrier itself also leads to growth factor release. Because the latter is more likely to happen in vivo than the former, it is preferred that the release carrier be prepared from biodegradable polymers. This complexation protects the growth factor from denaturation and enzymatic degradation in vivo.

   As the material for growth factor release, we have selected gelatin because it has the desired physicochemical properties and has been extensively used for industrial, pharmaceutical, and medical purposes. The biosafety of gelatin has been proven through its long clinical uses. Another unique advantage is the electrical nature of gelatin, which can be changed by the processing method. For example, an alkaline process of collagen results in hydrolysis of amide groups of the asparagine and glutamine residues, having a high density of carboxyl groups, which makes the gelatin negatively charged. The p*I* of gelatin is about 5.0.

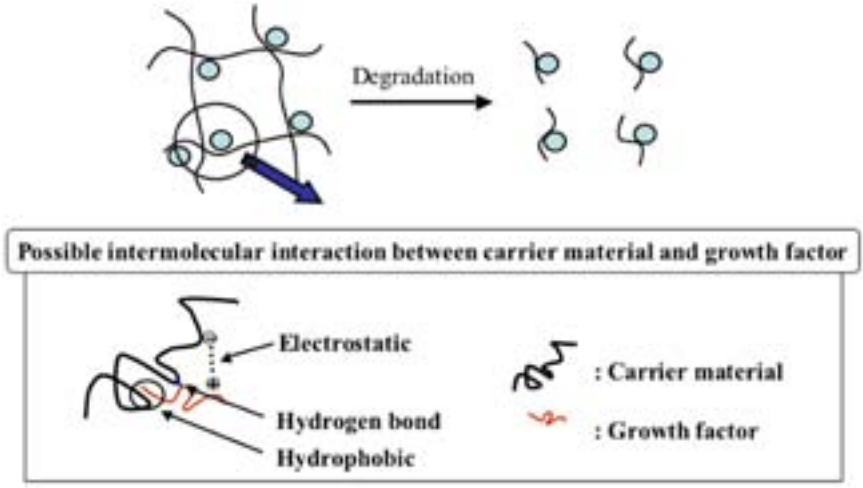

Fig. 2. Conceptual illustration of growth factor release from a biodegradable hydrogel based on physical interaction forces.

By contrast, the nature of a gelatin is much different for an acidic process. A positively charged gelatin of "basic" type is prepared and the p*I* is about 9.0. If a growth factor to be released has the positively charged site in the molecule that interacts with acidic polysaccharides present in the ECM, the negatively charged gelatin of "acidic" type is preferable as the carrier material. Considering the electrostatic interaction, the "basic" gelatin is preferable for release of molecules with the negative charged site. It was found that, as expected, bFGF, transforming growth factor-β1 (TGF-β1), or platelet-derived growth factor was sorbed into the acidic gelatin hydrogel mainly owing to the electrostatic interaction ***(22)***.

Animal experiments revealed that the hydrogels prepared from the acidic gelatin were degraded in the body ***(23)***. The degradation period of hydrogels depends on their water content, which is a measure of crosslinking extent: the higher the water content of the hydrogels, the faster their in vivo degradation. The water content of hydrogels increased with increasing concentrations of chemical crosslinking agents and gelatin concentration used in the preparation of the hydrogels. The time profile of in vivo bFGF retention was in accordance with that of hydrogel degradation, irrespective of the hydrogel biodegradability. It seems reasonable to suppose that bFGF was released from the gelatin hydrogel along with degraded gelatin fragments in the body as a result of hydrogel degradation. These findings strongly indicate that growth factor release is governed mainly by hydrogel degradation, as described in **Fig. 2**. As a result, in this release system, the release period is not influenced by the hydrogel's shape at all, but controllable only by changing the degradation rate of hydrogel ***(24)***. Note that gelatin hydrogels can be formed into different shapes of disks, tubes, sheets, granules, and microspheres ***(24,25)***.

2. *Tissue regeneration by gelatin hydrogel incorporating bFGF*. As described in the previous section, the gelatin hydrogel was found to be a superior carrier for the controlled release of growth factor. Hereafter, concrete experimental results on angiogenesis, bone regeneration, and adipogenesis are described as achieved by this release system alone or in combination with stem cells.
3. *Angiogenesis*. bFGF has been reported to have a variety of biological functions ***(26)*** and to be effective in enhancing wound healing through induction of angiogenesis and regeneration of bone, cartilage, and nerve tissue. Among its biological actions, the gelatin hydrogel was effective in enhancing the in vivo angiogenic effect of bFGF. When gelatin hydrogels incorporating bFGF were subcutaneously implanted into a mouse's back, the angiogenic effect was observed around the implanted site, in marked contrast to the sites implanted with bFGF-free, empty gelatin hydrogels or injected with an aqueous solution of bFGF ***(22)***. No angiogenesis was induced by the injection of bFGF solution even when the dose was increased to 1 mg/site. This result must be owing to a rapid elimination of bFGF from the injection site ***(27)***. By contrast, the gelatin hydrogel incorporating bFGF induced significant angiogenesis even when the dose was as low as 30 μg/site. The maintenance period of the hydrogel-induced angiogenic effect could be changed by prolonging the hydrogel's water content as the water content became lower ***(27)***. It is likely that the hydrogels with lower water contents were more slowly degraded and consequently released bFGF of biological activity in vivo less rapidly than those with higher water contents, leading to a prolonged angiogenic effect. A similar enhanced and prolonged angiogenic effect was also observed when using gelatin hydrogels incorporating bFGF of the microsphere type ***(24)***.

   The technology to induce artificially in vivo angiogenesis is indispensable for tissue engineering. Two objectives of angiogenesis induction include the therapy of ischemic disease and advanced angiogenesis for cell transplantation. As an example of the former, the therapy of ischemic myocardium by gelatin hydrogels incorporating bFGF is introduced here. Myocardial infarction was induced by ligating the left anterior descending (LAD) coronary artery of dog heart. Gelatin microspheres incorporating bFGF were intramuscularly injected into both sides of the LAD 10 mm distal from the ligated site. As a control, an aqueous solution of bFGF at the same dose level was injected. Injection of the gelatin microspheres containing bFGF induced regeneration of collateral coronary arteries at the site of ligated LAD and increased the blood flow in the left circumflex coronary artery (LCX) (**Fig. 3**). More interesting, the injection of microspheres was also effective in recovering the motion of myocardium in the ischemic region. Neither of these therapeutic effects were observed for the injection of bFGF solution at the same dose level ***(1)***.

   There is no doubt that a sufficient supply of nutrients and oxygen to the cells transplanted in the body is indispensable for cell survival and the maintenance of biological functions. Without sufficient supply, cells preseeded in a scaffold for tissue regeneration would hardly survive following implantation of the scaffold

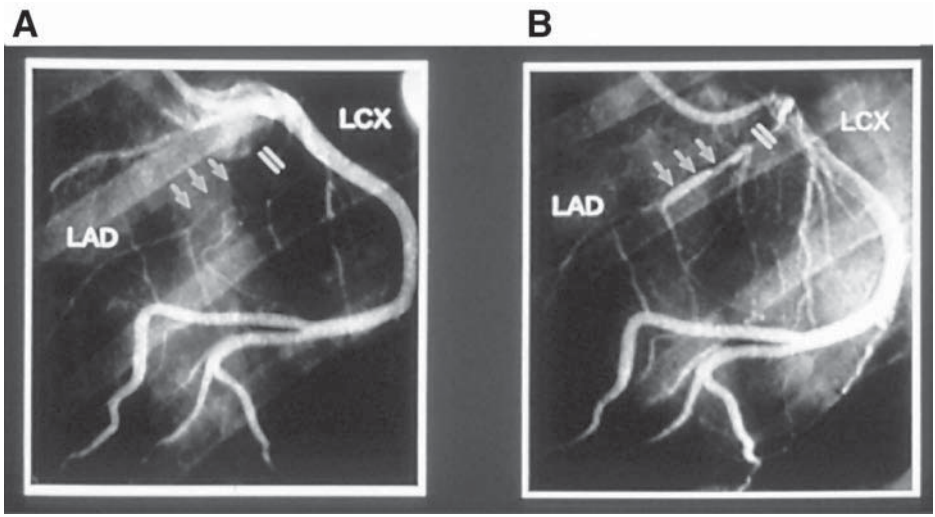

Fig. 3. Left coronary angiograms of ischemic dog heart 1 wk after intramyocardial injection of **(A)** bFGF solution and **(B)** gelatin microspheres incorporating bFGF. bFGF was bilaterally injected at the distal side of the LAD ligated portion (indicated by Mark II) at a dose of 100 μg/heart. The hydrogel water content was 95.0 wt%.

into the body. Such a situation is caused by allo- or xenogeneic cells transplanted into the body for organ substitution. For successful cell transplantation, the nutrient and oxygen supply is the dominant challenge relative to immunoisolation. For both of the supplies, it is promising to induce angiogenesis throughout the transplanted site of cells by using angiogenic growth factors. In a recent study, pancreatic islets were encapsulated by a hydrogel bag effective for immunoisolation and implanted into the sc tissue of streptozotocin-induced diabetic mice. Advanced angiogenesis at the site of cell transplantation induced by gelatin microspheres containing bFGF enabled the encapsulated islets to improve the survival rate, resulting in a prolonged maintenance period of normal glucose level in the blood (**Fig. 4**) ***(28)***. This finding demonstrates that in vivo angiogenesis induced by the gelatin microspheres containing bFGF could be achieved in the subcutis of even diabetic mice, which have an inferior injury-repairing capability relative to healthy mice. It is of prime importance to induce tissue regeneration even in the bodies of patients who have diseases or are elderly. Little tissue engineering research has been performed on aged animals. This area of study will undoubtedly become important when considering clinical applications for tissue engineering. This angiogenic effect for the prolonged cell survival was observed for transplantation of hepatocytes ***(29)*** and cardiomyocytes ***(30)***.

4. *Bone regeneration*. Gelatin hydrogels incorporating bFGF were found to have a promising potential for bone repair ***(31,32)***. For example, when implanted into a monkey skull defect, the gelatin hydrogel incorporating bFGF promoted bone

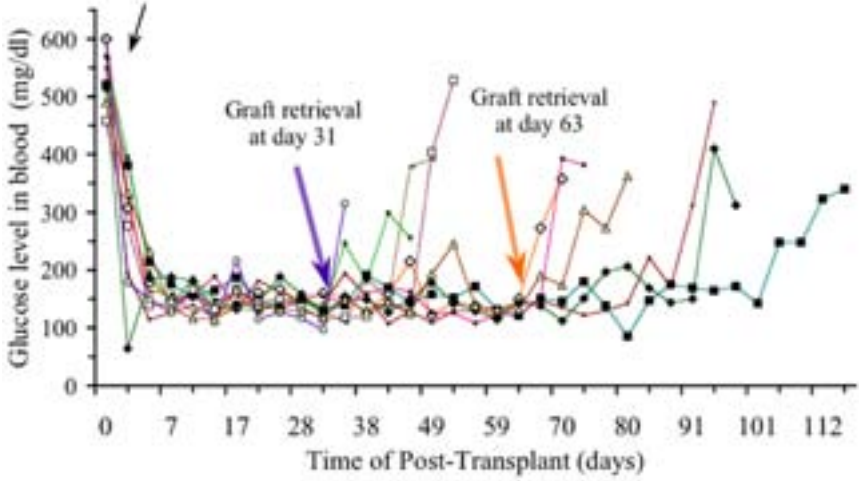

Fig. 4. Time course of glucose level in blood of diabetic mice after sc xenograft of rat pancreas islets encapsulated by an agarose/PSSa hydrogel membrane into vascularized site induced by advance injection of gelatin microspheres containing bFGF (small arrow, transplantation). Two of 10 recipients became hyperglycemic again when the grafts were respectively retrieved at d 31 (○, *, and □) and d 63 (◇, ×, △, +, ◆, and ■) (large arrows). This strongly indicates that the encapsulated islets functioned normally in the subcutis of diabetic mice. The average normoglycemic period was 68.4 ± 25.6 d.

regeneration at the defect and closed the defect by 21 wk after implantation. By contrast, both the use of bFGF-free gelatin hydrogels and the use of a similar dose of bFGF in the solution resulted in a total lack of bone regeneration, and a remarkable ingrowth of soft connective tissues at the bone defect. Measurement of bone mineral density (BMD) at the skull defect revealed that gelatin hydrogels containing bFGF enhanced the BMD to a significantly higher extent than did free bFGF, irrespective of the hydrogel water content. The BMD resulting from the bFGF-free gelatin hydrogel implant was similar to that of the untreated group, indicating that the presence of hydrogel did not impair bone healing at the defect. In a histological study, hydrogel implantation increased the number of osteoblasts residing near the edge of the bone defect and retained it at a significantly high level over the time range studied.

It is known that both TGF-β1 and bone morphogenetic protein (BMP) also promote bone regeneration ***(33–36)***. We have succeeded in repairing bone and skull defects of rabbits and monkeys by the controlled release of TGF-β1 from gelatin hydrogels, in marked contrast to the use of free TGF-β1 even at higher doses ***(34)***. However, the degree of repair depended on the water content of hydrogels, which could be reduced or increased. It is possible that too rapid degradation of the hydrogel causes a short period of bFGF release, resulting in no

induction of bone regeneration. Conversely, a long-term residue of hydrogels owing to slow degradation would physically hinder bone regeneration. As a result, it is likely that the hydrogel with an optimal biodegradability induced complete bone regeneration at the skull defect ***(34)***. As with previously described studies, hydrogels function as carriers of growth factors as well as barriers to prevent the ingrowth fibrous tissues into bone defects. Balance of the time course between the two hydrogel functions would result in better bone repairing. We have recently succeeded in the controlled release of BMP-2 by hydrogels of a gelatin type. This controlled-release system enabled BMP-2 to induce formation of bone tissue ectopically or orthotopically at doses lower than used for the application of free BMP-2 in solution.

There are some cases in which a combination of the controlled release of growth factor and stem cells is effective in achieving bone repair. In one trial, we utilized cells with osteogeneic potentials and combined them with the growth factor release system. MSCs were isolated from the bone marrow of a rabbit fibula. We demonstrated that application of a combination of MSCs and gelatin microspheres containing TGF-β allowed completely repaired defects in rabbit skulls by newly formed bone tissue, in marked contrast to that of either material used alone ***(37)***. In this case, however, the TGF-β release system when used alone was not effective because the dose was too low.

5. *Adipogenesis*. When gelatin microspheres incorporating bFGF were mixed with a basement membrane extract (Matrigel) and subcutaneously implanted into a mouse's back, *de novo* formation of adipose tissue was observed at the implanted site ***(38)***. Recently, we also succeeded in inducing *de novo* adipogenesis by combining preadipocytes isolated from fat tissues, gelatin microspheres incorporating bFGF, and a collagen sponge (**Fig. 5**). When the preadipocytes and the microspheres were placed into the collagen sponge and implanted into the back subcutis of a mouse, *de novo* formation of adipose tissue was observed at the implanted sponge site. Combination of all three materials was needed to induce this adipogenesis ***(39)***. These results experimentally justify the strategy of in vivo tissue engineering asserting that tissue regeneration can be achieved by creating a suitable environment in the body site to be regenerated.
6. *Conclusion*. For regeneration of body tissues, a variety of growth factors act on cells by forming a complex network while the action timing, action site, and concentration of growth factors are delicately regulated in the body. It is likely that the mechanisms of tissue regeneration in living systems will be clarified with rapidly advancing progress in cell biology, molecular biology, and embryology. Even so, it will be impossible to imitate living systems by solely making use of the scientific knowledge and technologies currently available. However, clarification of living mechanisms will help researchers to understand which growth factor is key to induce the regeneration of a target tissue. If such a key growth factor is supplied to the necessary site at a suitable time period and concentration, I believe that the living body will be stimulated toward the process of natural tissue regeneration. Once the right direction toward tissue regeneration is

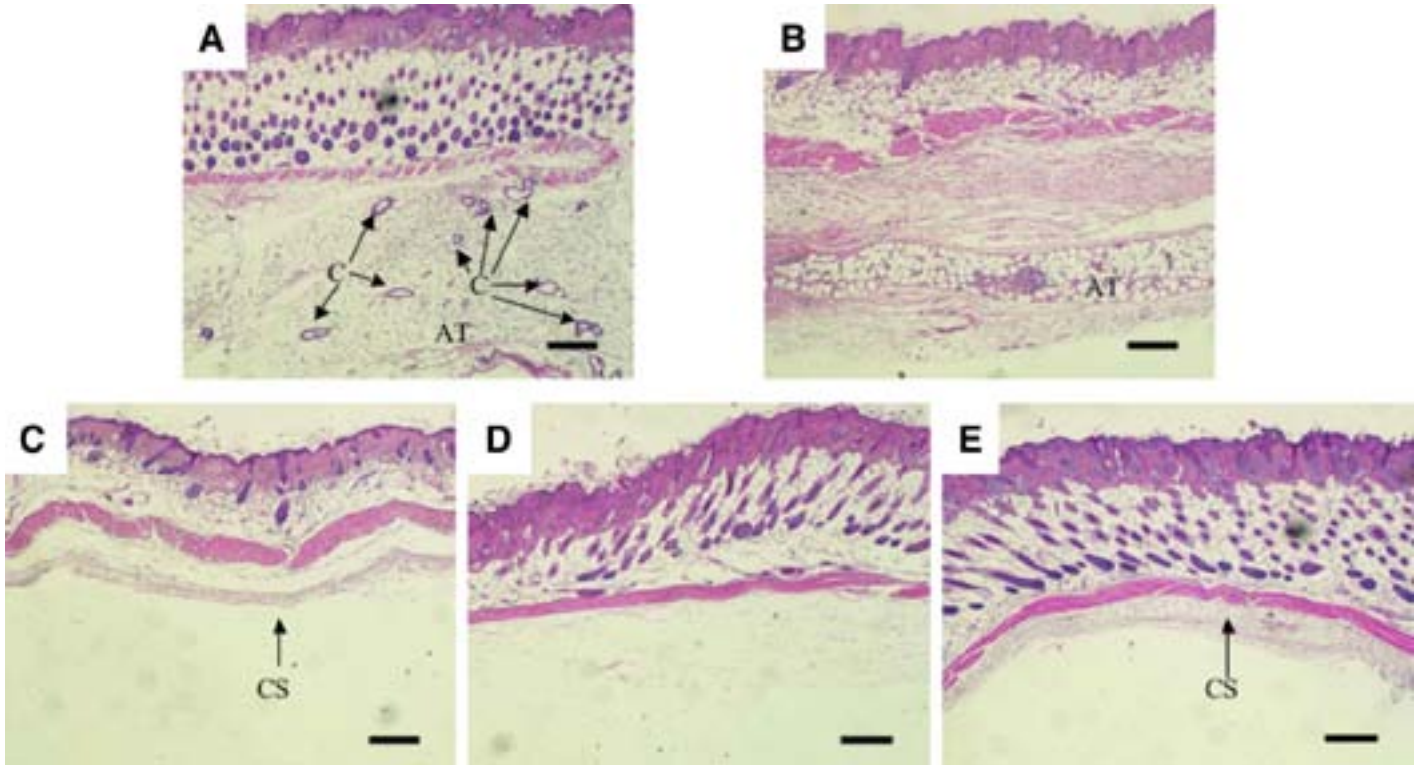

Fig. 5. *De novo* formation of adipose tissue in mouse subcutis 6 wk after implantation of a collagen sponge containing a mixture of preadipocytes and gelatin microspheres incorporating bFGF: **(A)** collagen sponge containing mixture of preadipocytes and gelatin microspheres incorporating bFGF; **(B)** collagen sponge containing mixture of preadipocytes and free bFGF; **(C)** collagen sponge containing preadipocytes; **(D)** mixture of preadipocytes and gelatin microspheres incorporating bFGF; **(E)** collagen sponge containing gelatin microspheres incorporating bFGF. (Magnification: ×100; Sudan III staining). The gelatin microspheres incorporating bFGF were completely degraded to disappear from the injected site. The bFGF dose was 10 µg/site and the hydrogel water content was 95.0 wt%. Bar = 300 µm. CS, collagen sponge; AT, adipose tissue newly formed; C, capillary newly formed.

taken, the intact system of the body will start to act and function, resulting in automatic achievement of tissue regeneration. There is no doubt that as long as growth factors are used, their controlled release will be an essential technology in the future. Recently, bFGF has been on the Japanese market as a therapeutic agent for skin ulcer and decubitus. I expect that this will be a cue to encourage clinical application of tissue regeneration based on growth factor DDS technology.

If tissue engineering matures to be the third choice of therapeutic medicine relative to reconstructive surgery and organ transplantation, it will give patients many therapeutic choices and privileges. To this end, substantial collaborative efforts among researchers in materials, pharmaceutical, biological, and medical sciences, and clinical medicine are needed to reach academic and technical maturity in tissue engineering. Because tissue engineering is still in its infancy, it will take much more time before its full potential is realized. Without the cell scaffold and DDS technologies to induce tissue regeneration, any developmental results of medicine, biology, and molecular biology regarding stem cells will never be realized in medical therapy for patients, which is the final goal of "regenerative" medicine. Tissue engineering is one of the indispensable tools to make regenerative medicine clinically available. Little DDS research aiming at tissue regeneration as well as organ substitution has been conducted. I am confident that the majority of the readers of this chapter will get a better understanding of the magnified significance of biomaterials as well as DDSs in the future progress of tissue engineering. It is hoped that this article will increase readers' interest in this research field.

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