Chapter 11

Tissue Regeneration Based on Drug Delivery Technology

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Summary

Tissue engineering (TE) is one of the biomedical technologies developed to assist the regeneration of body tissues to treat large size defects that are not possible to self-repair. TE may also help to substitute the biological functions of damaged organs by making use of cells. Although there is no doubt that cells are important for this purpose, an artificially created site to induce repair of the defect is a key factor for successful tissue regeneration. This can be achieved only by utilizing an artificial scaffold of 3-dimensional structure for cell proliferation and differentiation as well as growth factors. Growth factors are often required to promote tissue regeneration. They also can induce angiogenesis which is required to supply oxygen and nutrients for the survival of the transplanted cells. However, one cannot always expect the biological effects of growth factors to be fully exerted because of poor in vivo stability, unless growth factor delivery technology is applied. This paper describes recent experimental data on tissue regeneration that emphasize the role of drug delivery technology in tissue engineering, briefly overviewing biodegradable polymers used for this purpose.

Keywords: Controlled Release, Drug Delivery, Growth Factor, Tissue Engineering, Tissue Regeneration

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What is Tissue Engineering?

When a tissue or organ is severely injured, largely lost or functionally impaired, it is clinically treated either by reconstructive surgery or organ transplantation. Reconstructive surgery is accomplished with the use of bio-materials or biomedical devices. Biomedical devices which are made from man-made materials alone cannot perform all of the functions of a single organ and therefore, cannot prevent progressive deterioration. Although these therapies have saved and improved countless lives of patients, they remain imperfect solutions. One of the largest problems for organ transplantation is the lack of donor tissues or organs. Additionally, the use of immuno-suppressive agents causes various side-effects, such as virual infections and carcinogenesis. One promising approach to tackle these problems is to enable the self-healing potential of the body to regenerate lost or damaged tissues and organs. A biomedical field of engineering to achieve this new therapeutic approach is "tissue engineering" (Figure 1). The objective of tissue engineering (TE) is to regenerate tissues as well as creating biological substitutes for defective or lost tissues and organs by making use of cells.

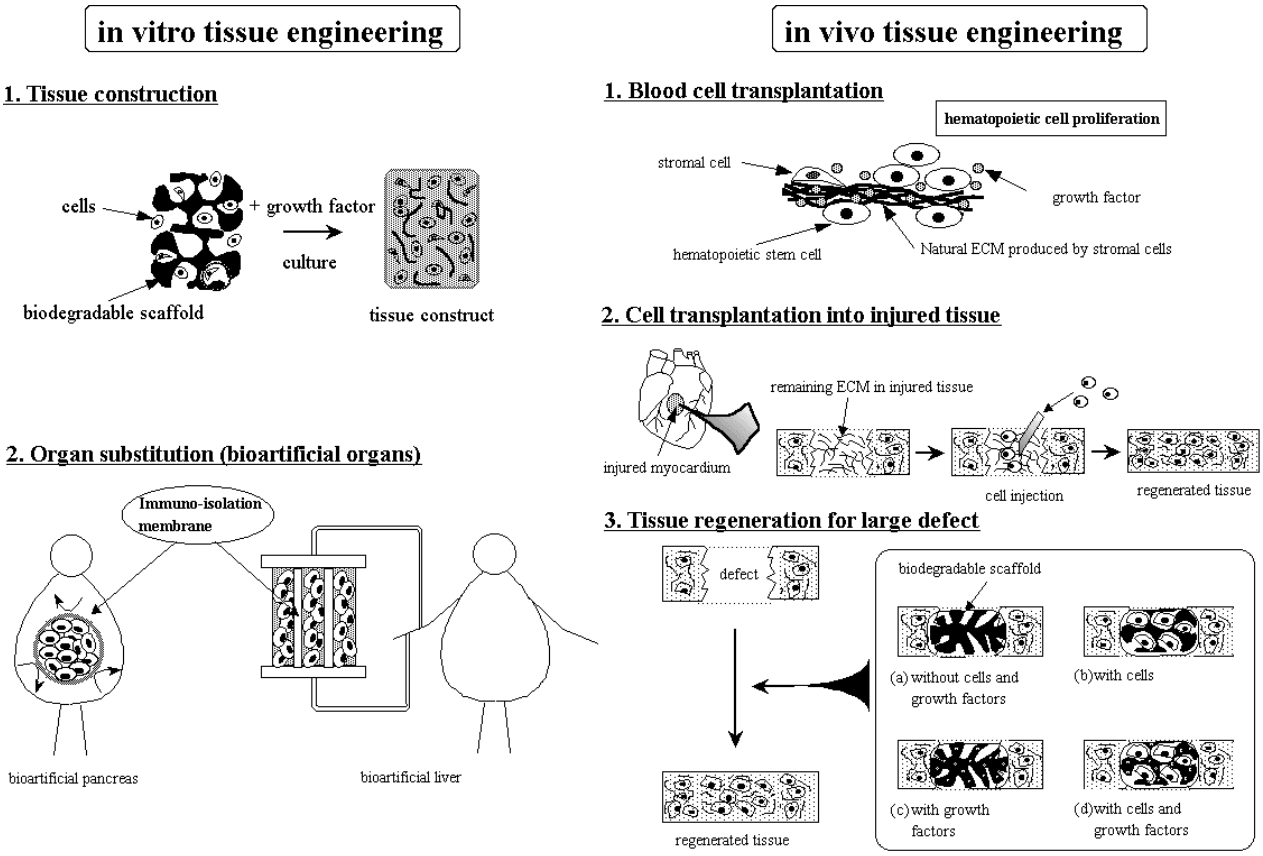


Fig. 1. Basic principle of TE

Factors Necessary for Tissue Engineering

How can a defective or lost tissue be regenerated? It is undoubtedly necessary for tissue regeneration to increase the number of cells constituting the tissue as well as re-construct a structure to support proliferation and differentiation of cells, so-called extracellular matrix (ECM). In addition, growth factors are often required to promote tissue regeneration, depending on the type of tissues to be regenerated. The mechanism of tissue regeneration has been currently elucidated at the cellular and genetic levels and found to be fundamentally similar between newt hydra and human (1). In either case, the stem cells of multipotential and proliferation ability play a key role in tissue regeneration.

Factors necessary for TE include cells, scaffolds for cell proliferation and differentiation, and growth factors. Cells currently used for tissue regeneration do not need always to be stem cells. It is possible to utilize precursor or blastic cells (2-4) as a cell stage which is intermediate between stem cells and maturing cells. It would be ideal if we can obtain cells from patients themselves, but cell harvest from individual patients is not always easy. Consequently, one can use allogenic cells although care should be taken for immuno-isolation. Embryonic stem (ES) cells and clone-engineered cells are also of high potentiality, but the ethical issue should be carefully considered for their clinical use. It has been well recognized that the ECM is not only a physical support of cells but it also has an important influence on the cell proliferation and differentiation or morphogenesis which contributes to tissue regeneration and organogenesis. Thus, it is unlikely that a large-size tissue defect will be naturally regenerated and repaired only by supplying cells to the defect. For example, one possible way is to provide a site suitable for induction of tissue regeneration in the defect by placing a scaffold as an artificial ECM which supports cell attachment and the subsequent proliferation and differentiation, resulting in promoted induction of tissue regeneration. Some researches have demonstrated (5, 6) that endogenous cells residing around the scaffold or cells pre-seeded in the scaffold proliferate and differentiate within it if the artificial ECM is appropriate for the cells. Once a new tissue is regenerated, it should eventually produce its own healthy ECM. The scaffold remains *in situ* for some time and it may causes physical hindrance during the process of tissue regeneration. The third factor necessary for TE is the use of growth factors. However, direct injection of growth factors in

the solution form into the regeneration site is generally not effective, as the injected growth factors diffuse rapidly out from the site or get decactivated. To enable the growth factors to efficiently exert their biological effects, the use of technologies of drug delivery that are practically available is warranted. One promising technique is the controlled release of growth factors at their intended site of action over an extended period of time by incorporating the growth factors into an appropriate carrier. It is also highly possible that the growth factors are protected against proteolysis, as long as, at least, they are incorporated in their carrier, prolonging retention of their activity *in vivo*. The release carrier should be degradable in the body since it is not needed any more after the growth factors release is completed. Thus, it is a key to success of TE to create an environment suitable for induction of tissue regeneration by the use of scaffolds and drug delivery technologies. Regenerative medicine for patients based on the idea of TE will be difficult to achieve without the control of the regeneration environment. Many materials have been investigated for medical and pharmaceutical applications. Considering the scaffold and drug delivery applications described above, it is undoubtedly preferable to take advantage of available biodegradable materials for TE.

Biodegradable Materials for Tissue Engineering

Table 1 summarizes synthetic and natural polymers of biodegradable nature. The synthetic biodegradable polymers that have been clinically used are the homopolymers and copolymers of lactide as well as copolymers of glycolide and lactide. Their biodegradation pattern can be controlled by changing the molecular weight and copolymer compositions, which is described in another chapter of this book in more details. One type of poly(anhydrides) and poly(ε-cyanoacrylates) is being currently used as the carrier of an antitumor agent and as a surgical adhesive, respectively. Other synthetic polymers have been experimentally investigated aiming at their biomedical and pharmaceutical applications. Among natural polymers, proteins (collagen, gelatin, fibrin, and albumin) and polysaccharides (chitin, hyaluronic acid, cellulose and dextran) have been medically and pharmaceutically employed. Generally the degradation of polymers is driven by hydrolytic and enzymatic cleavage of their main chain. Most of synthetic polymers are

fundamentally degraded by hydrolysis although poly(amino acids) show enzymatic degradation. On the contrary, the natural polymers are all degraded enzymatically. There is a degradation manner in which a polymer becomes water-soluble with the chemical elimination of side chains, and consequently disappears from the implanted site. Natural polymers are often used as hydrogels that are prepared through the crosslinking of these polymers. There are some biodegradable ceramics, for example tricalcium phosphate and calcium carbonate. Hydroxyapatite is not degraded in the body because of its extremely low solubility in water.

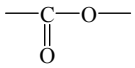
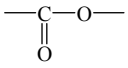
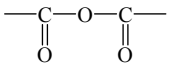
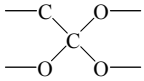
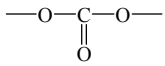
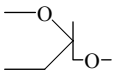
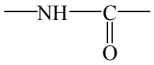
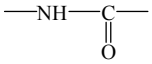
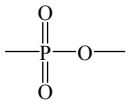
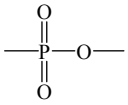
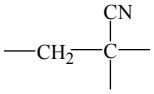
Synthetic polymer		Natural polymer (animals · plants · microbes)	
The name and structure of chemical bond	Example	The name and structure of chemical bond	Example
Ester 	polylactide, polyglycolide, lactide-glycolide copolymer, poly(e-caprolactone), poly(p-dioxane), poly(ß-malic acid)	Ester 	poly(ß-hydroxybutyrate), poly(malic acid)
Anhydride 	poly(anhydrides)		
Ortho ester 	poly(ortho esters)		
Carbonate 	polycarbonates	Glycoside (Polysaccharide) 	chitin, chitosan, hyaluronic acid, pectin, pectic acid, galactan, starch, dextran, pullulan, agarose, heparin, alginate, chondroitin-6-sulfate
Phosphazene	poly(phosphazenes)		
Peptide 	poly(amino acids)	Peptide (Protein) 	collagen, gelatin, fibrin, albumin, gluten, polypeptides, elastin, fibroin, enzyme
Phosphoric ester 	poly(phosphoric ester-urethanes)	Phosphoric ester (Nucleic acid) 	deoxyribonucleic acid (DNA) ribonucleic acid (RNA)
Carbon-carbon 	poly(cyanoacrylates)		

Table 1. Chemical structure of biodegradable synthetic and natural polymers

Classification of Tissue Engineering

TE is classified into two categories in terms of the place to perform TE: *in vitro* and *in vivo* TE (Table 1). The *in vitro* TE involves tissue regeneration and organ substitution (bioartificial organs) outside the body, in the lab.

In Vitro Tissue Engineering

If a tissue can be constructed *in vitro* in factories or laboratories on a large scale, we can supply tissue constructs to patients whenever needed. This is an ideal therapy and available for commercialization. However, for the *in vitro* TE, it is highly indispensable to arrange an environment required for tissue reconstruction by providing all the essential materials. This is in contrast to the *in vivo* TE, where most of the materials necessary for tissue regeneration are automatically supplied by the host living body. Therefore, reconstruction of only a few tissues has been attempted *in vitro*, e.g. skin (dermis and epidermis), articular cartilages, and arteries. An artificial skin product composed of human dermal and epidermal layers has been prepared *in vitro* (7). This is applicable not only to skin regeneration but also to the skin analogue used for drug testing.

Another application of *in vitro* TE is the substitution of organ functions by the use of allo- or xeno-geneic cells. Such engineered organs are called bioartificial organs because they are composed of heterogenic cells and man-made membranes or porous constructs for immuno-isolation to protect the cells from the host response and maintain the cell function. Liver and pancreas in particular have attracted the attention of researchers working on bioartificial organs (8).

In Vivo Tissue Engineering

In vivo TE can be achieved with or without the use of biodegradable scaffolds. If the healthy ECM is still available in the body, no artificial scaffolds are needed. Following intravenous injection into patients with leukemia, hemotopoietic stem cells isolated from the human blood can differentiate into functional cells in the bone marrow (blood cell transplantation). Stem cells are also being explored for use for regeneration of defective cornea and retina (9) while the transplantation of

myocardial cells has been attempted for the therapy of myocardial infarction (10). However, for the regeneration of a large-size defect, it is necessary to use a biodegradable scaffold. The scaffold is implanted with or without cell seeding. One of the most popular scaffolds used in TE is the collagen sponge (11). The sponge functions as a biodegradable scaffold for the proliferation of cells originating from the surrounding healthy tissue with subsequent secretion of natural collagen. Collagen scaffolds should degrade in the body in a way that does not lead to a physical hindrance of the newly-regenerated tissue. TE approaches using collagen sponges or other biodegradable polymeric sheets with no cell seeding have been tried for regeneration of the skin dermis (12), trachea (13), esophagus (14), and dura mater (15).

Most of the body tissues are not able to regenerate unless the scaffold used is seeded with cells. Regeneration of epidermis and cartilage necessitates seeding of the scaffold with keratinocytes and chondrocytes, respectively. Cells isolated from blood vessels or small intestine combined with biodegradable scaffolds were used for *in vivo* regeneration of the respective organs (16, 17). Bone regeneration readily takes place by using bone marrow cells together with scaffolds (18). This is because bone marrow cells contain mesenchymal stem cells that can differentiate into osteocytic lineage (19). It is possible to seed more than one cell type for regeneration of tissues composed of several cell types. For example, phalanges and small fingers could be reconstructed by seeding three different scaffolds with periosteum, chondrocytes, and tenocytes for bone, cartilage, and tendon (ligament), respectively (20).

There are some cases in which growth factors are required for the *in vivo* TE. The type of growth factors used depends on the type of tissue to be engineered and on the site to which the tissue needs to be used. Further addition of growth factors to scaffolds seeded with cells will accelerate the process of tissue regeneration. Examples of this approach are described below.

When a body defect develops, the defect space will be soon filled with fibrous tissue (repair) produced by fibroblasts which are ubiquitously present in the body and can rapidly proliferate. Once this ingrowth of fibrous tissue into the defect space takes place, no tissue regeneration can be expected to take place any more. To prevent such a fibrous tissue ingrowth, barrier (guided tissue regeneration) membranes can be used. The objective of such membranes is to make space for

tissue regeneration and prevent the undesirable fibrous tissue ingrowth, resulting in simultaneously preferred regeneration of the defective tissue. Examples include guided regeneration of peripheral nerves (21) and guided regeneration of lost periodontal tissues and alveolar bone (22). Membranes used for such purpose should generally be prepared from biodegradable materials (Table 1), as they are no longer needed after the completion of tissue regeneration.

Tissue Engineering by the Use of Growth Factor Release

If the tissue to be repaired has a high regenerative ability, new tissue will be formed within the biodegradable scaffold matrix by active, immature or precursor cells infiltrating from the surrounding healthy tissues. However, additional means are required when the regeneration potential of the tissue is very low, because of, for instance, low local concentration of the cells and growth factors required for new tissue generation. The simplest method is probably to supply growth factors to the site of regeneration for cell differentiation and proliferation. There are some reports that indicate that the use of growth factors in solution form was effective in enhancing tissue regeneration. However, the dosage is too high to accept clinically for possible therapeutic trials. As described above, it is possible for growth factors to be supplied using a controlled release system. Table 2 summarizes research on tissue regeneration where growth factors are combined with various carriers. Every study without exception indicates that it is necessary to combine growth factors with a carrier to maintain their activity and support tissue regeneration. However, in spite of this, there has been little investigation of the kinetic of growth factor release from carriers and its effect on tissue regeneration.

Growth factor	Carrier	Animal	Tissue regenerated	Reference
BMP	PLA	Dog	Long bone	23
	Collagen sponge	Rat	Long bone	24
		Dog, Monkey	Periodontal ligament and cementum	25
	b-TCP	Rabbit	Long bone	26
	Porous HA	Rabbit	Skull bone	27
rhBMP□2	Porous PLA	Dog	Spinal bone	28
		Rat	Skull bone	29
	PLA microsphere	Rabbit	Skull bone	30
	Collagen sponge	Dog	Periodontium	31
	Gelatin	Rabbit	Skull bone	32
	PLA-coating gelatin sponge	Dog, Monkey	Long bone, Jaw bone, Skull bone	33
	Porous HA	Monkey	Skull bone	34
	PLA-PEG copolymer	Rat	Long bone	35
rhBMP□7	Collagen	Dog	Spinal bone	36
		Dog	Long bone	37
EGF	Agarose	Hamster	Angiogenesis	38
	PVA	Rat	Dermis	39
aFGF	PVA	Mouse	Angiogenesis	40
	Alginate	Mouse	Angiogenesis	41
bFGF	Alginate	Mouse	Angiogenesis	41
	Agarose/heparin	Mouse, Pig	Angiogenesis	42, 43
	Amylopectin	Mouse	Angiogenesis	44
	Gelatin	Mouse	Angiogenesis, Dermis, Adipogenesis	45, 46, 47
		Rabbit, Monkey	Skull bone	48, 49
		Dog	Nerve	50
	Fibrin gel	Mouse	Angiogenesis	51
	Collagen minipellet	Rabbit	Long bone	52
	Collagen	Mouse	Cartilage	53
	Poly(ethylene-co-vinyl acetate)		Rat	Nerve
NGF	Collagen minipellet	Rabbit	Nerve	55
	PLGA	Rat	Nerve	56
TGF□b1	PEG	Rat	Dermis	57
	Gelatin	Rabbit	Skull bone	58
	Plaster of Paris, PLGA	Rat	Skull bone	59
	TCP	Dog	Long bone	60
	Porous HA	Dog	Long bone	61
	Collagen	Baboon	Skull bone	62
		Mouse	Dermis	63
PDGF-BB	Porous HA	Rabbit	Long bone	64
	Collagen	Rat	Dermis	65
	Chitosan	Rat	Periodontal bone	66
VEGF	Collagen	Mouse	Angiogenesis	67
	Alginate	Mouse	Angiogenesis	68
HGF	Gelatin	Mouse	Angiogenesis	69
IGF-I	PLGA-PEG	Rat	Adipogenesis	70
IGF-I/bFGF	PLGA-PEG	Rat	Adipogenesis	71
PDGF/IGF-I	Titanium implant	Dog	Jaw bone	72

aFGF: acid fibroblast growth factor, bFGF: basic fibroblast growth factor, BMP: bone morphogenetic protein, rhBMP: recombinant human bone morphogenetic protein, EGF: epidermal growth factor, HGF: hepatocyte growth factor, HA: hydroxyapatite, IGF-I: insulin-like growth factor-I, 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), TCP: tricalcium phosphate, TGF: Transforming growth factor, VEGF: vascular endothelial growth factor

Table 2. Experimental tissue regeneration studies using a combination of growth factors and carrier

Recently there has been attempts to use genes encoding for growth factors for TE (73). Transfection of a growth factor gene into cells at the site of regeneration could result in secretion of this growth factor for a certain period of time, hence promoting tissue regeneration. Treatment of ischemic diseases (74) and bone tissue regeneration (75) has been achieved by the use of growth factor genes.

Controlled Release of Growth Factors from Biodegradable Hydrogel

One of the largest problems in protein release technology is the loss of biological activity of the protein released from a protein-polymer combinational formulation. It has been demonstrated that this loss of activity results from denaturation and deactivation of the protein during the formulation process with polymer matrix. Therefore, a new preparation method of polymers should be exploited to minimize protein denaturation. From this viewpoint, a polymer hydrogel may be a preferable carrier for protein release because of its biosafety and its high inertness. However, it will be impossible to achieve controlled release of protein over a long period of time from hydrogels since the rate of protein release is generally diffusion-controlled through aqueous channels inside the hydrogel. Thus, one possible approach could be to immobilize a growth factor in a biodegradable hydrogel, allowing the immobilized factor to be released as a result of hydrogel biodegradation. In such release system, growth factor release can be controlled only by changing the *in vivo* hydrogel degradation.

To immobilize growth factors into hydrogels, chemical bonding or physical interaction forces between the growth factor and hydrogel polymer have been used. Since the chemical methods often result in the denaturation and lowering of protein activity, it is preferable to use physical methods. Actually, physical immobilization can be observed for growth factors existing in the body (76). It is known that some growth factors possess a positively charged site on their molecular surface, and they are normally stored in the body ionically bound to the acidic polysaccharides of the ECM, such as heparan sulfate and heparin. Being bound/complexed protects growth factors from

denaturation and enzymatic degradation *in vivo*. Growth factors are released form the ECM complex as a result of polysaccharide degradation according to need.

We have developed a release system of growth factors which mimics that occurring in the living body. Figure 2 shows the idea of controlled release of growth factor from 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. Growth factors with a positively charged site interact electrostatically with the polymer chain so that they can be physically immobilized in the hydrogel carrier. If environmental changes, such as increased ionic strength, occurs, the immobilized growth factor will be released from the factor-carrier formulation. Even if such an environmental change does not take place, degradation of the carrier itself will also lead to growth factor release. Because the latter is more likely to happen *in vivo* than the former, it is preferable to prepare the release carrier from biodegradable polymers.

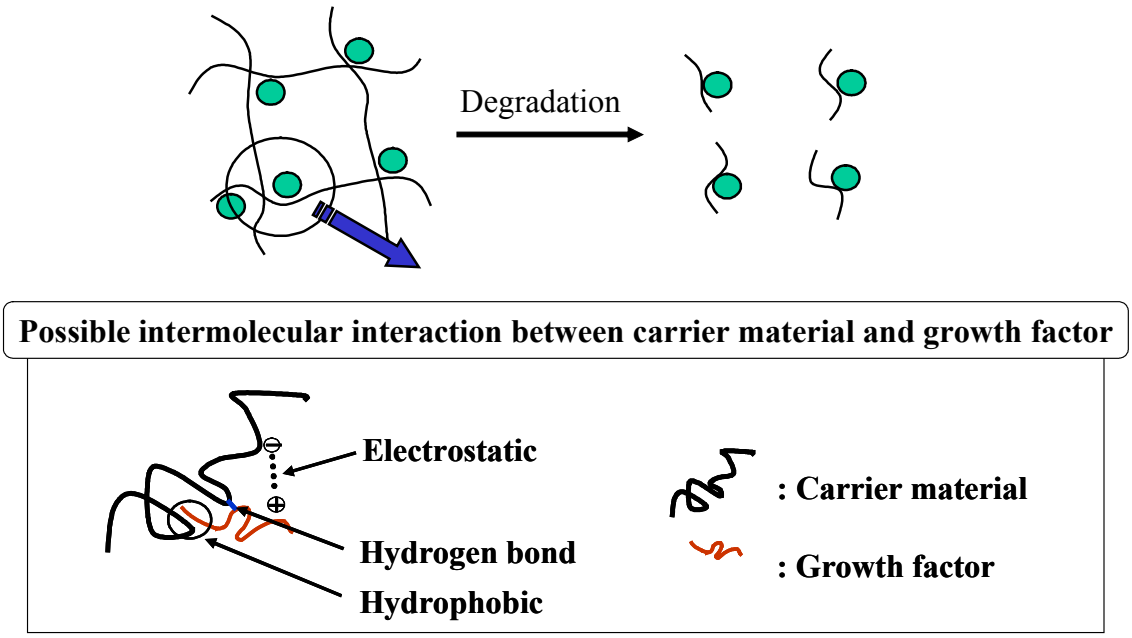


Fig. 2. Concept illustration of growth factor release from biodegradable hydrogel based on physical interaction forces

For growth factor release based on the physical interaction forces, it is absolutely necessary to employ a bio-safe polymer as a carrier material. In addition the material used should be a natural polymer with charged groups. Therefore, we have selected gelatin, as it has been extensively used for industrial, pharmaceutical, and medical purposes. The biosafety of gelatin has been proved through its long clinical use. Another unique advantage is the electrical nature of gelatin which changes according to the collagen processing method. For example, the alkaline process through hydrolysis of amide groups of collagen yields gelatin having a high density of carboxyl groups, which makes the gelatin negatively charged. If the growth factor to be released has positively charged sites that will interact with acidic polysaccharides present in the ECM, a negatively charged gelatin of "acidic" type is preferable as a carrier material. It was found that basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), hepatocyte growth factor (HGF) or platelet-derived growth factor (PDGF) incorporation into the acidic gelatin hydrogel are mainly due to the electrostatic interactions as expected (77).

Animal experiments revealed that hydrogels prepared from the acidic gelatin were degraded in the body (78). The degradation period of hydrogels depends on their water content which is a measure of crosslinking extent. The higher the water content of a hydrogel, the faster is its *in vivo* degradation. Growth factor release was monitored in mice after implanting gelatin hydrogels subcutaneously containing ^{125}I-labeled bFGF or gelatin hydrogels labeled with ^{125}I containing bFGF. In both cases residual radioactivity decreased with implantation time and the decreasing rate increased with the decreasing water content of the hydrogel. The time course of bFGF radioactivity remaining in the hydrogel depended on the hydrogel degradability and was in good accordance with the radioactivity remaining in the hydrogel (78). These findings strongly indicate that growth factor release is governed mainly by hydrogel degradation as described in Figure 2. As a result, the release period is not influenced by the hydrogel shape and can be controlled only by changing the degradation rate of hydrogel (79). It should be noted that gelatin hydrogels can be formulated into different shapes of disks, tubes, sheets, granules, and microspheres (79, 80).

Tissue Engineering by Gelatin Hydrogels Incorporating Growth Factors

As described in the previous section, the gelatin hydrogel was found to be a good carrier for the controlled release of growth factor. In this section, several experimental results on angiogenesis, bone regeneration, and adipogenesis, achieved by this release system are described to emphasize the significance of drug delivery in TE.

Angiogenesis

bFGF was originally characterized as a growth factor for fibroblasts and capillary endothelial cells *in vitro* and as a potent mitogen and chemoattractant for a wide range of cells *in vivo*. In addition, bFGF has been shown to have a variety of biological activities (81) and to be effective in enhancing wound healing through induction of angiogenesis and regeneration of bone, cartilage, and nerve. Gelatin hydrogels are effective in enhancing the *in vivo* angiogenic effect of bFGF. When gelatin hydrogels containing bFGF were subcutaneously implanted into the back of mice, an angiogenic effect was observed around the implanted site, in contrast to sites implanted with bFGF-free gelatin hydrogels or injected with aqueous solution of bFGF (77). No angiogenesis was induced by the injection of bFGF solution even when the dose was increased to 1 mg/site. This must be due to a rapid elimination of bFGF from the injection site (82). In contrast, gelatin hydrogels incorporating bFGF induced significant angiogenesis at a dose as low as 30 μg/site. As is shown in Figure 3, the period of maintained angiogenic effect of hydrogels could be modulated by changing the water content of the hydrogel and was prolonged when their water content is/was decreased (83). It is likely that *in vivo* hydrogels with lower water content are degraded more slowly and consequently release active bFGF at a slower rate than those with higher water content, leading to a prolonged angiogenic effect. Similarly enhanced and prolonged angiogenic effect was also observed upon using gelatin hydrogels continaing bFGF microspheres (79).

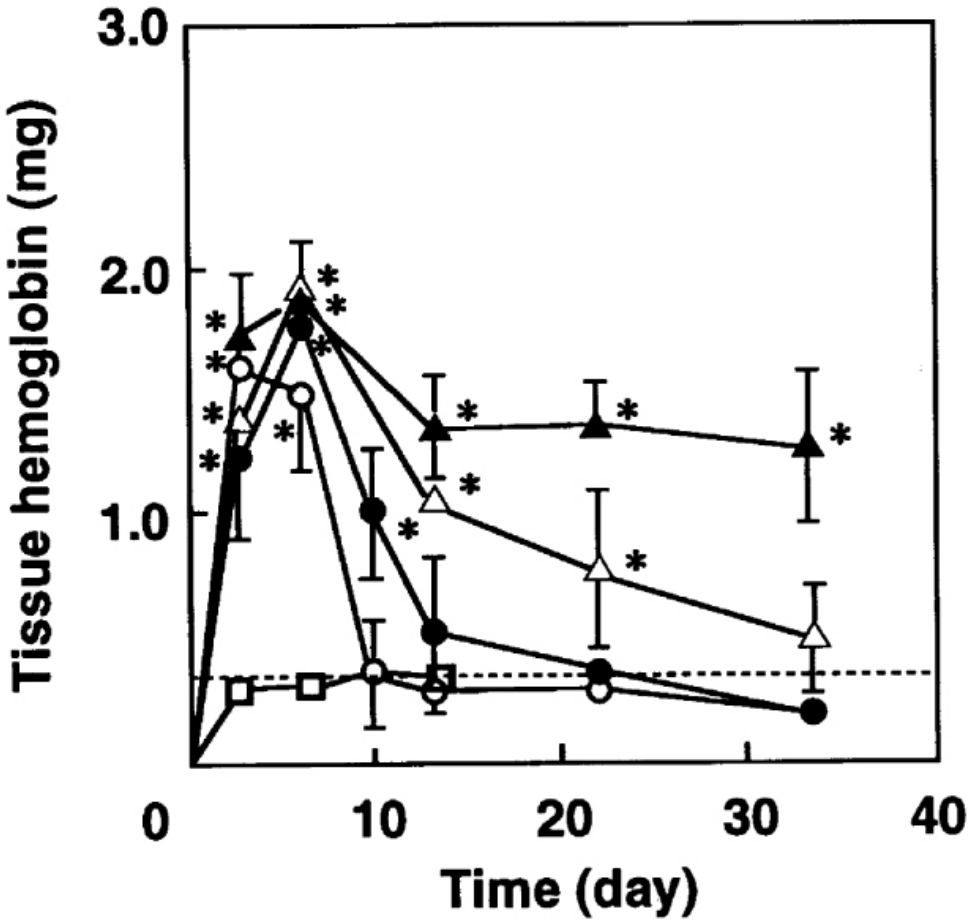


Figure 3. The time course of angiogenesis induced by gelatin hydrogels incorporating bFGF with a water content of gelatin hydrogels: 98.0 (clear circle); 94.1 (filled circle); 90.4 (clear triangle); and 85.5 wt% (filled triangle); a non-crosslinked gelatin hydrogel incorporating bFGF (square). The bFGF dose was 100 μg/mouse. The dotted line indicates the weight of tissue hemoglobin in the corresponding area of untreated, normal mice. The asterisks indicate significance at $p<0.05$ against the value of control mice on the corresponding day.

Techniques to induce *in vivo* angiogenesis are very important for TE. Angiogenesis induction is important for the treatment of ischemic disease and is beneficial prior to cell transplantation. To give an example of the therapeutic effect, treatment of ischemic myocardium by using gelatin hydrogels incorporating bFGF is shown. Myocardial infarct was induced by ligation of the left anterior descending (LAD) coronary artery of dog heart and gelatin microspheres containing bFGF injected

at the infarcted site. Regeneration of collateral coronary arteries as well as improvement of the contraction of the myocardium in the ischemic region was observed (Figure 4).

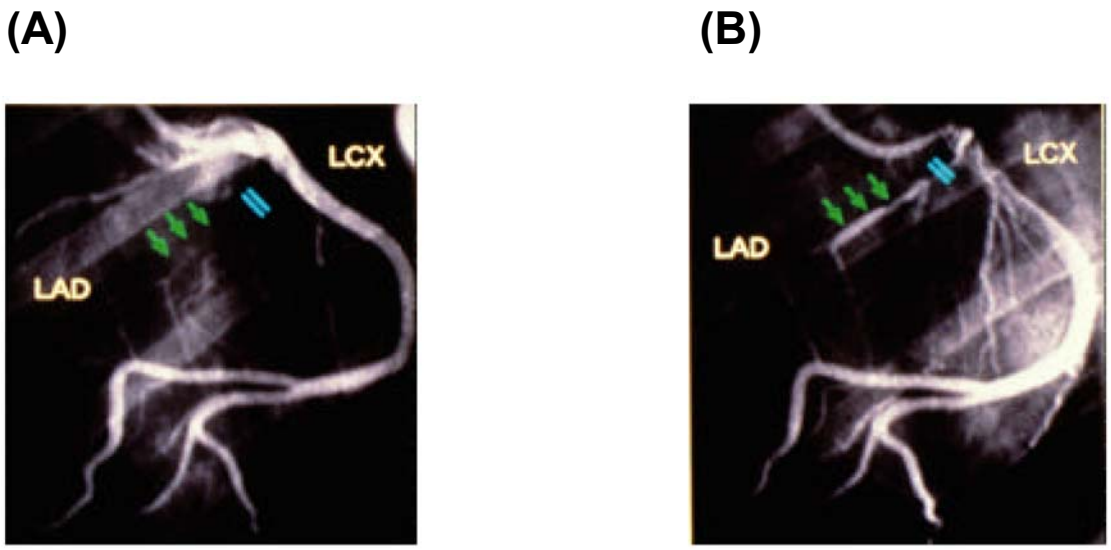


Figure 4. Left coronary angiograms of dog ischemic heart one week after intramyocardial injection of bFGF solution (A) and gelatin microspheres containing bFGF (B). bFGF was bilaterally injected into the distal side of the left anterior descending (LAD) artery ligated portion (indicated by the mark II) at the dose of 100 μg/heart. The hydrogel water content was 95.0 wt%.

No such therapeutic effects were observed with the injection of bFGF solution at the same dose. It should be noted that sufficient supply of nutrients and oxygen to cells transplanted in the body is indispensable for cell survival and the maintenance of biological functions. Without such support, cells pre-seeded in a scaffold would hardly survive following implantation of the scaffold in the body. Such situation is seen during allo- or xenogeneic cell transplantation into the body. For successful cell transplantation, the supply of nutrients and oxygen is as a great problem as that of the immuno-isolation technology for cells transplanted. In both cases, a promising method to induce angiogenesis throughout the cell transplantation is to make use of angiogenic growth factors. After implantation into the subcutaneous tissue of diabetic rats (streptozotocin-induced diabetes), allogeneic pancreatic islet encapsulated by a poly(vinyl alcohol) (PVA) bag effective for immuno-isolation did not always contribute to the normalization of blood glucose level for a long period because of islet death due to poor blood supply in the subcutaneous tissue as compared to that implanted in the peritoneal cavity. However, prior induction of angiogenesis at the intended site of cell transplantation induced by gelatin microspheres incorporating bFGF enabled the encapsulated islets to have improved survival rate. This resulted in a prolonged period of normal(ized) glucose level in the blood (Figure 5). This angiogenic effect in prolonging cell survival was observed also in hepatocyte transplantation. A biodegradable sponge of L-lactic acid and ε-caprolactone copolymer containing gelatin microspheres loaded with bFGF was implanted into the peritoneal cavity of rats. Gelatin microspheres incorporating bFGF were placed into the sponge for in advance to inducetion of angiogenesis within the sponge inside. One week after bFGF-induced angiogenesis, of allogeneic rat hepatocytes were injected into the sponge where they remained alive up to two months, led to their prolonged survival. In the case of control sponges that did not contain bFGF, the transplanted hepatocytes did not survive for such a long time (1 week) (83). These findings clearly indicate that the induction of angiogenesis prior to cell transplantation was effective in achieving successful cell transplantation.

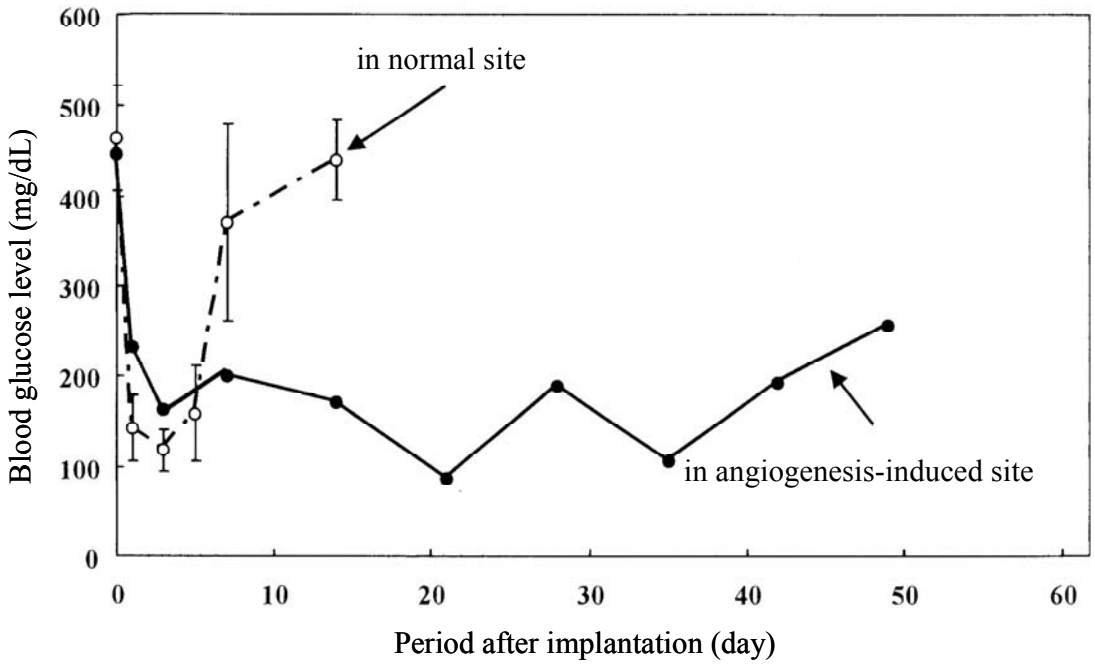


Figure 5. The time course of blood glucose level of diabetic rats following subcutaneous implantation of allogeneic pancreatic islet encapsulated by a poly(vinyl alcohol) hydrogel bag. The bFGF dose was 100 μg/site and the hydrogel water content was 95.0 wt%.

Bone Regeneration

Gelatin hydrogels incorporating bFGF were found to have a promising potential for bone repair (48, 49). For example, when implanted into monkey's cranial defect, gelatin hydrogels containing bFGF promoted bone regeneration at the defect and the defect closed 21 weeks after implantation. On the contrary, use of bFGF-free gelatin hydrogels or a similar dose of bFGF but in solution did not result in bone regeneration, and significant ingrowth of fibrous tissues was observed in the bone defect 21 weeks after application (Figure 6). Measurement of the bone mineral density (BMD) at the site of the skull defect in monkeys revealed that gelatin hydrogels containing bFGF enhanced the BMD to a significantly higher extent than free bFGF, irrespective of the hydrogel water content. The BMD of empty gelatin hydrogels was similar to that of the untreated group, indicating that the hydrogel presence did not disturb bone healing at the defect. In a histological study, hydrogel implantation was associated with a significant increase in the number of osteoblasts residing near the bone edge of the defect that was retained over the studied range of time, such as for 6 week or longer. The period of maintained increase in cell number was prolonged with the decrease in the hydrogel water content. These findings indicate that controlled release from the gelatin hydrogel enabled bFGF to activate osteoblasts for an extended period, resulting in induced regeneration of skull bone.

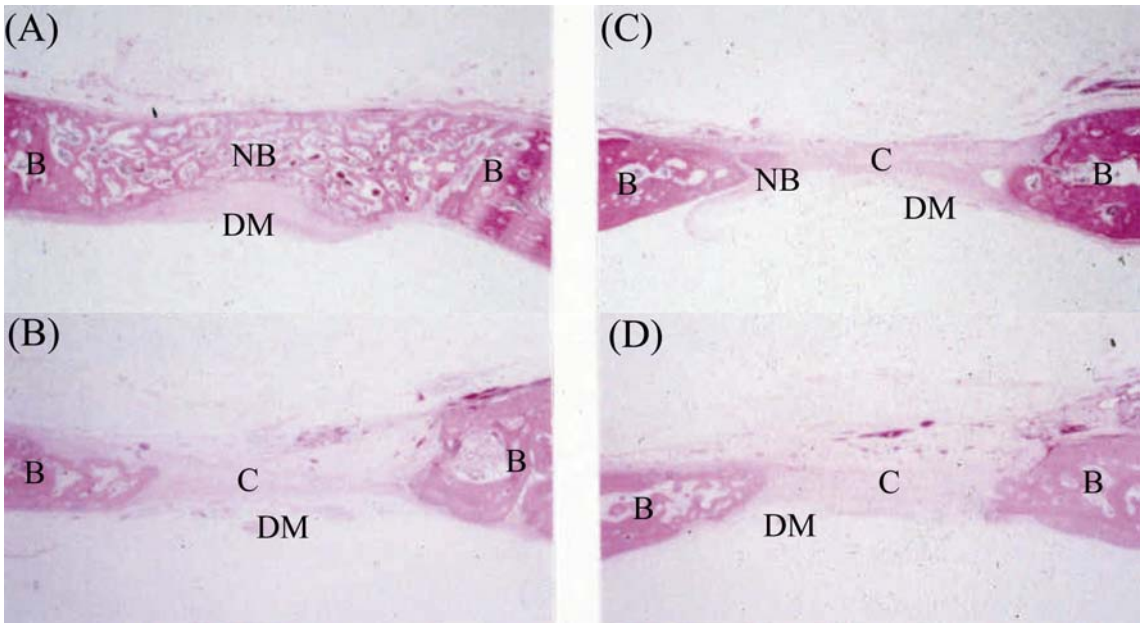


Figure 6. Histological cross-sections around skull defects of monkey 21 weeks after treatment with a gelatin hydrogel incorporating bFGF (A), free bFGF (B), an empty gelatin hydrogel (C), and phosphate-buffered saline solution (PBS) (D): b; bone, d; dura mater, c; fibrous tissue, and nb; new bone. (A, B, C, and D; HE staining, x 40) The bFGF dose was 100 μg/defect and the hydrogel water content was 85.0 wt%.

It is known that both TGF-β1 and bone morphogenetic protein (BMP) also promote bone regeneration (32, 58, 84, 85). We have succeeded in achieving bone of skull defects in rabbits and monkeys by the use of controlled release of TGF-β1 from gelatin hydrogels. Such repair is not observed when free TGF- β1 even at higher doses is used (58). However, the extent of tissue regeneration depended on the water content of hydrogels and was reduced with increasing or decreasing hydrogel water content. It is possible that too rapid degradation of the hydrogel causes the release of bFGF over a short period of time, resulting in no induction of bone regeneration. Contrary to this, the long-term presence of hydrogels due to slow degradation would physically hinder bone regeneration. As a result, it is likely that controlled release of growth factor for a time period from hydrogels with an optimal biodegradability induce complete bone regeneration of the skull defect (58). In this case, the hydrogel functions as a carrier of growth factor as well as the barrier that prevents the ingrowth of fibrous tissue into the bone defect. A correct balance of these

hydrogel functions over time would result in better control of bone regeneration. We have recently succeeded in achieving controlled release of BMP-2 by gelatin hydrogels. In these hydrogels the time course of BMP-2 release can be regulated by changing the hydrogel biodegradability (86). This controlled release system enabled BMP-2 to induce bone formation ectopically or orthotopically at lower doses than when applied in solution.

There are some cases in which the sole use of controlled release of growth factors will not always lead to bone regeneration in large-size defects. In one attempt to improve repair, we have utilized cells with osteogeneic potential and combined them with the growth factor release system. Among them were mesenchymal stem cells (MSC) derived from the bone marrow. We have demonstrated that the application of combined MSCs and gelatin microspheres incorporating TGF-β1 led to complete closure of large-size defects in rabbit skulls by newly formed bone tissue, whereas such a defect closure was not observed for what has occurred with the use of either material alone (87).

Adipogenesis

When gelatin microspheres containing bFGF were mixed with a basement membrane extract (Matrigel) and subcutaneously implanted into the mouse back, *de novo* formation of adipose tissue was observed at the implanted site (47). A histological study revealed that mature adipocytes were observed in the newly-formed tissue mass following implantation of mixed microspheres and Matrigel, whereas no adipogenesis was observed with the use of of either component alone. It is possible that the controlled release of bFGF induced both angiogenesis and efficient proliferation and maturation of the adipose precursor cells that migrated in the vascularized Matrigel. Recently we succeeded in regenerating adipose tissue by using preadipocytes isolated from fat tissues, gelatin microspheres incorporating bFGF, and a collagen sponge (Figure 7) (88). When the preadipocytes and the microspheres were placed into the collagen sponge and implanted into the back subcutis of mice, *de novo* formation of adipose tissue was observed in the sponge. Combination of all of the three was required for the development of adipogenesis. It is likely that the released bFGF increases the number of preadipocytes and the rate of differentiation into mature adipocytes in the collagen sponge that was used as a scaffold, resulting in achievement of *de novo* adipogenesis.

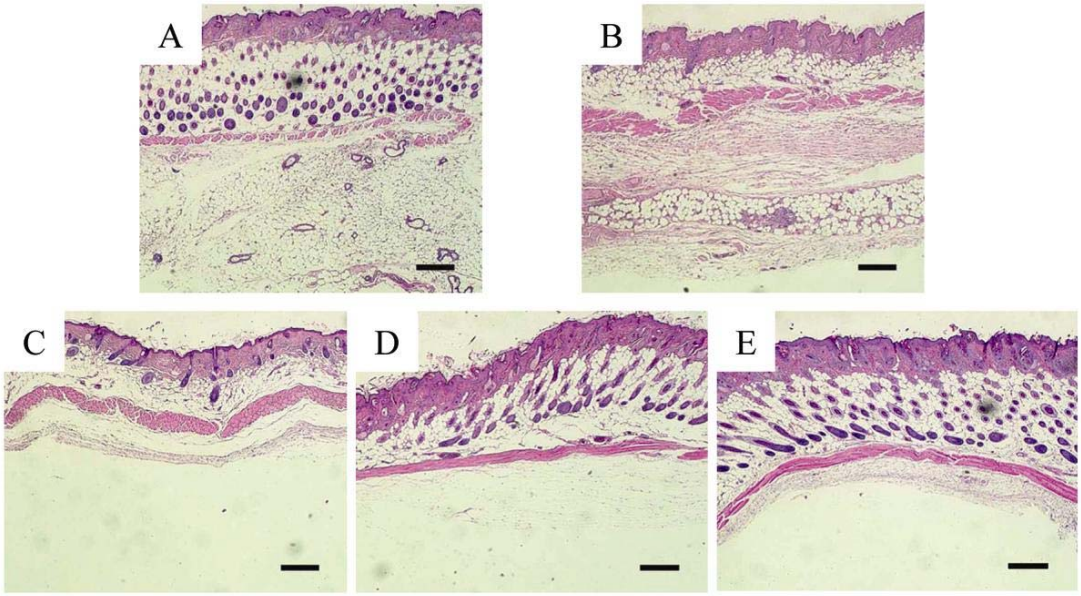


Figure 7. *De novo* formation of adipose tissue in the mouse subcutis six weeks after implantation of a collagen sponge including the mixture of preadipocytes and gelatin microspheres incorporating bFGF: (A) a collagen sponge including a mixture of preadipocytes and gelatin microspheres incorporating bFGF, (B) a collagen sponge including a mixture of preadipocytes and free bFGF, (C) a collagen sponge including preadipocytes, (D) a mixture of preadipocytes and gelatin microspheres incorporating bFGF, and (E) a collagen sponge including gelatin microspheres incorporating bFGF. (Magnification x 100, Sudan III staining). The bFGF dose was 10 μg/site and the hydrogel water content was 95.0 wt%. The bar length corresponds to 300 μm.

Concluding Remarks

For regeneration of body tissues, a variety of growth factors act on cells with forming their mutual networks. Timing, site, and concentration of action of growth factors are delicately regulated in the body. It is likely that the basic mechanisms of growth factor actions in the living systems controlling cell behaviour will be clarified by rapid advances in cell biology, molecular biology, and embryology. Nonetheless, it will still be impossible to replicate living systems only by the technologies that are currently available. It will be essential to understand which growth factors are the key players for regeneration of a certain tissue. If a key growth factor is supplied to the

target site at the right time for the appropriate period of time and at the right concentration, we believe that the living body system will be naturally directed toward the process of tissue regeneration. Once the right direction is taken, the intact system of the body will start to function, resulting in automatic achievement of tissue regeneration. There is no doubt that when growth factors are used *in vivo*, their controlled release is essential. However, the present technology does allow accurate regulation of the amount and length of time of growth factor release. Additionally, it is not always enough to control the amount and time period of growth factor release. Therefore, at present, one practical approach is to release sufficient growth factor to increase the number of precursor, blastic or stem cells *in vivo*. It is practically impossible, however, to control cell differentiation by the currently available technology of growth factors release.

Regenerative medicine, which is induction therapy of tissue regeneration based on TE, is the third field after reconstructive surgery and organ transplantation. To achieve clinical TE, substantial collaborative research between material, pharmaceutical, biological, and clinical scientists is needed. There is so far, little research on drug delivery aiming at tissue regeneration and the biological substitution of organ functions. This is probably due to poor availability of growth factors on a large scale and their high cost. As an alternative to controlled drug release, prolongation of drug half-life, improvement of drug absorption, and drug targeting could be used. For example, a promising approach is to promote tissue regeneration by targeting a growth factor with an extended half-life to the tissue site to be regenerated. The technology of drug delivery will also be applicable to create non-viral vectors to prepare genetically-engineered cells for TE.

As TE is still in its infancy, it will take a long time to become well established Increasing significance of drug delivery in future will further help progress of TE. We will be happy if this chapter stimulates the reader's interest in this research field.

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