Biomaterial technology for tissue engineering applications

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Tissue engineering is a newly emerging biomedical technology and methodology to assist and accelerate the regeneration and repairing of defective and damaged tissues based on the natural healing potentials of patients themselves. For the new therapeutic strategy, it is indispensable to provide cells with a local environment that enhances and regulates their proliferation and differentiation for cell-based tissue regeneration. Biomaterial technology plays an important role in the creation of this cell environment. For example, the biomaterial scaffolds and the drug delivery system (DDS) of biosignalling molecules have been investigated to enhance the proliferation and differentiation of cell potential for tissue regeneration. In addition, the scaffold and DDS technologies contribute to develop the basic research of stem cell biology and medicine as well as obtain a large number of cells with a high quality for cell transplantation therapy. A technology to genetically engineer cells for their functional manipulation is also useful for cell research and therapy. Several examples of tissue engineering applications with the cell scaffold and DDS of growth factors and genes are introduced to emphasize the significance of biomaterial technology in new therapeutic and research fields.

Keywords: biomaterials; drug delivery system; biosignalling molecules; tissue engineering; tissue regeneration

1. SIGNIFICANCE OF BIOMATERIAL TECHNOLOGIES IN TISSUE ENGINEERING APPLICATIONS

Advanced surgical therapies currently available consist of reconstruction surgery and organ transplantation. Although there is no doubt that these therapies have saved and improved countless lives, they have several therapeutic and methodological limitations. In the case of reconstruction surgery, biomedical devices cannot completely substitute the biological functions even for a single tissue or organ, and consequently cannot prevent the progressive deterioration of injured or damaged tissues and organs. One of the biggest issues for organ transplantation is the shortage of donor tissues or organs. Additionally, the continuous and permanent use of immunosuppressive agents to prevent immunological rejection responses often causes side effects, such as the high possibility of bacterial infection, carcinogenesis and virus infection. To resolve these issues in the two advanced therapies, a new therapeutic solution that is clinically mild to patients is required.

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In this clinical situation, a new therapeutic trial, in which disease healing can be achieved based on the natural healing potential of patients, has been explored. This trial is termed tissue regeneration therapy where the regeneration of tissues and organs is naturally induced to therapeutically treat diseases by artificially promoting the potential of cell proliferation and differentiation. To realize this cell-induced regeneration therapy, there are two approaches. One is cell transplantation where cells with a high potential of proliferation and differentiation are transplanted to induce tissue regeneration based on their potentials. The other is the therapeutic approach with biomaterials and technologies. In the latter approach, an in vivo local environment that enables cells to promote their proliferation and differentiation is created by making use of biomaterials and technologies. If the environment efficiently manipulates the cells inherently present in the body to enhance the biological potentials of tissue regeneration, cell-induced natural healing of tissues and organs will be achieved without cell transplantation. This approach is called tissue engineering. This basic concept of biomaterial-based tissue engineering was originally introduced by Langer & Vacanti (1993). Cell scaffold and biosignalling molecule
delivery technologies with biomaterials have been demonstrated to create cell environments suitable for tissue regeneration (Saltzman & Olbricht 2002; Bach et al. 2003; Bannasch et al. 2003; Chen & Mooney 2003; Hubbell 2004; Langer & Tirrell 2004; Silva & Mooney 2004; Kuo et al. 2006; Leo & Grande 2006; Tabata 2008). For the former approach, generally, cells are transplanted into the body by the bolus injection or infusion method. However, few cells are retained at the transplanted site and their grafted rate is very low because of their excretion and death. In addition, the cells infused are hardly accumulated and grafted at the site to be regenerated. This low grafting rate of cells transplanted and their consequent poor functions often cause low therapeutic efficacy of cell transplantation. To overcome these problems, it is necessary to give the cells an environment suitable to their survival and functional achievement. Biomaterials play a key role in creating the environment for cells. The scaffold to promote the proliferation and differentiation is prepared from biomaterials, while the biomaterial is used as the delivery carrier of biosignalling molecules as the cell nutrients to biologically activate cells. Combination with biomaterials enables an angiogenic factor to efficiently induce in vivo angiogenesis, which gives nutrients and oxygen to the transplanted cells. Biomaterials need to assist the approach of cell transplantation and enhance the therapeutic efficacy.

This new regeneration therapy cannot always therapeutically substitute the reconstructive surgery and organ transplantation clinically available, and has advantages and disadvantages. However, it is clinically expected as the third therapeutic choice. If this tissue regeneration therapy is realized, it will enable us to create new therapeutic strategies as well as increase the therapeutic choice of clinicians, which consequently brings about large therapeutic benefits for patients who have not been able to receive clinically effective therapies. There are three objectives of regeneration therapy. The first objective is to create a new therapeutic strategy of surgery and internal medicine, which is generally well known. The second objective is to enlarge the clinical application of therapies conventionally available. Conventional surgical therapy is not always effective in treating patients who are aged or suffer from other diseases, such as diabetes and hyperlipaemia, or cannot be applied because the number of key cells is small and their potential for proliferation and differentiation is low. In this case, it is practically possible that combination with the technology and methodology to promote cell-based self-healing potentials improves the therapeutic efficacy even for patients who have been clinically treated. The third objective is to suppress the progressive deterioration of diseases. The deterioration and progress of disease conditions are suppressed by artificially promoting cell potentials to induce tissue regeneration. For example, in chronic fibrosis diseases, the fibrous tissue of excessive collagen fibres and fibroblasts causes the impairment of natural healing processes at the disease site. If the fibrosis can be loosened and digested, and additionally the natural healing potential of the surrounding healthy tissue can be augmented, it is highly expected that the disease deterioration and progression can be suppressed in a physiologically natural manner.

At present, there is no effective medical therapy for chronic fibrosis diseases, such as lung fibrosis, cirrhosis, dilated cardiomyopathy and chronic nephritis. For these diseases, the injured site is normally occupied with fibrous tissue of excessive collagen fibres and the fibroblasts excessively proliferate. It is possible that this tissue occupation causes the physical impairment of healing processes at the disease site. Even in adults, the natural healing potential for tissue regeneration still remains, but cannot operate naturally for certain reasons in disease conditions. For example, therefore, if the fibrosis can be loosened and digested to disappear by any method of drug treatment, it is highly expected that the disease site will be regenerated and repaired based on the natural regeneration potential of the surrounding healthy tissue. This trial is a new and possible therapy for chronic fibrosis diseases and defined as ‘tissue regeneration of internal medicine’ because of the drug treatment application of internal medicine (figure 1). This therapeutic approach is similar to the surgical regeneration therapy where the cell, the scaffold and the growth factors, or their combination, are surgically applied to the tissue defect for generation therapy, because both approaches are based on the natural healing potential of patients. We have demonstrated that the controlled release of a matrix metalloproteinase (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 (Aoyama et al. 2003). The intraperitoneal release of hepatocyte growth factor (HGF) histologically cured the liver fibrosis of rats with liver cirrhosis (Oe et al. 2003). A biodegradable hydrogel could achieve the controlled release of small interfering RNA (siRNA) for transforming growth factor (TGF)-β1 type II receptor and regenerate and repair the fibrosis of chronic renal sclerosis (Kushibiki et al. 2006).

The basic idea of tissue regeneration therapy is to take advantage of the natural healing potentials of patients themselves. Thus, this is applicable for another therapy of internal medicine. For conventional catheter treatment, an aneurysm occlusion with blood clots has been clinically performed. However, sometimes the recurrence of aneurysm due to clot lysis is clinically problematic. As one trial to overcome the problem, aneurysm occlusion with tissue organization has been achieved by using coils incorporating basic fibroblast growth factor (bFGF; Kawakami et al. 2005). The bFGF release promoted the cell proliferation inside the aneurysm to allow occlusion by the tissue organized. The tissue regeneration strategy will be applied to the therapy of internal medicine.

One of the therapeutic advantages is the ability to accelerate the natural healing of body injury through promoted angiogenesis or the infiltration and recruitment of key cells at the injured site. This will enable patients to shorten the healing period and suppress the deterioration process of disease even under inflammation and infection conditions. A disadvantage of this...
therapy is that, generally, at least a few days are required to induce and activate cell-based tissue regeneration. Consequently, it cannot be expected that tissue regeneration therapy alone will achieve the rapid healing of wounds or diseases. Depending on the clinical situation, it is necessary for better medical treatment to combine the conventional therapies with the tissue regeneration strategy.

2. FUNDAMENTAL BIOMATERIAL TECHNOLOGY AND METHODOLOGY FOR TISSUE ENGINEERING-BASED REGENERATIVE THERAPY

Basically, body tissue is composed of two components: cells and the surrounding environment. The latter includes the extracellular matrix (ECM) for cell proliferation and differentiation (natural scaffold) as the living place of cells and biosignalling molecules as the nutrients of cells. There are some cases where tissue regeneration is achieved by the single or combinational use of the components in an appropriate way. However, since successful tissue regeneration cannot always be expected only by their simple combination, it is necessary to biomedically contrive the way to combine. To this end, proper and positive assistance of biomaterial technology will be practically promising. Biomaterials play a key role in designing and creating substitutes for ECM and the drug delivery system (DDS) of biosignalling molecules to enhance their biological activities. In addition to therapeutic applications, biomaterials are also useful in the progress of research and development of stem cell biology and medicine.

<table>
<thead>
<tr>
<th>synthetic polymers</th>
<th>natural polymers</th>
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<tr>
<td>poly(t-lactic acid) (PLLA)</td>
<td>collagen</td>
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<tr>
<td>poly(glycolic acid) (PGA)</td>
<td>gelatin</td>
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<tr>
<td>poly(e-caprolactone) (PCA)</td>
<td>fibrin</td>
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<tr>
<td>copoly(LL-CA)</td>
<td>hyaluronic acid*</td>
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<td>copoly(LLA-ethylene glycol (EG))</td>
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<tr>
<td>copoly(fumarate-EG)</td>
<td>chitosan, chitin</td>
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*There are no enzymes in the body to directly degrade these polymers. They are washed out by body fluids to disappear from the implanted site.

As the biomaterials, various synthetic and natural materials, such as polymers, ceramics and metals or their composites, have been investigated and used in different manners. Among them, biodegradable biomaterials are explained here. From the practical viewpoint, metals and ceramics except for calcium carbonate and tricalcium phosphate are not biodegradable. On the other hand, some polymers are biodegradable materials (table 1). The word ‘biodegradation’ is defined to be the phenomenon where a material is degraded or water solubilized by any process in the body to disappear from the site implanted. There are two ways of material disappearance. First, the main chain of the material is hydrolysed or enzymatically digested to decrease the molecular weight, and finally disappears. Second, the material is chemically cross-linked to form a hydrogel insoluble in water. When the cross-linking bond is degraded to generate water-soluble fragments, the...
Figure 2. Role of biomaterials in tissue engineering-based regeneration therapy. (a) Biomaterials for cell scaffold to induce in vivo tissue regeneration. Bioabsorbable scaffold: (i) without cells and growth factors, (ii) with cells, (iii) with growth factors, (iv) with cells and growth factors. (b) Biomaterials to protect a space and induce angiogenesis for in vivo tissue regeneration. (c) Biomaterials for DDS of biosignalling molecules (growth factors and genes): (i) controlled release of signalling molecule, (ii) proliferation of signalling molecule lifetime, (iii) absorption acceleration of signalling molecule, (iv) signalling molecule targeting. (d) Biomaterials for in vitro cell manipulation to obtain cells and cell constructs for transplantation. (e) Biomaterials for engineering biological functions of cells.

fragments are washed out from the site implanted, resulting in the disappearance of material. Synthetic polymers are generally degraded by simple hydrolysis while natural polymers are mainly degraded enzymatically. The synthetic polymers can be modified with ease to change their chemical composition and molecular weight, which affect the physicochemical property of the materials. Natural polymers of proteins, polysaccharides and nucleic acids are available. Their degree of freedom for property modification is small when compared with that of synthetic polymers, but they can be chemically modified to produce various derivatives. The natural polymers are normally used in the formulation of hydrogels prepared by chemical cross-linking. Generally, the synthetic polymers are hydrophobic and mechanically strong when compared with natural ones; in other words, their degradation rates are comparatively slow. For the purpose of material application to tissue regeneration, the retention of biomaterials implanted in the body often causes the physical impairment of tissue regeneration. On the other hand, an appropriate mechanical strength of materials is also required. Generally, the mechanical strength of materials weakens as their degradation becomes faster. The two opposite properties should be balanced by material design and combination.

There are five key technologies or methodologies that are necessary for biomaterial-based tissue regeneration therapy and the basic stem cell research that scientifically supports the future regeneration therapy of cell transplantation. The first key technology is for the preparation of cell scaffolds to promote cell proliferation and differentiation for in vivo tissue regeneration (figure 2a). ECM is not only a physical support for cells, but also provides a natural environment for cell proliferation and differentiation or morphogenesis, which contributes to cell-based tissue regeneration and organogenesis. Generally, it is difficult to naturally regenerate and repair a large-size tissue defect only by supplying cells to the defective site, because both the cells and the ECM as well as the surrounding environment are lost. Therefore, to induce tissue regeneration at the defective site, one possible way is to artificially build a local environment for cells, which is a three-dimensional scaffold of artificial ECM to initially assist their attachment and subsequent proliferation and differentiation, inducing cell-based tissue regeneration. It is expected that cells residing around the implanted scaffold infiltrate into the scaffold and consequently proliferate and differentiate therein if the artificial ECM is biologically compatible. Biomaterials play an important role in the preparation...
of cell scaffolds. Basically, the scaffold should be porous and biodegradable. The pore structure is necessary for the infiltration of cells into the scaffold, the supply of oxygen and nutrients to cells proliferated therein and the washout of cell wastes. Once the cell-induced tissue regeneration is naturally initiated, cells eventually produce the ECM of natural scaffold. The scaffold is a temporary platform of cell activities. The long-term retention of cell scaffold sometimes causes physical hindrance against the natural process of tissue regeneration. It is a key for successful tissue regeneration to control the time profile of scaffold biodegradation at the defect as well as the three-dimensional structure. The cell scaffold developed initially is a physical entity that does not have any functions to positively promote the proliferation and differentiation of cells. However, a new type of cell scaffold has been investigated to combine biomaterials with biological ligands reactive for cell receptors, biosignalling molecules and cell-adhesive substances for artificially promoted cell-based tissue regeneration (Lutolf & Hubbell 2005; Chan & Mooney 2008). The mechanical property of biomaterial scaffolds is also important. If the mechanical strength is low, the scaffold readily deforms in the body. As a result, the regeneration of bulky tissue cannot be always expected. An appropriate mechanical design is much required. For example, the incorporation of fibres and ceramic granules enabled biomaterial sponges to mechanically reinforce and enhance in vivo tissue regeneration following the implantation of sponges combined with stem cells (Hiraoka et al. 2003; Takahashi et al. 2005).

The second key technology is to provide the space for cell-based tissue regeneration and supply nutrients and oxygen to cells by angiogenesis (figure 2b). When a body defect is generated, the defect space is generally occupied rapidly by the fibrous tissue produced by fibroblasts, which are ubiquitously present in the body and can proliferate rapidly. This is one of the typical wound healing processes to temporarily fill and repair a body defect in case of emergency. 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 can no longer be expected. To prevent tissue ingrowth, a barrier membrane to provide a space for tissue regeneration is required. The scaffold (figure 2a) sometimes functions to provide the space for tissue regeneration. To expect cell-based tissue regeneration, the site at which the cells are to be transplanted should be physiologically arranged. For the transplantation of cells, if there are no supplies of nutrients and oxygen, it is not practically expected that the cells transplanted would survive and function well to induce tissue regeneration. A similar problem is observed for the transplantation of stem cells although their potential for proliferation and differentiation is superior to that of matured cells. For example, as one possible strategy to tackle the issue, it is promising to induce in vivo angiogenesis by biomaterial technology and methodology (Tabata & Ikada 1999; Tabata 2003a, 2005a, b; Tambara et al. 2005; Soto-Gutierrez et al. 2006; Takehara et al. 2008).

When the tissue around a defect does not have the inherent potential to regenerate, tissue regeneration cannot always be expected if only the scaffold or the space-providing membrane is supplied. The cell scaffold and membrane of biomaterials should be used in combination with the cells or/and signalling molecules (e.g. growth factors, cytokines, chemokines) that have the potential to accelerate cell-induced tissue regeneration. Currently, several stem cells with a high potential for proliferation and differentiation are being prepared and applied to a tissue defect to induce tissue regeneration therein (Salgado et al. 2006; Fernyhough et al. 2008; Mountford 2008). Although there are some cases where a growth factor is required to promote tissue regeneration, 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 diffuses from the injected site and is enzymatically digested or deactivated. To enable the growth factor to efficiently exert its biological function, a new technology is required. This comes in the form of the third key technology of tissue engineering: DDS (figure 2c).

Although every DDS technology is available for tissue engineering, the release technology of growth factors and genes has been mainly applied to induce tissue regeneration. For example, the controlled release of growth factor at the site of action over an extended period of time is achieved by incorporating the factor into an appropriate biomaterial carrier. It is also possible that when incorporated in the release carrier, the growth factor is protected against proteolysis so as to prolong the activity retention in vivo. The release carrier should be degraded in the body, because it becomes useless after the release function is completed. In addition to the controlled release, the improvement of signalling molecule stabilization, the prolongation of the in vivo half-life and the targeting to the site of action will enhance the factor-induced tissue regeneration.

Over time, DDS has been investigated and developed as the only technology or methodology to enhance the in vivo efficacy of therapeutic drugs. Based on fixed ideas and historical background, it has been thought that DDS cannot be scientifically and technologically applied to tissue regeneration therapy. There has been little investigation of DDS-based tissue regeneration. Considering that drugs applicable for regenerative therapy include proteins and genes effective in promoting the proliferation and differentiation of cells to induce tissue and organ regeneration, generally, they are biologically unstable in vivo. Therefore, upon in vivo administration of biosignalling molecules, it is necessary for enhanced in vivo biological activity to make use of the DDS technology and methodology. Many types of biomaterials have been used for DDS applications. The application of DDS to tissue regenerative therapy will be described later.

As mentioned earlier, it is necessary to prepare cells for transplantation therapy. Therefore, basic research of cell biology and medicine is actively ongoing to select candidate cells suitable for cell therapy. Among the candidates, cells that have proliferation and differentiation potentials higher than matured cells, so-called stem cells, are promising from the viewpoint of cell-based
tissue regeneration. For example, in addition to haemo-
poietic stem cells, mesenchymal stem cells (MSC) are
present in adult bone marrow. It has been elucidated
that the MSC of adult stem cells have a proliferative
ability and an inherent potential to differentiate into
osteogenic, chondrogenic, adipogenic and myocardial
cell lineages. Presently, human MSC are isolated and are
commercially available (Pittenger et al. 1999) while
clinical experiments have been begun. If it is possible to
clinically use a differentiated type of a patient’s own
MSC, immunological rejection will no longer be a
question. Many researchers of tissue regeneration
therapy with stem cells, especially MSC, have reported
their therapeutic feasibility in tissue regeneration
(Leo & Grande 2006; Docheva et al. 2007; Fibbe et
al. 2007; Picinich et al. 2007; Shanti et al. 2007). In addition,
nerval stem cells (Hsa et al. 2007; Kornblum 2007) and
stem cells isolatable from fat tissue (Strem & Hedrick
2005; Gimble et al. 2007; Schaffer & Buchler 2007) have
been extensively investigated. They can be prepared
from the foetus and adult and have exhibited plasticity
for cell differentiation and are expected to be promising
cell candidates for regenerative medical therapy.
Embryonic stem (ES; Mountford 2008) and inducible
pluripotent stem (iPS) cells (Yamanaka 2007) have
been established and expected as cell sources for
transplantation therapy and the research and development
of drugs.

However, one of the problems is the shortage of cells
clinically available. Therefore, it is necessary to develop
a technology or methodology for the preparation of a
large number of stem cells of high quality. For this
purpose, isolation, induction and in vitro culture
technologies of stem cells are required. The fourth
technology is for the efficient preparation and prolifer-
ation of cells (figure 2d), which are achieved by
providing a cell culture substrate as the artificial
ECM. The cell scaffold for in vivo tissue regeneration
mentioned previously can be used for the purpose
of culture substrate. The three-dimensional substrate can
be designed and prepared from biomaterials of cyto-
compatibility. From the viewpoint of nutrients and
oxygen supplies, research and development of cell
culture methods and bioreactors are required (Holtorf
et al. 2006; Mironov et al. 2008).

The fifth technology is to genetically engineer cells
for their functional manipulation and basic biology.
There are some cases where cells transplanted do not
function well to induce cell-based tissue regeneration.
As one trial to tackle this issue, cells are genetically
engineered with biomaterials to activate the biological
functions. It is necessary for the genetic engineering of
cells to develop a carrier of gene transfection and cell
culture system for efficient gene expression. Generally,
viral vectors have been scientifically used for gene
transfection because of their high efficiency. However,
viruses cannot be used to treat patients; thus, non-viral
gene carrier biomaterials are required to be developed
from the clinical viewpoint of cell therapy. This
technology is also applicable for the basic research of
stem cell biology and medicine, which gives important
knowledge and results for cell therapy. This combina-
tion of cell scaffold, space protection and DDS

3. CLINICAL ASPECTS OF TISSUE
ENGINEERING-BASED TISSUE
REGENERATION

Tissue engineering for clinical regeneration therapy can
be classified as either in vitro or in vivo depending on the
site where tissue regeneration or organ substitution
is performed. In vitro tissue engineering involves tissue
reconstruction by cell culture methods and organ
substitution with functional cells—termed bioartificial
hybrid organ. If a tissue can be reconstructed in vitro in
factories or laboratories on a large scale, it can be
supplied to patients when required. For example,
human skin fibroblasts are cultured in a collagen
sponge to prepare an artificial dermis for skin grafting
(Kuroyanagi et al. 2001; Kumagai 2002; Ichikawa
et al. 2005; Takemoto et al. 2008). To prepare pulmonary
vein and bone substitutes for human treatment, the
cells of blood vessel and bone marrow-derived stem cells
are cultured in the porous scaffolds of tube-shape
polylactide (Shin’oka et al. 2001) and cubic hydroxyl
apatite (Ohgushi & Caplan 1999). However, it is
difficult to reproduce the in vivo event completely
in vitro by using the basic knowledge of biology and
medicine or cell culture technologies currently avail-
able. At present, it is difficult to realize in vitro tissue
engineering because the artificial arrangement of a
biological environment to induce cell-based tissue
reconstruction is practically impossible. Even if a
three-dimensional tissue-like construct is prepared
in vitro, it is practically difficult to allow the construct
to survive and function in vivo after grafting.
In addition, the construct does not always connect
with surrounding natural tissue biologically. The
in vivo environment for the construct implanted should
be designed. Another application of in vitro tissue
engineering is the substitution of organ functions by the
use of allo- or xenogeneic cells. The cells are combined
with an immunoisolation membrane of biomaterials to
substitute the physiological functions of liver and
pancreas (Falqui et al. 1991; Olle et al. 1996; Yamashita
et al. 2002; Kobayashi et al. 2003; Ehashi et al. 2006).
Biomaterials have been investigated to design and
create a biological environment that can assist the
proliferation and differentiation of cells and maintain
their biological functions.

Distinct from the in vitro tissue engineering, in vivo
tissue engineering is advantageous from the viewpoint
of the environment to induce tissue regeneration. It is
likely that most of the biological components necessary
for tissue regeneration, such as growth factors and
cytokines, are naturally supplied by the body. Based on
this advantage, almost all the approaches of tissue

engineering have been performed in vivo with or without biodegradable cell scaffolds. There are several examples where in vivo tissue regeneration is achieved by use of the scaffold with or without cells (Tabata 2003b, 2005b, 2008). As described previously, if patients are young and healthy, and the tissue to be repaired has a high potential to induce regeneration, active and immature cells infiltrate the matrix of an implanted biodegradable scaffold from the surrounding healthy tissue, resulting in the formation of new tissue. However, additional means are required if patients are aged and/or suffer from other diseases, such as diabetes and hyperlipaemia, or if the regeneration potential of tissue is low as a result of, for example, a low concentration of cells and growth factors. The simplest method is to supply a growth factor to the site of regeneration for cell differentiation and proliferation in a controllable fashion. As described previously, to allow a growth factor of in vivo instability to efficiently function in the body, it is necessary to make use of the DDS technology.

One of the largest problems in the release technology of growth factor protein is the loss of biological activity of protein released from a protein-carrier formulation. It has been demonstrated that this activity loss mainly results from denaturation and deactivation of protein during the preparation process of the formulation (Gombotz & Wee 1998; Tabata 2003a). Therefore, a method to prepare the formulation of protein release with biomaterials should be exploited to minimize protein denaturation. From this viewpoint, a polymer hydrogel may be a preferable candidate as a protein release carrier because of its biosafety and its high inertness towards protein drugs. However, it will be practically impossible to achieve the controlled release of protein over a long period of time from hydrogels only when the protein is simply combined in the hydrogels. The protein combined is normally localized in the water phase inside the hydrogel and the release is generally diffusion controlled through the water pathway of hydrogels. The release profile is regulated by modifying the cross-linking density of hydrogel polymers. However, there is a limitation for the regulation of protein diffusion by the cross-linking density. Therefore, it is practically impossible to achieve protein release for more than a few days. Considering protein-induced activation of cell functions, protein release for at least 7 days or longer is required. Thus, it is necessary to contrive a method of protein release. A possible approach is to immobilize a growth factor in a biodegradable hydrogel. It should be noted that chemical immobilization is not suitable for this purpose, because the chemical reaction to growth factor often causes a loss of biological activity. Physical immobilization is preferable. The immobilized factor is not released by simple diffusion, but only by the solubilization of the factor in water accompanied with the generation of water-soluble hydrogel fragments as a result of hydrogel biodegradation. In such a release system, the time profile of growth factor release is governed only by that of the in vivo hydrogel degradation. The requirements of a hydrogel polymer for this release system are to have biodegradability and the ability to physically interact with protein for immobilization. In addition, from the viewpoint of therapeutic applications, it is preferable that the polymer has been in previous clinical use.

There have been several reports on the controlled release of proteins (Andrianov & Payne 1998; Fujioka et al. 1998; Gombotz & Wee 1998; Tessmar & Gopferich 2007). Some hydrogels were effective in enhancing the in vivo biological activity of growth factors to induce tissue regeneration (Lutolf & Hubbell 2005; Coviello et al. 2007; Van Tomme & Hennink 2007). Based on the requirement described above, we have selected gelatin to prepare a biodegradable hydrogel protein release carrier, because it has been clinically used in medical and pharmaceutical applications and proven to be biocompatible. As expected, the hydrogel of gelatin immobilized the growth factor through the physicochemical interaction between the gelatin and factor. The growth factor could be released from the hydrogel accompanied with the degradation of carrier hydrogel (Tabata et al. 1994, 1999; Tabata & Ikada 1999). Gelatin is easily chemically derivatized to change the molecular nature, which is susceptible to physical interaction with protein. Using the gelatin hydrogel, the controlled release of bioactive growth factors over a time range of 5 days to three months was possible. The controlled release of various growth factors has succeeded in the regeneration therapy of various tissues (figure 3). bFGF is one of the angiogenic factors and has the ability to enhance wound healing through an induction of angiogenesis and induce the regeneration of bone, cartilage, skin, nerve and fat tissues (Tabata 2007, 2008). When human recombinant bFGF (Fibrast spray, Kaken Pharmaceutical Co., Tokyo; http://www.kaken.co.jp) was incorporated into a gelatin hydrogel and subcutaneously implanted into a mouse back, significant angiogenic effect was observed around the implanted site, in marked contrast to the injection of bFGF solution even at higher doses (Ikada & Tabata 1998). Vascular endothelial growth factor (VEGF) is another angiogenic biosignalling molecule and has been extensively investigated to demonstrate the potential to induce angiogenesis in different forms of DDS modifications (Tabata et al. 2000a; Zisch et al. 2003; Patel et al. 2008). In general, however, blood vessels regenerated by VEGF are fine when compared with those of bFGF and VEGF often causes tissue oedema. Comparing the points, it is preferable to use bFGF for angiogenic therapy from the viewpoint of the therapeutic necessity of wide blood vessels.

There are two important objectives of angiogenesis in tissue engineering: the therapy of ischaemic disease and ‘in-advance angiogenesis’ for cell transplantation. As a first example, when injected into the ischaemic site of myocardial infarction (Iwakura et al. 2003) or leg ischaemia (Nakajima et al. 2004), gelatin microspheres incorporating bFGF induced angiogenesis to a significantly greater extent than the bFGF solution. This angiogenic therapy for leg ischaemia has been clinically shown to demonstrate good therapeutic efficacy (Marui et al. 2007; figure 4). This is the first report on clinical angiogenic therapy by using the DDS technology of growth factor without any cell transplantation.
The granules of gelatin hydrogels incorporating bFGF were injected into the femoral muscle of the patient’s ischaemic leg. bFGF is released locally around the injected site over two weeks and no bFGF in the blood circulation was detected. When compared before and after the bFGF treatment, the pain score, the tissue oxygen and the blood flow were all significantly improved. In addition, the walking distance of patients treated increased by a clinically significant extent. We have performed the bFGF-induced angiogenic clinical treatment for 25 patients in four university hospitals in Japan (ages: 27–73; male/female ratio: 0.8). Currently, approximately 360 collaborations with the release technology of various growth factors are being performed by clinicians and researchers.

Figure 3. Examples of tissue regeneration with biodegradable hydrogels for growth factor release. (a) Regeneration of coronary artery: (i) bFGF solution, and (iii) diastole, (iv) systole; (ii) gelatin microspheres incorporating bFGF, and (v) diastole, (vi) systole (LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery). (b) Bone regeneration: (i) BMP-2 solution, (ii) hydrogel incorporating BMP-2. (c) Promotion of hair shaft elongation (*p<0.05 versus water-soluble form; **p<0.05 versus other VEGF concentrations in a released form). (d) Articular cartilage regeneration: (i) CTGF solution, (ii) gelatin microspheres incorporating CTGF. (e) Fat tissue regeneration: gelatin microspheres incorporating bFGF.

Figure 4. Examples of clinical tissue regeneration for ischaemic ASO and diabetic foot ulcer of intractable disease with biodegradable hydrogels for bFGF release. Intractable diseases could be repaired only by the intramuscular injection or implantation of hydrogel granules incorporating bFGF. bFGF was locally released over two weeks at the site injected to induce in vivo angiogenesis resulting in promoted wound healing. The first clinical case worldwide: (a) 27 years, male, (b) four weeks later, (c) 12 weeks later; (d) 73 years, female, (e) four weeks later, (f) 16 weeks later. Before treatment, the patients could not walk due to their severe pain. But angiogenic therapy allowed them to walk without any problem.
The sufficient supply of nutrients and oxygen to cells transplanted is indispensable for their survival and maintenance of biological functions in vivo (figure 1b). For successful cell transplantation, it is beneficial to induce angiogenesis in advance throughout the site where cells are transplanted, by using the bFGF release system. This technology of in-advance angiogenesis efficiently enhanced the grafting rate and improved the biological functions of pancreatic islets (Balamurugan et al. 2003), hepatocytes (Ogawa et al. 2001), cardiomyocytes (Sakakibara et al. 2002) and kidney cells (Saito et al. 2003), as well as the engrafting of a bioartificial dermis–epidermis skin-tissue construct (Tsujii-Saso et al. 2007). Consequently, the therapeutic efficacy induced by the cells and constructs implanted was significantly augmented compared with that of no angiogenesis. The release system enabled the enhanced activity of various growth factors, such as bFGF, TGF-β1 and bone morphogenetic protein-2 (BMP-2) to induce bone regeneration and bone healing, as well as to synergistically promote bone regeneration induced by MSC of bone marrow (Tabata et al. 2006). It is well known that insulin-like growth factor (IGF)-1 suppresses the apoptosis of nerve cells. Controlled release of IGF-1 from the gelatin hydrogel inhibited the ageing of acoustic nerve, resulting in suppressed deterioration of hearing hardness (Iwai et al. 2006). This IGF-1 treatment has been started clinically to demonstrate good therapeutic efficacy (table 2). In addition, the hydrogel system can release not only one type of growth factor but also two or more types in different concentrations and release profiles. Upon applying a hydrogel incorporating a low dose of either bFGF or TGF-β1 to a bone defect of rabbit skulls, no bone tissue was regenerated at the defect. However, a synergistic effect on bone regeneration was observed by the simultaneous release of two factors (L. Hong, Y. Tabata, S. Miyamoto, K. Yamada, Y. Ikada 2000, unpublished data). The synergistic angiogenesis of bFGF and HGF was observed (Marui et al. 2005). The platelet contains a cocktail of autologous growth factors. The controlled release of the cocktail by the hydrogel enabled the regeneration of bone (Hokugo et al. 2005), knee meniscus (Ishida et al. 2007) and intervertebral disc (Nagae et al. 2007), in marked contrast with the use of cocktail alone.

4. FURTHER EXPERIMENTAL ASPECTS OF TISSUE ENGINEERING-BASED REGENERATION THERAPY

The cell scaffolding technology can not only be applied to the in vivo cell-based tissue regeneration, but can also assist and promote the basic sciences in vitro proliferation and differentiation of stem cells. The latter is for the preparation of cells with a good quality applicable for cell therapy and the research development of stem cell biology. To manipulate the proliferation and differentiation of stem cells in vitro, there are two scientific and technological approaches: the modification of culture medium and cell substrate. There have been several trials to add various soluble factors in the culture medium to manipulate cell behaviour. Considering that normally most cells cannot survive and biologically function without attachment on to the culture substrate, there is no doubt that the substrate greatly affects the profiles of cell proliferation and differentiation. For example, it has been demonstrated that the direction of cell differentiation can be modified by the softness (Engler et al. 2006) and size (Nakamura et al. 2005) of cell substrates and the surface modification of biosignalling molecules (Nagaoka et al. 2006; Benoit et al. 2008). In addition, there have been reports on a three-dimensional scaffold that has a steric gradient of bioactive molecule concentration inside the material (Yamamoto et al. 2007). It has been well recognized that a biological niche is the local environment of stem cells to naturally regulate their proliferation and differentiation in vivo (Arai & Suda 2007). When the molecular mechanism of stem cell niche is biologically clarified and the key components can be elucidated and used, the combination with the conventional cell scaffold will enable stem cells to artificially manipulate their potentials for tissue regeneration.

<table>
<thead>
<tr>
<th>disease or operation</th>
<th>growth factor</th>
<th>effect</th>
<th>no. of facilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>vascular graft surgery for heart</td>
<td>bFGF</td>
<td>angiogenesis</td>
<td>1</td>
</tr>
<tr>
<td>arteriosclerosis obliterans (ASO)</td>
<td>bFGF</td>
<td>angiogenesis</td>
<td>4</td>
</tr>
<tr>
<td>and buerger diseases</td>
<td>bFGF</td>
<td>angio genesis</td>
<td></td>
</tr>
<tr>
<td>diabetic skin ulcer</td>
<td>bFGF</td>
<td>angio genesis, dermatogenesis</td>
<td>3</td>
</tr>
<tr>
<td>periodontal disease</td>
<td>bFGF</td>
<td>regeneration of alveolar bone</td>
<td>3</td>
</tr>
<tr>
<td>hardness of hearing</td>
<td>IGF-1</td>
<td>inhibition of acoustic nerve ageing</td>
<td>2</td>
</tr>
<tr>
<td>meniscus injury</td>
<td>PRP</td>
<td>chondrogenesis</td>
<td>1</td>
</tr>
<tr>
<td>facial plastic surgery</td>
<td>bFGF</td>
<td>chondrogenesis, soft tissue regeneration</td>
<td>1</td>
</tr>
<tr>
<td>chest plastic surgery</td>
<td>bFGF</td>
<td>regeneration of sternum bone, angiogenesis</td>
<td>1</td>
</tr>
<tr>
<td>plastic surgery of soft tissues</td>
<td>bFGF</td>
<td>angiogenesis</td>
<td>1</td>
</tr>
<tr>
<td>facial nerve paralysis</td>
<td>bFGF</td>
<td>nerve function recovery</td>
<td>1</td>
</tr>
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</table>

Table 2. Ongoing clinical experiments of tissue regeneration with the release technology of growth factors.
Future development of stem cell biology and substantial collaboration with biomaterials technology will open a new research field of stem cells and consequently lead to a promising strategy of cell therapy.

DDS technology and methodology play an important role in the basic research of stem cell biology. Biosignalling molecules to regulate the functions and fate of stem cells have been elucidated. In this connection, for the future, the necessity for DDS will be undoubtedly increased to develop the basic research of tissue regeneration and consequently realize stem cell-based regeneration therapy. It is practically impossible to allow biosignalling proteins to function in vivo without DDS assistance. In addition to the growth factors, genes have been used for tissue regeneration therapy. There are two future directions of gene therapy. The first direction is the conventional gene therapy where a plasmid DNA and adenovirus are directly injected to give the therapeutic effect. For gene therapy, it is practically important to enhance the efficiency of gene transfection. Generally, the efficacy of viral vectors is higher than that of non-viral carriers. However, the viral vectors are not clinically suitable because of their toxicity and immunogenicity. Therefore, it is necessary to improve the in vivo efficacy of gene transfection. One of the possible ways is to use DDS technologies. Several gene carrier biomaterials, such as cationic liposomes and cationic polymers, have been investigated to enhance the level of gene expression (Kushibiki et al. 2003; Kushibiki & Tabata 2004; Jo & Tabata 2008). In addition, the release technology is also effective in enhancing gene transfection and expression. The release of plasmid DNA from a biodegradable hydrogel of cationized gelatin derivative enhanced the level of gene expression as well as prolonged the time period of expression (Kushibiki et al. 2003; Kushibiki & Tabata 2004). The injection of the cationized gelatin hydrogel incorporating plasmid DNA enhanced the in vivo therapeutic effects to a significantly greater extent than plasmid DNA solution alone (Kushibiki et al. 2004). The microspheres incorporating plasmid DNA also enhanced the gene expression of cells to genetically activate their biological functions and consequently increased the efficacy of cell therapy. Using the microspheres incorporating plasmid DNA, intracellular controlled release of plasmid DNA was achieved to enhance the efficiency of gene transfection to a higher level than that of adenovirus transfection. The cells genetically engineered functioned well to achieve higher therapeutic efficacy (Yamamoto & Tabata 2006; Jo & Tabata 2008). With the recent development of genomics researches, the DNA sequence of the human genome has been elucidated and disease therapy on the genetic level will develop in the future. As proteins, genes are also unstable in vivo. Therefore, it is no doubt that DDS technology will have an important role in gene therapy.

The second direction is to genetically engineer cells for their functional activation, which is applicable for cell transplantation therapy and stem cell biology. There are some cases where the transplantation of stem cells alone does not always induce a clinically acceptable therapeutic effect. A promising and practical way to overcome this problem would be to genetically engineer stem cells by gene transfection for the activation of their biological functions. So far, such cell activation has been tried by using virus vectors (Lai et al. 2008). This trial has been of great success, but the good results cannot be applied to clinical therapies because of the virus use. Therefore, it is necessary to develop a system of non-virus gene transfection. The DDS technology and methodology are very effective in developing a non-viral system of gene transfection with the efficiency of gene transfection as high as that of the viral system (Yamamoto & Tabata 2006; Jo & Tabata 2008). In addition to the microspheres for the intracellular release of plasmid DNA, a new non-viral carrier has been prepared from cationized polysaccharides. The carrier of gene transfection enabled plasmid DNA to enhance the level of gene expression for stem cells with less cytotoxicity than commercially available cationized liposomes. Gene transfection with the cationized polysaccharide was effective in enhancing the gene expression of stem cells to genetically engineer the biological function (Jo & Tabata 2008). In addition, the stem cells engineered showed the efficacy of cell therapy greater than the original cells (Jo et al. 2007). The level of gene expression by the non-viral carrier was also enhanced by contriving the methodology of gene transfection culture, such as a reverse transfection method and bioreactor combination (Okazaki et al. 2007; Nagane et al. in press). Thus, the gene engineering technology with biomaterials is effective in enhancing stem cell-based regeneration therapy and also developing the basic research of stem cell biology and medicine. The technology of gene transfection will be available for non-viral induction of iPS cells and also allow several bioactive substances to internalize into cells for an investigation of their biological functions in stem cell biology. The efficient action of siRNA by non-viral carriers is effective in genetically regulating the cell function. The biomaterial carrier enables siRNA to enhance the silencing effect in vitro and in vivo, resulting in the artificial modification of cell functions, which is applicable for stem cell biology and future cell therapy.

5. CLOSING REMARKS

Tissue regeneration therapy—a new therapy based on the natural induction of tissue regeneration through cell transplantation and tissue engineering—is a third therapy following reconstructive surgery and organ transplantation. To achieve the regeneration therapy by use of tissue engineering technologies, substantial collaborative research between material, pharmaceutical, biological and clinical scientists is needed. Even if superior stem cells can be practically obtained, it is impossible to therapeutically treat patients only by transplanting the cells prepared even combined with the scientific knowledge of cells and related substances, unless a local environment of cells suitable to promote the proliferation and differentiation is created and provided properly. To create the environment, there is no doubt that the biomaterials and the technology are needed.
However, one of the main problems to create the regeneration environment at present is the absolute shortage of biomaterial researchers who investigate the cell scaffold, the DDS, the protective barrier and cell culture, aiming at tissue regeneration and the biological substitution of organ functions. Such researchers must have knowledge in medicine, dentistry, biology and pharmacology, in addition to material sciences. It is indispensable to educate researchers of an interdisciplinary field, who have an engineering background and can also understand basic biology, medicine and clinical medicine. One of the representative interdisciplinary research fields is DDS technology, which is also applicable for producing non-viral vectors in the preparation of genetically engineered cells for regeneration therapy. The development of non-viral vectors with a high efficiency of gene transfection for stem cells is of a high priority.

Tissue engineering technology is not only used surgically for tissue defects, but also applied to develop a therapeutic method for chronic fibrosis diseases by making use of internal medical treatment. Tissue engineering is still in its infancy, although some research projects have already come close to the level of clinical applications. The increasing significance of biomaterials for cell scaffolding and DDS in future will help progress in basic and applied tissue engineering. We will be happy if this review stimulates readers’ interest in the idea and research field of tissue engineering to assist in understanding how important biomaterials are to develop the basic research of stem cell biology and medicine as well as realize tissue regeneration therapy.

The ethics committee of Kyoto University and Nippon Medical School approved the study protocol. Patient enrolment began in February 2005.

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