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REVIEW

Challenges in tissue engineering

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Almost 30 years have passed since a term 'tissue engineering' was created to represent a new concept that focuses on regeneration of neotissues from cells with the support of biomaterials and growth factors. This interdisciplinary engineering has attracted much attention as a new therapeutic means that may overcome the drawbacks involved in the current artificial organs and organ transplantation that have been also aiming at replacing lost or severely damaged tissues or organs. However, the tissues regenerated by this tissue engineering and widely applied to patients are still very limited, including skin, bone, cartilage, capillary and periodontal tissues. What are the reasons for such slow advances in clinical applications of tissue engineering? This article gives the brief overview on the current tissue engineering, covering the fundamentals and applications. The fundamentals of tissue engineering involve the cell sources, scaffolds for cell expansion and differentiation and carriers for growth factors. Animal and human trials are the major part of the applications. Based on these results, some critical problems to be resolved for the advances of tissue engineering are addressed from the engineering point of view, emphasizing the close collaboration between medical doctors and biomaterials scientists.

Keywords: cell source; scaffold; growth factor; carrier; animal studies; human trials

1. INTRODUCTION

When tissues or organs have been so severely diseased or lost by cancer, congenital anomaly, or trauma that conventional pharmaceutical treatments are no more applicable, artificial organs (including tissues) or organ transplantation are the first choice to reconstruct the devastated tissues or organs. However, these surgical treatments have been facing a number of challenges at moment. Artificial organs have been improved by remarkable advances in the biomedical engineering in the past decades, but still need better biocompatibility and biofunctionality. Problems in current organ transplantation include shortage of donated organs and immune rejection, although immunosuppressive therapy has recently much advanced.

Approximately three decades ago a new paradigm emerged as an alternative approach to tissue and organ reconstruction. That is tissue engineering. A distinctive feature of tissue engineering is to regenerate patient's own tissues and organs that are entirely free of poor biocompatibility and low biofunctionality as well as severe immune rejection. Owing to the outstanding advantages, tissue engineering is often considered as an ultimately ideal medical treatment. To regenerate new tissues, this biomedical engineering utilizes three basic tools; cell, scaffold and growth factor. These three are not always simultaneously used. For instance, it is sufficient for some bone tissue engineering to use only bone morphogenetic protein (BMP), while dermal tissue can be regenerated simply by placing a porous collagen sheet on a full-thickness skin wound without cell seeding and growth factor delivery. In this case, fibroblasts are recruited from the surrounding healthy skin tissue, migrate into the pores of the sheet and secrete proteins and glycosaminoglycans which construct a dermal tissue, the sheet being simultaneously absorbed into the body.

The earliest clinical application of human cells in tissue engineering may be for the skin tissue using fibroblasts, keratinocytes, or a scaffold (template). It started around 1980. A little later, periodontal and alveolar bone tissues were attempted to regenerate with use of membranes that ensure the maintenance of the site for tissue regeneration by preventing fibroblasts from invasion there (guided tissue regeneration (GTR) and guided bone regeneration (GBR)). Vacanti *et al.* (1988) studied the cell transplantation using bioabsorbable synthetic polymers as matrices, while Wakitani *et al.* (1989) reported the repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. A review article presented by Langer & Vacanti (1993) with title 'Tissue Engineering' has greatly contributed to the promotion of tissue engineering research worldwide.

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A new term 'Regenerative Medicine' seems to have appeared when two US research groups announced the success in establishment of human embryonic stem (ES) and embryonic germ (EG) cell lines in 1998 (Thomson *et al.* 1998; Shamblott *et al.* 1998). Since the epoch-making achievements, research focus seems to have shifted from tissue engineering to ES and other stem cells. Apparently, the bankruptcy of two US venture companies on tissue engineering in 2002 has further discouraged the research and development of tissue engineering.

It is likely that public and medical communities have great expectations on regenerative medicine or tissue engineering, although reported human clinical trials are still scanty in tissue engineering. It may be good timing to think about the reasons for the delayed progress in clinical applications of tissue engineering in comparison with tremendous amounts of basic studies concerned with this field. The objective of this article is to provide a brief overview on the current tissue engineering research covering from fundamental technologies, including cell source, scaffold fabrication, growth factor delivery to preclinical and clinical studies related to tissue engineering. The purpose of tissue engineering research is very clear; that is, to establish a new clinical technology that makes possible medical treatments for diseases that have been too difficult to be cured by existing methods. To avoid ambiguous description and broad bibliographical survey, attention will be given on suggestions of various challenges associated with tissue engineering that typically involves interdisciplinary science and technology ranging from biology to engineering.

2. KEY MATERIALS FOR TISSUE ENGINEERING

As mentioned above, the cell, scaffold and growth factor are the three key materials for tissue engineering. The cell synthesizes matrices of new tissue, while the scaffold provides the appropriate environment for cells to be able to effectively accomplish their missions. The function of growth factors is to facilitate and promote cells to regenerate new tissue. Although numerous investigations have been undertaken to regenerate various kinds of tissue, there are still many critical factors involved in this regenerative program, including cell source, scaffold construction, cell seeding, culture environment, matrix production analysis, mechanical properties of cell–scaffold construct and suitable animal models. However, it may be possible someday in the future to isolate patient's cells by means of a small biopsy, expand the cell number in the culture, seed cells onto a three-dimensional scaffold and implant to the same patient.

2.1. Cells

The cell source has an enormous influence on the success of tissue engineering. Based on the living species difference, cells applicable to tissue engineering may be classified into autologous (patient's own), allogenic (human other than patient) and xenogenic (animal origin). Autologous cells are the most appropriate for tissue engineering so far as their activity remains high, whereas allogenic and xenogenic cells are immunogenic and will need an immunosuppressive therapy when a new tissue is engineered from these heterogenous cells. After publishment of reports that have revealed a presence of porcine endogenous retrovirus in pigs (Patience *et al.* 1997), the frequency of pig use as the cell source has dramatically reduced. A problem associated with autologous cells is the difficulty in harvesting a sufficient amount of cells, especially when a patient is aged or has severely been diseased. For instance, it is extremely difficult to harvest cardiac cells from a patient suffering from myocardial infarction. If the amount of harvested cells is not sufficient enough for clinical treatment, the cells should be expanded by cell culture. This procedure requires not only a clean cell-processing centre to avoid contamination, but also is time-consuming. In addition, possible viral infection will accompany the fetal calf serum (FCS), which is most commonly used in cell culture. Allogenic cells are useful for skin tissue engineering, because even the allogenic engineered skin tissue serves as better wound cover than non-biological ones, for instance, owing to secretion of powerful growth factors from the engineered tissue. Xenogenic feeder cells have usually been utilized for engineering of epidermal tissue from keratinocyte, because of their high epidermal growth activity, although they have a risk of viral infection.

Cells can be also classified on the basis of the difference in the extent of differentiation. Non-differentiated cells are ES and EG cells that are able to differentiate into all kinds of cells present in the body and have potential to expand without limitation. These are the major reasons for why the pluripotent stem cells have attracted so much attention. However, the pluripotent cell involves a number of problems when the cell is used for medical treatments of patients. If ES cells are obtained from fertilized eggs that have remained not used after the infertile therapy of couples, the ES cells are allogenic to the patient who will receive the cell transplantation. Somatic cell nuclear transfer to an enucleated egg is an alternative approach to circumvent this immune issue because of gene matching, but this technology is controversial as one cannot deny the possible risk of clonal human reproduction through abuse of this novel technology.

In the adult body, stem cells exist that can differentiate into many lineages under appropriate conditions. The haematopoietic stem cell (HSC) found in bone marrow is most extensively studied, providing eosinophils, erythrocytes, megakaryocytes, osteoclasts and B and T cells. The bone marrow contains also mesenchymal stem cells (MSCs) that are capable of differentiating into several connective tissue cell types, including osteocytes, chondrocytes, adipocytes, tenocytes, myocytes and bone marrow stromal cells (Kotobuki *et al.* 2004). On the other hand, several tissues in the adult contain progenitor cells that can proliferate and then differentiate to provide organ-specific cell types. Examples include the proliferative keratinocytes found in skin, hepatocytes responding to liver damage, intestinal crypt cells that replenish the absorptive epithelium cells and osteoblasts actively forming new bone and becoming osteocytes. These

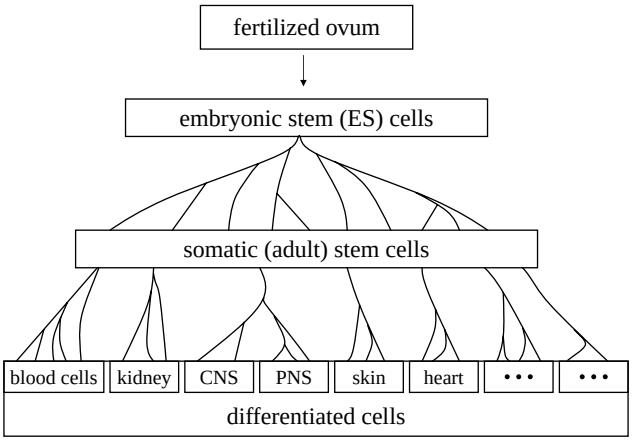

Figure 1. A long way from undifferentiated to differentiated cells. CNS, central nervous system; PNS, peripheral nervous system.

progenitor cells appear to have their differentiation limited to a defined lineage. No unique marker has been found that positively identifies the human MSCs. Often they express surface markers associated with distinctive differentiated cell types.

Those which are much more attractive and practical for tissue engineering are somatic (or adult) stem cells that have been believed to exist in tissues of adult, as shown in figure 1. The most extensively studied stem cell is the HSC that exists in the bone marrow. The stem cell of the epidermal tissue is thought to be present in the basement membrane, but has not yet been identified. The MSC may be the stem cell that has been studied most extensively next to the HSC. The capability of MSC to differentiate into various tissues, including bones, cartilages, adipose, blood vessels, nerves and skin, has attracted much attention of tissue engineers. An advantage of MSC is the high safety compared with the ES cell that elicits teratoma when transplanted before differentiation into a certain lineage. This suggests that complete differentiation and purification of the cells modified from ES cells are prerequisites for the clinical application of ES cell. By contrast, no tumorigenesis has been reported on bone marrow cells when they have clinically been used. It is interesting to note that bone marrow regenerates only the tissue specific to the site where the bone marrow cells have been transplanted, although the bone marrow must involve different kinds of stem cells.

Bone marrow cells are relatively easy to harvest and expand, and if they can transdifferentiate into virtually any cell type when exposed to the right set of conditions, they could become a 'universal stem cell' (Pittenger *et al.* 2002). However, there is strong evidence that much of what has been considered to be transdifferentiation of bone marrow cells is actually attributable to fusion of these cells to host differentiated cells, particularly to the central nervous system, cardiac and liver cells. Fusion does not necessarily mean 'artefact', because it is a normal developmental process in at least several tissues, and thus may be clinically useful as well. Whether the observed developmental potential of bone marrow cells has a single or multiple explanations matters little, if the end result restores tissue structure and function. That is why the apparent ability of bone marrow cells to tissue differentiation has caused so much excitement. It has been reported that the fat tissue contains MSCs (Zuk *et al.* 2001). This tissue may be easier for harvesting than bone marrow, and hence numerous studies have been undertaken to isolate MSCs from the fat tissue.

When combined with knowledge of growth factor and cytokine that promote differentiation, and suitable scaffold or carrier for delivery to a particular tissue site, MSC may appear to be the ideal candidate cell for development of therapeutic tissue regeneration and tissue engineering. Neither unpredictable nor unwanted cell types have been detected among the differentiated human MSCs. This differs from all reports investigating the differentiation of ES cells which form multiple and unpredictable cell types as they differentiate.

If the adult stem cell of each tissue becomes readily available to tissue engineering investigators as a result of great advances in cell biology and biotechnology, clinical application of tissue engineering will be remarkably accelerated. However, in many cases, the number of harvested stem cells is not sufficient enough for clinical applications. We need to multiply the harvested cells by cell culture. This is a challenge because their proliferative ability is generally not high and de-differentiation will eventually take place during the proliferation of stem cells. When stem cells are cultivated on a two-dimensional cell culture dish, cell proliferation proceeds at a reasonable rate but accompanies de-differentiation. On the contrary, de-differentiation does not readily take place when stem cells are cultured on three-dimensional substrates, but the cell proliferation rate is reduced to very low levels. Therefore, it has been attempted to switch the de-differentiated state of cells to their original state by three-dimensional culture, but the switching efficiency is not as high as expected. The most desirable approach is to multiply stem cells at a high rate keeping the undifferentiated original state.

The FCS that has most frequently been used for cell culture contains xenogenic species that might induce some infection (Louet 2004). Replacement of the FCS with synthetic sera using a range of recombinant growth factors has been explored for a long time, but the proliferative capability is still lower than FCS. However, it was recently found that the proliferative ability of human serum increased to the level similar to that of FCS, if platelets involved in the human blood had been in advance broken before serum preparation and the platelet contents were joined to the serum (Kawaguchi *et al.* 2005). The conventional human serum is prepared after removal of the whole platelets.

2.2. Scaffold

It would be very convenient to both patients and physicians if devastated tissues or organs of patients can be regenerated by simple cell injection to a target site, but such cases are relatively rare, including haematopoietic diseases, cardiovascular diseases with malfunction of capillary or small blood vessels like arterioles, diseases due to deficiency of physiologically

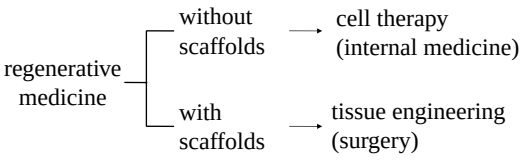

Figure 2. Classification of regenerative medicine based on the use of scaffolds.

active substances (e.g. insulin, dopamine and adenosine deaminase) and lost sensory (e.g. retina and cochlear cells). Most of large-sized tissues and organs with distinct three-dimensional form will require support for their formation from cells. The support is called scaffold, template, or artificial extracellular matrix (ECM). The major function of scaffold is similar to that of the natural ECM that assists proliferation, differentiation, and biosynthesis of cells. In addition, a scaffold placed at the site of regeneration will prevent disturbing cells from invasion into the site of action.

A few technical terms have been used without clear definition. They include 'tissue engineering', 'regenerative medicine', 'cell (cellular) therapy' and 'cell transplantation'. For convenience, they are classified here depending on the scaffold use, as depicted in figure 2. According to this definition, regenerative medicine involves two concepts, cell therapy without any use of scaffold and tissue engineering that needs scaffold as a support of tissue regeneration.

To fulfil the functions of a scaffold in tissue engineering, the scaffold should meet a number of requirements. First, it should have interconnected micropores, so that numerous cells can be seeded, migrate into the inside, increase the cell number and should be supplied by sufficient amounts of nutrients. Micropores make both vascular formation and waste transport possible. This is important for the survival of cells inside the scaffold. An optimal pore size is in the range between 100 and 500 μm. Furthermore, scaffold should have an optimal porosity with adequate surface area and mechanical strength. The absorption kinetics of scaffold is also critical and depends on the tissue to be regenerated. If a scaffold is used for the tissue engineering of skeletal system, degradation of the scaffold biomaterial should be relatively slow, as it has to maintain the mechanical strength until tissue regeneration is almost completed. For the skin tissue engineering, the scaffold does not need to stay longer than one month. If the scaffold remains for a longer time than desired, the remaining material may retard the tissue regeneration rather than promote it. This indicates that the absorption kinetics of scaffold material will profoundly affect the success rate of tissue engineering.

Poly(α-hydroxyacid)s, especially glycolide and lactide polymers, have been widely used as biomaterials for scaffold fabrication (Morita & Ikada 2002). In general, polyglycolide (PGA) and its copolymers, such as lactide–glycolide copolymer (PLGA), degrade too quickly when used as a scaffold, because their tensile strength reduces to the half within two weeks. In contrast, poly(L-lactide) (PLLA) degrades too slowly, requiring 3–6 years for complete resorption. Owing to

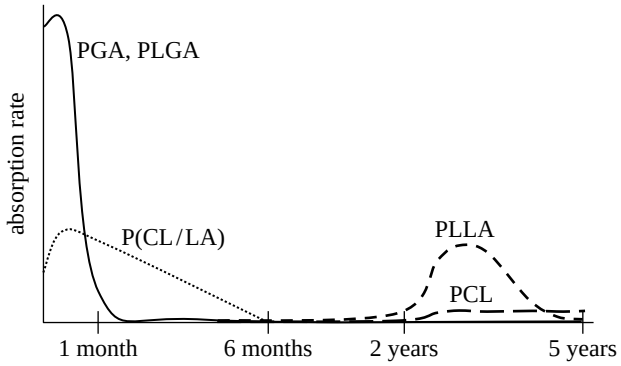

Figure 3. Schematic for absorption rate of absorbable polyesters. PGA, polyglycolide; PLGA, lactide–glycolide copolymer; P(CL/LA), ε-caprolactone–lactide copolymer; PLLA, poly-L-lactide; PCL, poly-ε-caprolactone.

this inadequate resorption property of PGA and PLLA, lactide copolymers, such as lactide-ε-caprolactone copolymers (LA–CL cop), have been preferably employed in recent studies on tissue engineering. Figure 3 represents relative resorption rates of PGA, PLGA, LA–CL cop [P(CL/LA)], PLLA and poly-ε-caprolactone (PCL).

Poly(α-hydroxyacid)s degrade through non-enzymatic hydrolysis, whereas naturally occurring biomaterials, including collagen, hyaluronic acid and chitin, undergo enzymatic hydrolysis. Except for chitin, most of naturally occurring polymers are hydrophilic and yield products with low mechanical strength in comparison with poly(α-hydroxyacid)s. This leads to limited applications of these biopolymers. Alginates do not contain any hydrolysable bonds, but are often used as a resorbable biomaterial. This is because alginates that have been made water-insoluble through ionic crosslinking with divalent cations such as Ca^{2+} will return to water-soluble polymer in the body as the ionic crosslinks are released through exchange of Ca^{2+} with Na^{+} in the body. Other water-soluble biopolymers are mostly rendered water-insoluble through covalent crosslinking with use of glutaraldehyde or carbodiimide.

A variety of methods have been applied for fabrication of porous scaffolds. Among them are freeze-drying and porogen leaching (Whang & Healy 2002). In addition, sophisticated technologies have been employed for scaffold fabrication in recent years. They include solid-free prototype and electrospinning. Any expensive apparatus is not required for electrospinning, but solid-free prototype needs high-cost devices. It should be noted that even if refined patterns have been designed on a scaffold, the pattern will soon disappear as the material undergoes degradation. The pore size of nanofibre sheets made by electrospinning is generally too small for cell migration into the material, if the sheet layer is as thick as 1 mm.

Inorganic scaffolds have been used in addition to polymeric scaffolds, specifically for the bone tissue engineering. The biomaterials used for this purpose include hydroxyapatite and β-tricalcium phosphate (TCP). These inorganic scaffolds with interconnected pore structure are brittle, and hence are mixed with soft

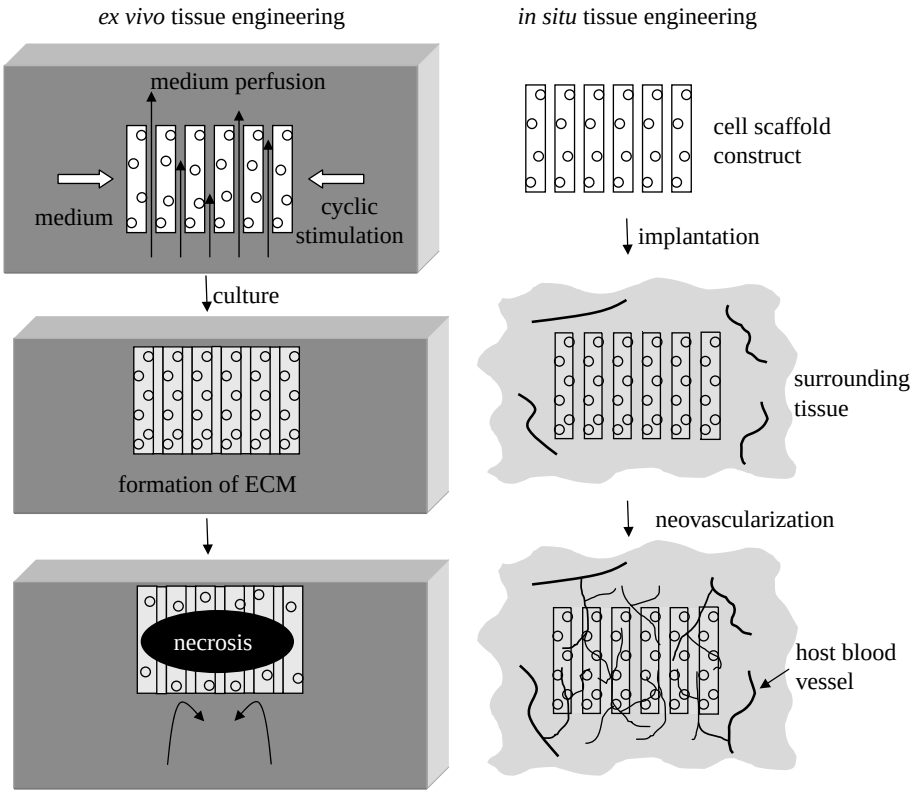

Figure 4. Differences in biological reaction between *ex vivo* and *in situ* tissue engineering.

polymers to yield tough hybrid scaffolds, so that they still maintain high bioactivity (Laurencin *et al.* 2002).

Two extreme cases are well known for applications of the cell–scaffold construct made by cell seeding to a scaffold. One is to place the construct in a bioreactor to reconstruct an engineered tissue *in vitro*. The other is to implant the construct in the body until a new tissue is regenerated *in vivo*. The former is called *in vitro* (or *ex vivo*) tissue engineering and the latter *in vivo* (or *in situ*) tissue engineering. The *in vitro* tissue engineering can produce multiple engineered tissues from a single cell source. Therefore, much effort has been paid to the *in vitro* tissue engineering with expectation to create new tissue engineering business. Indeed, mass production of engineered tissues offers products that can be delivered to medical centres on their demand. In this case, the cell source used for tissue engineering is not cells from a patient, but may be originated from healthy adults or children under an active growth condition. Since this cell source is allogenic, the clinical application must be limited to special cases, such as wound cover and life-threatening diseases. On the contrary, the *in vivo* tissue engineering serves only a certain single patient. It appears that a big business opportunity cannot be expected for this sort of tissue engineering. However, the *in vivo* tissue engineering also needs cell expansion that may make a business chance. In addition, medical centres may purchase scaffolds that have been mass-produced in biomedical industry. When a cell–scaffold construct becomes a product for sale, some issues should be taken into consideration. First, the standardization for the safety assessment of the cell–scaffold construct will be required, although it is very difficult because the product involves human cells that have never been a therapeutic object of sales. In contrast, scaffold products containing no cells are similar to conventional medical devices, such as absorbable sutures and bone-fixation screws. When patient's own cells are used only for the patient, there is no infection but contamination risk alone.

Another big issue of tissue engineering is the neovascularization that is essential to supply oxygen and nutrients to the cells in constructs. It is virtually impossible to expect the neovascularization throughout a cell–scaffold construct in the case of *in vitro* tissue engineering. On the contrary, the *in vivo* tissue engineering will induce the neovascularization if the construct can provide adequate stimuli to the surrounding tissues, as illustrated in figure 4.

2.3. Growth factors

There are a range of proteins that play a key role in proliferation and differentiation of cells. These proteins are endogenously secreted in the body by cells themselves (autocrine) or as a result of communication with surrounding cells (paracrine). These proteins are called cell growth factor or simply growth factor. An advantage of an engineered skin tissue to non-biological wound covers, even though the engineered tissue is allogenic, is that the engineered tissue is able to provide growth factors to the wound site where the engineered tissue is placed.

The growth factors that have frequently been applied to tissue engineering include bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGF or FGF-2), vascular epithelial growth factor and transforming growth factor-β (TGF-β).

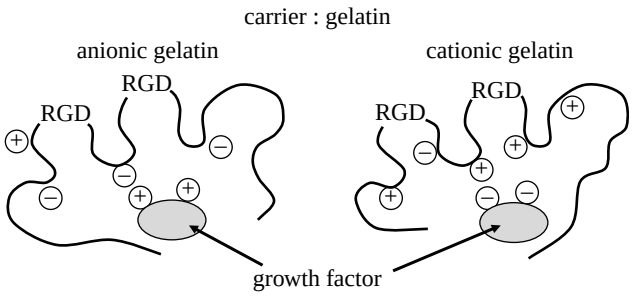

Figure 5. Entrapping of growth factor in bioabsorbable gelatin through ionic interaction. RGD, cell-adhesion oligopeptide consisting of arginine, glycine and aspartic acid.

The remarkable capability of growth factors may be imagined from the fact that BMP and bFGF alone can induce bone and vascular tissue regeneration, respectively, without the assistance of scaffold or seeded cells. Apparently, an addition of proper growth factors to a cell–scaffold construct must further promote the tissue regeneration in comparison with no use of growth factors.

What is the most critical in the application of growth factors to tissue engineering is how to deliver growth factors to the site of action. As is well known, bolus injection of growth factors in solution is not an effective means, because the injected protein molecules quickly scatter away from the injected site. Three methods have been attempted for the growth factor delivery. One is to use DNA plasmids that include the gene encoding the desired growth factor. When injected in the body, the plasmid will biosynthesize the growth factor and secrete it from the cells where the plasmid resides, so long as the plasmid remains active. The second method exploits also gene technology. A gene encoding the growth factor is transferred to a specific type of cells using a vector and the processed cells are transplanted into the body where the desired tissue is to be engineered. The growth factor encoded by the transferred gene will be released into the body as long as the gene stays active in the cells.

In the third method, growth factor protein is directly applied along with a carrier. This approach has most frequently been studied for the growth factor delivery in tissue engineering. The selection of carrier greatly influences the release kinetics of the growth factor. The requirements for carrier are to induce denaturation of growth factor molecules to a minimal extent during their incorporation or entrapment, to facilitate optimal release kinetics of growth factor, and to be absorbed into the body. Few studies have attempted to follow the *in vivo* release profile of growth factors in detail. It seems probable that most of the carriers widely used have not exhibited a steady constant release but a burst profile. Combination of BMP with collagen carrier has clinically been used in the US. The potent bioactivity of BMPs was first applied by Urist in 1965 to induce ectopic bone formation in muscle pouches of rabbits, rats, mice and guinea-pigs (Urist 1965). Since then, various BMPs have been isolated, characterized and cloned (Wozney *et al.* 1988). After numerous animal studies and human clinical trials

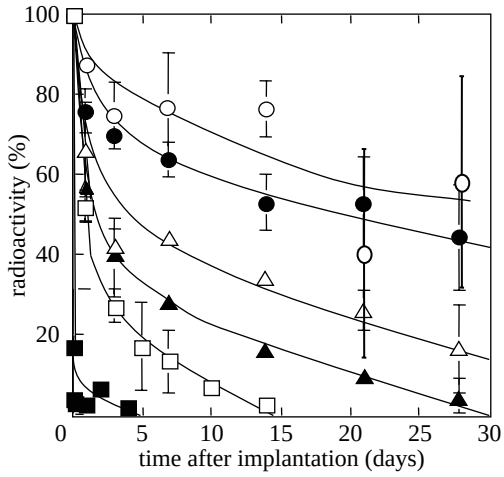

Figure 6. *In vivo* time profiles of the radioactivity remaining after the subcutaneous implantation of gelatin hydrogels incorporating ^{125}I-labelled bone morphogenetic protein-2 (BMP-2) into the back of mice. The hydrogel water contents are (open circles) 93.8, (closed circles) 96.9, (open triangles) 97.8, (filled triangles) 99.1 and (open squares) 99.7 wt%. The symbol (filled squares) indicates the remaining radioactivity after injection of ^{125}I-labelled BMP-2 solution.

demonstrating osteoinductive properties on a par or better than bone autograft, the US Food and Drug Administration recently approved recombinant human BMP-2 for use in spinal fusion procedures.

We have been using gelatin, a collagen derivative, as a carrier of growth factors. Two kinds of gelatin are commercially available, acidic and basic. Acidic gelatin can form ionic complex with basic proteins. It is therefore expected that bFGF will be released from the ionic complex formed upon mixing acidic gelatin with ionically basic bFGF, when implanted in the body, as a result of enzymatic degradation of gelatin molecules. A possible release mechanism is shown in figure 5. Indeed, an excellent release kinetics was observed when the ionic complex was implanted in mice, but a mixture of basic gelatin with bFGF did not exhibit any sustained release *in vivo* (Tabata *et al.* 1994). Interestingly, the mixture of BMP with basic gelatin represented sustained release profiles when subcutaneously implanted in mice, as shown in figure 6 (Yamamoto *et al.* 2003). In this case, the explanation of sustained release of BMP by complex formation may not be correct, because the isoelectric point of the BMP used in this study was around 8.5. Probably, another interaction different from electrostatic force may be prevalent between the gelatin and BMP molecules.

A single growth factor has been used mostly for the tissue engineering of one tissue. Apparently, it would be better to use plural growth factors for promotion of tissue regeneration. Currently, much effort has been made to induce neovascularization using various growth factors for supply of sufficient amounts of nutrients to the cells engaged in tissue regeneration. Application of growth factors in tissue engineering will be accelerated when growth factors become more readily available and less expensive.

3. ANIMAL STUDIES

When a new clinical technology is developed, its safety and efficacy should be examined using animals prior to human trials. This principle is also applied to tissue engineering. Tissue engineering research uses animals not only in primary experiments, but also in secondary testing before clinical application. The final preclinical animal models in which the new technology is tested should mimic the clinical situation as close as possible. Cell–scaffold constructs are often implanted in nude mice to avoid immunorejection. Because nude mouse is too small to evaluate an engineered tissue, a large animal should be used, but the cells in the cell–scaffold construct should be harvested from the same animal. It should be kept in mind that animal models play an essential role in tissue engineering.

3.1. Skeletal system

Numerous studies have demonstrated the feasibility of bone marrow-derived MSCs in rodents and large animal models, but investigations comparing tissue-engineered bone to autologous bone grafts in a clinically relevant model, or in controlled studies in primates, have not yet been reported. Bone is a self-repairing structural material, able to adapt its mass, shape and properties to changes in mechanical requirements and endues voluntary physical activity for life without breaking. Unloaded bone defects smaller than a critical size will heal spontaneously if appropriate conditions are available. Since mechanical environments markedly affect the bone tissue engineering, Lamerigts *et al.* (2000) developed a loaded implant model to study the effect of load on the bone formation in tissue-engineered constructs. They used controlled air pressure to load a piston on materials inserted in a distal femur defect.

Adult articular cartilage contains no blood supply, neural network and lymphatic drainage. As a result, articular cartilage defects larger than 2–4 mm in diameter rarely heal even with continuous passive motion. Owing to the physiological and anatomical differences between the human joint and that of the experimental animals, it is thought that there is no animal defect model that is directly applicable to the human. However, final preclinical evaluation of an articular cartilage reconstruction technique requires confirmation in a large animal model. For the advancement of tissue engineering of articular cartilage, an understanding of the biomechanical properties of normal articular cartilage and the functional requirements for repaired articular cartilage is very important. Therefore, new techniques to measure the biomechanical properties of normal, diseased and engineered cartilage, including minimally invasive and non-invasive techniques for *in vivo* measurement, have been attempted to be developed.

Several products have been approved by FDA for treatment on tendon injuries, specifically for use in the supraspinatus tendon of the shoulder. One of these products is created from multiple layers of swine intestinal submucosa, while another is a porcine-derived engineered collagen matrix. Critical evaluation of these products' successful studies in animals leading to FDA approval may be helpful in planning for future devices and techniques.

3.2. Cardiovascular system

Diseases of the heart and blood vessels are the largest cause of mortality in developed countries. Although a great deal of experimentation has been performed *in vitro* and preliminarily *in vivo*, long-term studies in animals still need to be performed before cardiovascular tissue engineering constructs—blood vessels, cardiac tissue and heart valves—become commercially available. Lambs have been used to evaluate a tissue-engineered vascular construct. Shum-Tim *et al.* (1999) harvested ovine carotid arteries and extracted a mixed population of endothelial cells, smooth-muscle cells and fibroblasts, followed by growing *in vitro*. The mixed cell population was then seeded onto PGA–polyhydroxyalkanoate blend tubular scaffolds. These were implanted into lamb aorta and compared with controls of acellular polymer tubes. At five months, cell of the tissue-engineered grafts remained patent, whereas none of the acellular constructs did. Over time, collagen and DNA contents approached native levels, as did the histological appearance. Watanabe *et al.* (2001) used femoral veins of mongrel dogs to obtain cells, which were seeded onto 5 mm diameter resorbable scaffolds consisting of non-woven fabric sheets of PGA and a copolymer of L-lactide and ε-caprolactone, before elective sacrifice. On examination grossly, angiographically and immunohistologically, there was neither evidence of stenosis, dilation, nor thrombus formation.

The ideal cardiac tissue construct should display functional and morphological properties of native heart muscle. Thus, constructs should be contractile, electrophysiologically stable, mechanically robust yet flexible, vascularized or at least quickly vascularized after implantation and autologous. Rat has proven useful when studying cardiac muscle tissue engineering. Co-culturing neonatal cardiomyocytes, fibroblasts and collagen within bioreactors produced constructs that may promise for the repair of larger scar areas of the myocardium (Van Luyn *et al.* 2002). Seeded neonatal rat cardiomyocytes in a collagen matrix demonstrated contractility both spontaneously and in response to electrical and chemical stimulation (Kofidis *et al.* 2002). Further work on the tissue engineering of cardiac muscle suggested that such constructs had superior wall thickness and uniformity of tissue architecture when perfused (Carrier *et al.* 2002*a*). Constructs of 9.5 mm diameter and 2 mm thickness based on neonatal rat cardiac myocytes and fibrous resorbable scaffolds, when cultured by direct perfusion, showed improved special uniformity of cell distribution and enhanced expression of cardiac-specific markers, especially when oxygen levels were accurately regulated (Carrier *et al.* 2002*b*).

3.3. Nerve and spinal cord system

Injury to the adult spinal cord sets off a process known as secondary injury; a cascade of pathophysiological

events that results in loss of nervous tissue (secondary tissue loss), neuronal cell death and damage of spinal and supraspinal axonal circuitries. Patist *et al.* (2004) studied the effects of poly(D,L-lactide) macroporous scaffolds with or without brain-derived neurotrophil factors (BDNF) on tissue sparing, neuronal survival, axonal regeneration and behavioural improvements of the hindlimbs, following implantation in the transected adult rat thoracic spinal cord. The results demonstrated that the scaffolds were well tolerated in the transected adult rat spinal cord. The gliotic and inflammatory response was not beyond what is normally seen after transection/implantation in the cord and the amount of tissue loss was similar after implantation of scaffold or fibrin only. Moreover, the presence of BDNF in the scaffold had a neuroprotective effect on neurons in the rostral cord and resulted in a more rapid ingrowth of axons and in the formation of blood vessels in the porous scaffold. However, despite these effects, the overall regenerative response was low and none of the responding axons grew from the scaffold into the caudal cord.

With respect to peripheral nerve regeneration, recent studies have focused on nerve guides that are resorbed, preferably after they have fulfilled their guiding function, such as LA–CL cop and benzyl esters of hyaluronic acid. The sciatic nerve is the most commonly studied peripheral nerve. The main conclusions of investigations after sciatic nerve transection in rats are that resorbable nerve conduits lead to a considerable and relatively fast reinnervation of the muscles. However, reinnervation is at random, leading to inadequate activation of muscles, and at least temporarily, abnormal histochemistry of muscles.

3.4. Auricular cartilage

Auricular reconstruction for cartilage defects, such as for congenital microtia, remains one of the most difficult challenges in reconstructive surgery. Earlier work by Vacanti *et al.* (1988) demonstrated that bovine chondrocytes seeding onto synthetic resorbable scaffold could produce neocartilage after implantation into athymic mice. They further reported that cartilage could be created in predetermined shapes and dimensions using cell transplantation on appropriate templates (Vacanti *et al.* 1991) even in a complex three-dimensional architecture, like a human ear (Cao *et al.* 1997).

4. CLINICAL STUDIES

As mentioned in §1, the skin tissue engineering was first clinically applied around 1980. First clinical application of articular chondrocytes to reconstruct small defects in knee articular cartilage was reported by Brittberg *et al.* (1994). Despite tremendous number of investigations on tissue engineering since then, not many reports have been yet published on clinical application in this area. Vacanti *et al.* (1991) reported that synthetic polymers seeded with chondrocytes provide a template for new cartilage formation, but any detailed reports on clinical application of chondrocytes with synthetic polymers are not yet available. Lundburg's research group undertook intensive work on reconstruction of transected peripheral nerve using nerve guiding tubes in 1980s, but still a large number of animal studies have continuously been published. With respect to the bone tissue engineering, FDA has approved clinical use of BMP, but few clinical cases have been reported on the bone regeneration using scaffolds and osteogenic cells.

There may be many reasons for such slow advances in the clinical tissue engineering. On the contrary, one might state that it will take a long time, generally longer than 20–30 years, from the start of basic research to establishment of the new clinical technology associated to the basic research. Blood and bone marrow transplantation has evolved over the past 20 years into a successful therapy for a variety of malignant and non-malignant diseases. In recent years, researchers have to properly address ethical and animal right issues. The role of institutional review board has increasingly been becoming important. It may be difficult task to present clear evidence for the safety of cell–scaffold constructs to regulatory authorities.

Some clinical results of tissue engineering will be represented below. Although figure 2 clearly distinguished between cell therapy and tissue engineering to make clear different approaches of regenerative medicine, the following examples will include some of cell therapy that seem difficult to discriminate from tissue engineering.

4.1. Skin

Although numerous experimental strategies have been evaluated, there are currently no commercially available composite grafts consisting of dermal and epidermal components together in one grafting stage that can provide permanent autologous skin replacement for full-thickness wounds. Since the original use of the epithelialized cadaveric allografts to provide a dermal substitute onto which epidermis can be grafted, a small number of commercially available acellular dermal analogues have been used clinically for dermal replacement, including 'Integra' artificial skin. 'Integra', originally developed by Yannas and co-workers, is composed of a bovine type I collagen and glycosaminoglycan chondroitin-6-sulphate. The co-precipitate is lyophilized and subjected to dehydrothermal treatment, forming a highly porous matrix. Additional collagen crosslinking is achieved by exposure to glutaraldehyde. A silicone layer is applied to the surface and functions as a temporary epidermis to prevent trauma, dehydration and bacterial contamination.

Fibroblasts in selected connective tissues can express the gene for a muscle actin, α-smooth muscle actin (SMA) and contract. There is evidence that these cells, referred to as myoblasts, are responsible for dermal wound closure, and the organization of dense fibrous scar is a process that appears to interfere with regeneration. Up until a few years ago, there was virtually no consideration of whether similar processes occurred in other connective tissues. Recent work has

demonstrated that many connective tissue cells and their MSC precursor can also express SMA and can contract. Questions remain, however, about the specific roles of SMA-enabled connective cell contraction in normal physiological and pathological processes.

Following controlled injury, the epidermis regenerates spontaneously. A much deeper injury leads to excision of the dermis, which does not regenerate; instead, the severe wound closes by contraction and scar formation. The macroscopic force to contract a skin wound spontaneously is estimated as about 0.1 N. An individual dermal fibroblast in culture is capable of developing a force of order 1–10 nN. The number of contractile fibroblasts required to develop the macroscopic force that suffices to close the wound is, therefore, at least $10^{-1}/10\ \mathrm{nN} = 10^{7}$ cells, suggesting a factor of this magnitude to scale up from cell to organ. As is well known, the contraction is greatly reduced by placing an adequate scaffold in the skin wound. A cell type that plays a key role during contraction is the differentiated myofibroblast that has been credited with generation of most of the contractive forces in skin wounds. Myofibroblast differentiation is regulated by at least TGF-β1, the presence of mechanical tension and an ECM component. A possible mechanism for contraction blocking by a scaffold is as below (Yannas 2005). Once having migrated inside the scaffold and become bound on the extensive surface of the highly porous scaffold, the long axes of myofibroblast lose their in-place orientation, becoming almost randomly oriented. Accordingly, the contribution of the entire cell assembly to the macroscopic force can be reduced to a collection of pairs of vectors that are oriented at opposite directions from each other. In such a random assembly of force vectors, the sum of forces must be nearly zero. Cells that remain outside the scaffold are oriented in the plane and are free to generate their full contractile force.

4.2. Articular cartilage

Cartilage repair procedures have been developed to deliver autologous chondrogenic cells to the cartilage defect in the form of a cell suspension prepared by the expansion of cells obtained from a cartilage biopsy (Brittberg *et al.* 1994) or precursor cells derived from the periosteum (O'Driscoll 1999) or the perichondrium (Homminga *et al.* 1990), with the expectation that the cells will eventually undergo terminal differentiation to chondrocytes. While these procedures have been used in selected clinics for many years, there is not yet widespread implementation. Brittberg *et al.* were the first to publish their results on 23 patients treated in Sweden for symptomatic cartilage defects. Thirteen patients had femoral condylar defects, changing in size from 1.6 to 6.5 cm^2, due to trauma or osteochondritis dissecans. The results were very promising for the condylar defects. Patients were followed for 16–66 months. Initially, the transplants eliminated knee locking and reduced pain and swelling in all patients. After three months, arthroscopy showed that the transplants were level with the surrounding tissue and spongy when probed, with visible borders. A mean of 36 months after transplantation, the results were excellent or good in two of the seven patients with pattelar transplants, fair in three and poor in two: two patients required a second operation because of severe chondromalacia.

4.3. Central nervous system

Fetal human mesencephalic cells (which include dopaminergic neural stem cells) were the first cells to be transplanted in an attempt to cure Parkinson's disease (Rosenthal 1998). When injected into the striatum of Parkinson's patients, they differentiate into dopaminergic neurons (DANs) and make synaptic connections with host neurons, restoring the activity of the striatopallidothalamic output pathway toward normal. The results of such transplants, however, have been highly variable. In the best cases, there have been dramatic clinical improvements that have lasted 5–10 years. In other cases, improvements have been minimal, or patients have continued to deteriorate. Autopsies of two patients, who died, as well as transplant experiments on Parkinson's animals, indicate that this variation is due to differential survival of transplanted cells. It is thought that a minimum of 80 000 DANs (approx. 20% of the normal number of DANs in the human substantia nigra) are required to obtain a beneficial effect.

4.4. Myocardiac tissue

It has been reported that satellite cells transplanted into cryo-infarcted ventricular muscle of rats, rabbits and pigs integrated into the heart muscle, differentiated into cardiomyocytes and improved heart function. The first phase I trial transplanting satellite cells into the damaged human heart was carried out by Menasche on a 72-year-old patient suffering from severe congenital heart failure caused by extensive myocardial infarction (Menashe 2002). Satellite cells were isolated from a quadriceps muscle biopsy, expanded *in vitro* for two weeks and 800×10^{6} cells (65% myoblasts) delivered into the myocardial scar via 30 injections with a small-gauge needle. Simultaneously, a double bypass was performed in viable but ischaemic areas of the myocardium. Six months later, the patient's symptoms were dramatically improved. Echocardiogram showed evidence of new-onset contraction and fluoro-deoxyglucose positron emission tomography scan showed increased metabolic activity of the infarct. The improvement was considered unlikely to be due to increased collateralization from the bypass region, because this region was far from the infarct. Since this trial, several other cardiac patients have been transplanted with satellite cells.

4.5. Urethra and bladder

Acellular collagen-based matrices derived from the submucosa of small intestine and bladder have been widely used in animal studies. The results were confirmed clinically in a series of patients with hypospandias, an anatomic anomaly in which the urethral opening is not properly located, and urethral

stricture disease (Atala 1999). Cadaveric bladders were microdissected and the submucosal layers were isolated. The decellularized submucosa was used for urethral repair in patients with stricture disease and hypospandias. The matrix was trimmed to 2–16 cm and the neourethras were created by anastomosing the matrix in an onlay fashion to the urethral plate. After a 4–7-year follow-up, 34 of the 40 patients had a successful outcome. Six patients with a urethral stricture had a recurrence, and one patient with hypospandias developed a fistula, an opening along the newly developed urinary channel. The mean maximum urine flow rate significantly increased post-operatively.

The ideal substance for the endoscopic treatment of urinary incontinence and vesicoureteral reflux should be injectable, non-migratory and volume-stable. Alginate embedded with chondrocytes could serve as a synthetic substrate for the injectable delivery and maintenance of cartilage architecture *in vivo*. Two multicentre clinical trials were conducted using the engineered chondrocyte technology (Bent *et al.* 2001). Patients with vesicoureteral reflux were treated at 10 centres throughout the US. The patients had a similar success rate as with other injectable substances in terms of cure. Chondrocyte formation was not noted in patients who had treatment failure. Patients with urinary incontinence were also treated endoscopically with injectable chondrocytes at three different medical centres. Phase I trials showed an approximate success rate of 80% at both 3 and 12 months post-operatively.

In addition to the clinical studies described above, significant progress in the clinical trials of tissue engineering has been reported on the following tissues, organs, or diseases (Ikada 2006): finger bone (Vacanti *et al.* 2001), osteoarthritic ankle bone (Ohgushi *et al.* 2005), osteonecrosis of the femoral head (Gangji & Hauzeur 2005), jaw bone (Kinoshita & Amagasa 2002), maxillary sinus augmentation (Rodriguez *et al.* 2003), metacarpophalangeal joints (Honkanen *et al.* 2003), osteoarthritic knee cartilage (Wakitani *et al.* 2002), osteochondritis dissecans (Ochi *et al.* 2002; Peterson *et al.* 2003), myocardial infarction (Wollert *et al.* 2004), vascular conduits for extracardiac Fontan operation (Shin'oka *et al.* 2001; Naito *et al.* 2003; Matsumura *et al.* 2003) and peripheral nerve (Weber *et al.* 2000).

5. CONCLUDING REMARKS

The medical doctors who will make use of tissue engineering for medical treatments of patients are practically surgeons, because cell–scaffold constructs should be surgically implanted in the body of patients. It is also surgeons who evaluate the efficacy of cell–scaffold constructs using large animal models. As demonstrated in figure 2, regenerative medicine was divided here into cell therapy and tissue engineering, depending on the use of scaffold. However, tissue engineering is not therapy but engineering. It seems more reasonable to divide the regenerative medicine into cell therapy and regenerative surgery, as shown in figure 7. Cell therapy is supported by cell biology, while tissue engineering is an active collaborator of regenerative surgery. What is essential for this implantation

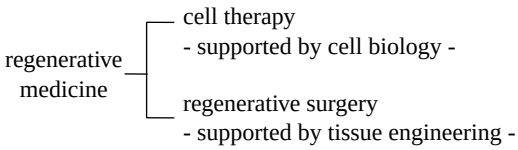

Figure 7. Importance of surgery in regenerative medicine.

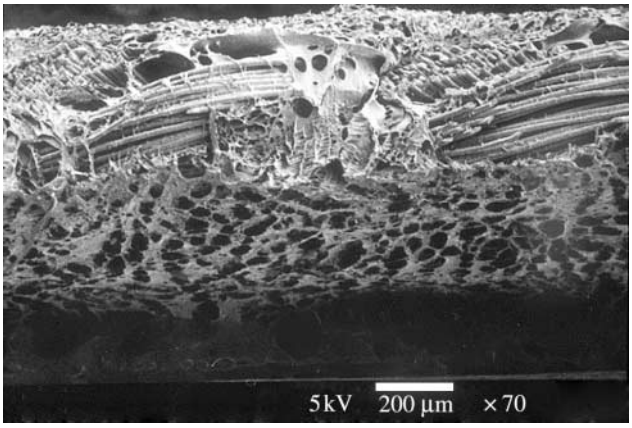

Figure 8. SEM photo of a sponge tube made from a ε-caprolactone–lactide copolymer (P(LA/CL)) and reinforced with polyglycolide (PGA) fibres.

and evaluation by surgeons is that the scaffold should have mechanical properties sufficiently enough for its fixation to the proper site of the body, preferably by suturing. Most of soft porous biomaterials like collagen are not able to endure fixation with suture because of their low tearing strength. In such a case, reinforcement, for instance, with resorbable fibres will be required, as shown in figure 8. Non-woven PGA fabrics that have frequently been used for scaffold fabrication are readily fixed by suturing, but have so high porosity that makes entrapment of sufficient amounts of cells difficult. ε-Caprolactone homopolymer also needs no reinforcement because of its excellent mechanical properties, but is not adequate as a scaffold material due to its too low resorption rate.

In addition to the fixability and resorbability, scaffolds should meet other several requirements. Among them is resistance against stricture. This is necessary when a scaffold is used for regeneration of tubular tissues like blood vessels and esophagus. Reinforcement of porous scaffolds with fibre, mesh, or stent will be effective for these cases. Figure 9 shows an illustration for the stent application to prevent stenosis of a tubular tissue under regeneration. Although such mechanical assistance must be critical especially for the tissue engineering with large animal models and humans, scaffolds with such reinforcement have ever rarely been reported. Handling of scaffolds is also essential for surgeons. Porous hydroxyapatite and β-TCP have been used for studies on bone tissue engineering, but these inorganic scaffolds have poor handling, because they are brittle and do not allow trimming in the operation theatre. It is often required to adjust the shape of scaffold to that of the tissue to be regenerated.

Instead of trying to address the requirements of surgeons, biomaterials scientists who are responsible

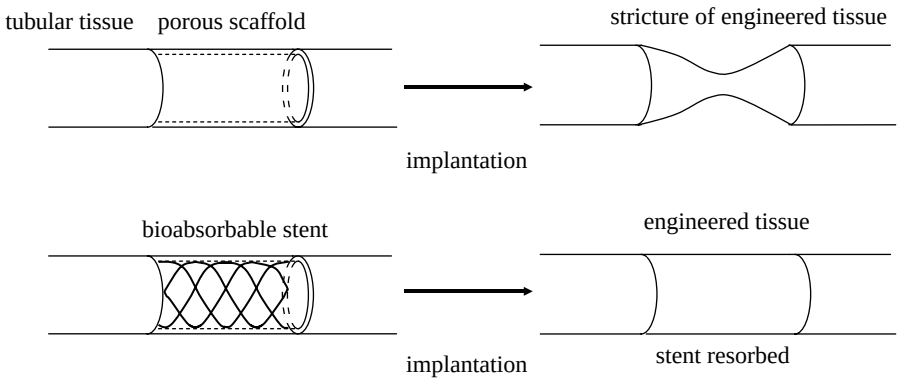

Figure 9. Protection by inserted, resorbable stent for tubular scaffold from stricture.

for scaffold fabrication seem to be busy with their own work. In recent years, a large number of investigations have been increasingly published on the scaffold fabrication by electrospinning. This interesting technique facilitates production of nanofibres from polymer solution by a simple procedure. However, the sheet made by deposition of nanofibres tends to offer too small pore sizes to allow for cell seeding to the inside when the sheet thickness increases to an adequate level for tissue engineering. Similar to electrospinning, a solid-free protocol technique has intensively been employed by biomaterials scientists for scaffold fabrication. This technique needs expensive, sophisticated equipments in contrast to electrospinning, but can yield scaffolds provided with pre-designed, three-dimensional porous structure. The geometrically refined patterning is reproducible and applicable to large-sized three-dimensional scaffolds. The designed, beautiful microstructure will remain for a long time in cell culture media or in the body so far as very slowly resorbable materials, such as PCL and PLLA, are used as material for the micropatterning. However, the patterns designed on resorbable biomaterials that are actually suitable for tissue engineering will be soon destroyed and disappear before completion of tissue regeneration. The same situation will happen for the scaffold that has been surface-modified to immobilize cell-adhesive proteins or peptides to facilitate quick cell adhesion. As is widely recognized, cells will sooner or later adhere to the surface of biomaterials, provided that they are neither extremely hydrophilic nor extremely hydrophobic, even if cell-adhesive moieties are lacking on the non-biological surface.

All the examples described above denote the importance of collaboration with medical doctors, especially surgeons, who conduct implantation and evaluation of the scaffolds provided by biomaterials scientists. Without intimate collaboration among different fields, it would be unlikely for tissue engineering to successfully respond to the expectation of patients who have been suffering from lost or severely diseased tissues or organs. It seems probable that a major reason for delayed clinical trials of tissue engineering be ascribed to insufficient responses of biomaterials group to the requirements of medical groups, apart from recent excessive regulations and stringent assessment levels of review board on tissue-engineered products.

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