Tissue Engineering

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The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in human health care. A new field, tissue engineering, applies the principles of biology and engineering to the development of functional substitutes for damaged tissue. This article discusses the foundations and challenges of this interdisciplinary field and its attempts to provide solutions to tissue creation and repair.

Every year, millions of Americans suffer tissue loss or end-stage organ failure (Table 1). The total national health care cost for these patients exceeds $400 billion per year (*1*, *2*). Approximately 8 million surgical procedures are performed annually in the United States to treat these disorders and 40 to 90 million hospital days are required (*2*). Physicians treat organ or tissue loss by transplanting organs from one individual into another, performing surgical reconstruction, or using mechanical devices such as kidney dialyzers (*3*). Although these therapies have saved and improved countless lives, they remain imperfect solutions. Transplantation is severely limited by a critical donor shortage. For example, fewer than 3,000 donors are available annually for the approximately 30,000 patients who die from liver failure (*4*). Donor shortages worsen every year and increasing numbers of patients die while on waiting lists for needed organs (*5*). Surgical reconstruction can result in long-term problems. For example, colon cancers often develop after surgical treatment of incontinence that directs urine into the colon (*6*). Mechanical devices cannot perform all of the functions of a single organ and therefore cannot prevent progressive patient deterioration.

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (*7*). Three general strategies have been adopted for the creation of new tissue:

1) Isolated cells or cell substitutes. This approach avoids the complications of surgery, allows replacement of only those cells that supply the needed function, and permits manipulation of cells before infusion. Its potential limitations include failure of the infused cells to maintain their function in the recipient, and immunological rejection.

2) Tissue-inducing substances. The success of this approach depends on the purification and large-scale production of appropriate signal molecules, such as growth factors, and, in many cases, the development of methods to deliver these molecules to their targets.

3) Cells placed on or within matrices. In closed systems, the cells are isolated from the body by a membrane that allows permeation of nutrients and wastes but prevents large entities such as antibodies or immune cells from destroying the transplant. These systems can be implanted or used as extracorporeal devices (Fig. 1). In open systems, cells attached to matrices are implanted and become incorporated into the body (Fig. 2). The matrices are fashioned from natural materials such as collagen or from synthetic polymers. Immunological rejection may be prevented by immunosuppressive drugs or by using autologous cells.

Investigators have attempted to engineer virtually every mammalian tissue. In the following summary, we discuss replacement of ectodermal, endodermal, and mesodermal-derived tissue.

Ectoderm

Nervous system. Brain diseases such as Parkinson's disease, where there is a loss of dopamine production, represent an important target for tissue engineering. Transplantation of normal fetal dopamine-producing cells by stereotaxically guided injection into the brain has produced significant reversal of debilitating symptoms in humans (*8*). Alternative methods have been tested in animal models. PC12 cells, an immortalized cell line derived from rat pheochromocytoma, have been encapsulated in polymer membranes and implanted in the guinea pig striatum (Fig. 3A). Dopamine release from the capsules was detectable for 6 months (*9*). Similarly, encapsulated bovine adrenal chromaffin cells have been implanted into the subarachnoid space in rats, where through their continuous production of enkephalins and cathecholamines they appeared to relieve chronic intractable pain (*10*).

Nerve regeneration has also been studied. Peripheral nerves are capable of regeneration after transection injury. Transected nerves can sometimes be clinically repaired by end-to-end approximation of the stumps with fine sutures. When nerve injury results in gaps that are too wide for healing, autologous nerve grafts are used as a bridge. Synthetic nerve guides (conduits) could help in these cases by protecting the regenerating nerve from infiltrating scar tissue or by directing new axons toward their target. Several laboratories have shown in animal models that synthetic guides composed of natural polymers (laminin, collagen, chondroitin sulfate) or synthetic polymers can enhance nerve regeneration (*11*). Initial results suggest that this process can be aided by placing Schwann cells derived from sciatic nerves in Matrigel® seeded in polymer membranes (*12*). In addition, polymers can be designed so that they slowly release growth factors, which may allow regrowth of the damaged nerve over a greater distance (*13*).

Cornea. More than 10 million people worldwide suffer from bilateral corneal blindness. Not only are transplant donors limited, but there is a risk of infectious agent transmission. Ideally, an artificial cornea would consist of materials that support adhesion and proliferation of corneal epithelial cells so that an intact continuous epithelial layer forms. These materials should also have appropriate nutrient and fluid permeability, light transparency, and no toxicity. Corneal epithelial cells have been preseeded on polyvinyl alcohol hydrogels and transplanted into rabbit corneas, where they remained adherent and proliferated for 1 to 2 weeks (*14*). Long-term studies of such materials are warranted; safe and effective methods of attaching these materials to the cornea must also be developed.

Skin. Approximately 150,000 individuals are hospitalized and 10,000 die each year in the United States because of burns. Several new types of tissue transplants are being studied for the treatment of burns, skin ulcers, deep wounds, and other injuries. In some cases, patients are implanted with a composite material whose upper layer consists of silicone (which prevents fluid loss) and whose lower layer consists of chondroitin-sulfate and collagen (which induces new blood vessels and connective tissue ingrowth). In essence, the patients

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receive a new dermis. After 3 weeks, the upper layer is replaced with an extremely thin epidermal graft. Clinical studies have shown good graft acceptance with minimal scarring (*15*). In a refinement of the procedure, the second skin graft was eliminated by seeding epidermal cells obtained from a small skin graft (0.003 inch thick) onto the lower layer prior to placement on the patient (*16*).

Table 1. Incidence of organ and tissue deficiencies, or the number of surgical procedures related to these deficiencies, in the United States. This is a partial list compiled from sources that include the American Diabetes Association, American Liver Foundation, Muscular Dystrophy Association, American Red Cross, American Kidney Foundation, The Wilkerson Group, Cowen and Co., American Academy of Orthopedic Surgery, American Heart Association, National Institute of Neurological Disorders and Stroke, Source Book of Health Insurance (Health Assurance Association of America), 1991, Federal Register, and Department of Health and Human Services (Medicare-based information).

Indication	Procedures or patients per year
Skin	
Burns*	2,150,000
Pressure sores	1,500,000
Venous stasis ulcers	500,000
Diabetic ulcers	600,000
Neuromuscular disorders	200,000
Spinal cord and nerves	40,000
Bone	
Joint replacement	558,200
Bone graft	275,000
Internal fixation	480,000
Facial reconstruction	30,000
Cartilage	
Patella resurfacing	216,000
Chondromalacia patellae	103,400
Meniscal repair	250,000
Arthritis (knee)	149,900
Arthritis (hip)	219,300
Fingers and small joints	179,000
Osteochondritis dissecans	14,500
Tendon repair	33,000
Ligament repair	90,000
Blood vessels	
Heart	754,000
Large and small vessels	606,000
Liver	
Metabolic disorders	5,000
Liver cirrhosis	175,000
Liver cancer	25,000
Pancreas (diabetes)	728,000
Intestine	100,000
Kidney	600,000
Bladder	57,200
Ureter	30,000
Urethra	51,900
Hernia	290,000
Breast	261,000
Blood transfusions	18,000,000
Dental	10,000,000

*Approximately 150,000 of these individuals are hospitalized and 10,000 die annually.

A second approach to skin grafts involves the in vitro culture of epidermal cells (keratinocytes). Small skin biopsies (1 cm^2) are harvested from burn patients and expanded 10,000-fold—a size comparable to an adult's body surface area. This expansion has been achieved by cultivating keratinocytes on a feeder layer of irradiated NIH 3T3 fibroblasts, which, in conjunction with certain added media components, stimulates rapid cell growth. An advantage of this approach is the ability of the grafts to cover extremely large wounds; a disadvantage is the 3- to 4-week period required for cell expansion, which may be too long for a severely burned patient. Cryopreserved allografts may help to circumvent the problem (*17*).

Another promising approach uses human neonatal dermal fibroblasts grown on degradable polyglycolic acid mesh (Fig. 3B). Because fibroblasts are easy to cryopreserve and grow, a uniform stock of cells can be maintained for these grafts. In deep injuries involving all layers of skin, the grafts are placed onto the wound bed and a skin graft is placed on top. The graft then vascularizes, resulting in the formation of organized tissue resembling dermis. Clinical trials have shown good graft acceptance with no evidence of immune rejection (*18*). Fibroblasts have also been placed on a hydrated collagen gel. Upon implantation, the cells migrate through the gel by enzymatic digestion of collagen, which results in reorganization of collagen fibrils (*19*). This approach has undergone limited clinical testing.

Endoderm

Liver. Most liver support systems remove toxins normally metabolized by the liver through dialysis, charcoal hemoperfusion, immobilized enzymes, or exchange transfusion (*20*). None of these systems, however, can offer the full spectrum of functions performed by a healthy liver. Investigators are now endeavoring to achieve liver replacement with isolated hepatocytes. The hepatocytes have been placed in suspensions, encapsulated in microcapsules or hollow fibers, placed on substrates such as microcarriers coated with extracellular matrix proteins, or attached to polymer networks (*20, 21*). In animal models, the transplanted hepatocytes have produced albumin and other liver function markers, and have cleared products of bilirubin and urea metabolism.

Hepatocyte systems are being studied for both extracorporeal and implantable applications. Extracorporeal systems, which would be used when a patient's own liver is recovering or as a bridge to transplant, offer several advantages: (i) better control of the medium surrounding the cell system (for example, the ability to achieve improved transport of oxygen, nutrients, and wastes); (ii) better control of the timing and duration of use; and (iii) a decreased chance of immune rejection because the patient's white cells can be separated from hepatocytes by plasmapheresis. Implantable hepatocyte systems, on the other hand, offer the

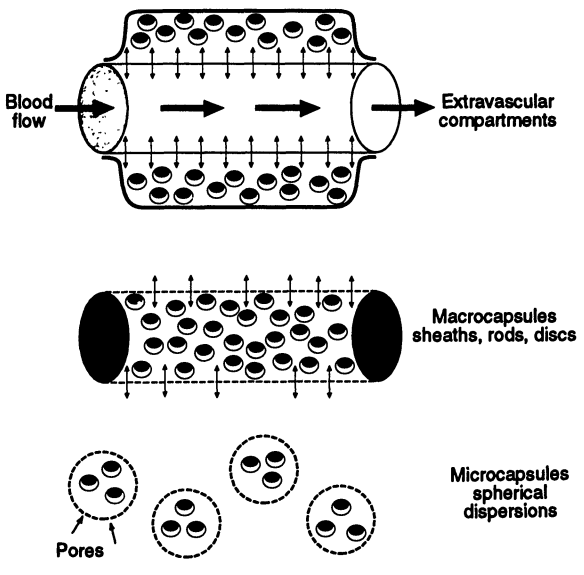

Fig. 1. There are three common closed-system configurations for cell transplant devices (*69, 70*) [figure adapted with permission from (*70*)]. In vascular devices, the cells are placed in an extracellular compartment surrounding a tubular membrane (i.d. ~1 mm) through which blood can flow. In macrocapsule systems, the cells are placed in sheaths, rods, or disks (diameter ≥0.5 to 1.0 mm). In microcapsule systems, the cells are placed in injectable spherical beads (diameter <0.5 mm). Device biocompatibility is critical because tissue reaction can block the flow of nutrients and wastes to and from the capsule. Microcapsules are commonly made of hydrogels—in particular, the polysaccharide alginate—because of the extremely mild conditions required for gel formation. The alginate can be further coated with polyanions, such as polylysine, and again with alginate if desired. Such coatings can affect the flow of nutrients and wastes, mechanical strength, and biocompatibility. Results of in vivo studies with alginate are not always consistent, possibly because of variations in alginate purity (*71*). Macrocapsules and vascular devices often consist of acrylonitrile–vinyl chloride copolymers (*69, 70*). Microcapsules have advantages over macrocapsules in that they impose fewer limitations on diffusional flow of nutrients and wastes and they can be administered by injection. Macrocapsules, on the other hand, are easier to retrieve should complications occur and are physically more stable than microcapsules.

possibility of permanent liver replacement if properly integrated into the patient. Furthermore, the vascular access required for extracorporeal use, which is sometimes associated with thromboembolic complications, may not be necessary with an implantable system (*20*).

Successful hepatocyte transplantation depends on a number of critical steps. First, the hepatocytes must be cultured in vitro prior to transplantation. Hepatocyte morphology can be maintained by sandwiching the cells between two hydrated collagen layers. Under these conditions, the hepatocytes secrete functional markers at physiological levels for at least 6 weeks (*22*). Second, the hepatocytes must be attached to the polymer substrata so that they maintain their differentiated function. By controlling the density of the extracellular matrix substrate used to coat microcarriers or polymer films, both the extent of differentiated function and cell proliferation can be regulated (*23*). Third, the viability of the transplanted hepatocytes must be maintained. This step can be accomplished by vascularizing the cell transplant region to provide oxygen and nutritional factors (*24*). Fourth, a sufficient mass of hepatocytes must become engrafted and functional to achieve metabolic replacement. For implantable systems, this problem has been addressed in animal models in which large numbers of hepatocytes are placed into vascularized areas of the body and supplied with hepatotrophic factors from the portal circulation (*25*). Finally, hepatocyte transplantation per se does not provide all cell types nor the delicate and complex structural features of the liver. For example, products normally excreted through bile may accumulate because of the difficulty in reconstructing the biliary tree solely from hepatocytes. This problem is not life-threatening, however, and resins that bind such products could be incorporated into an artificial liver (*20*). Hepatocytes placed on appropriate polymers can form tissue structures resembling those in the natural organ (Fig. 2) and have shown evidence of bile ducts and bilirubin removal (*25*).

Pancreas. Each year, over 728,000 new cases of diabetes are diagnosed and 150,000 Americans die from the disease and its complications; the total yearly cost in the United States is over $20 billion. Diabetes is characterized by pancreatic islet destruction, leading to loss of glucose control. Tissue-engineering approaches to treatment have focused on transplanting healthy pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Three general approaches (Fig. 1) have been tested in animal models. In the first, a tubular membrane was coiled in a housing that contained islets. The membrane was connected to a polymer graft that in turn connected the device to blood vessels. The membrane had a 50-kD molecular mass cutoff, which allowed free diffusion of glucose and insulin but blocked passage of antibodies and lymphocytes. In pancreatectomized dogs treated with this device, normoglycemia was maintained for more than 150 days (*26*). In a second approach, hollow fibers containing rat islets were immobilized in the polysaccharide alginate. When the device was placed intraperitoneally in diabetic mice, blood glucose levels were lowered for more than 60 days and good tissue biocompatibility was observed (*27*). Finally, islets have been placed in microcapsules composed of alginate or polyacrylates. In some cases, rodents treated with these microcapsules maintained normoglycemia for over 2 years (*28*). All of these transplantation strategies require a large, reliable source of donor islets. Porcine islets are used in many studies, although genetically engineered cells that overproduce insulin are also being examined.

Tubular structures. The current practice of using parts of other organs for reconstruction of the ureter, bladder, and urethra often leads to urinary reflux, infection, and dilation of the upper urinary tract, and, in some instances, electrolyte disturbance. Polymer or metal implants have been used to replace ureters but have generally failed because of poor biocompatibility, lack of peristaltic activity, and accumulation of salt deposits. Because the ureter has a good regenerative capacity, cell-polymer implants have also been explored as replacement therapy. In an initial study, cells derived from a bladder cell carcinoma were cultured on collagen sponges and implanted in rats and dogs for over 3 months. Although the rat implants showed substantial salt deposits on the sponge surface, the dog implants showed extensive urothelial cell regeneration on the collagen graft inner surface (*29*). More recently, urothelial cells were seeded onto degradable polyglycolic acid tubes and implanted in rats and rabbits. After 20 days, two to three layers of urothelial cells lined the polymers (*30*).

The concept of using tubular structures is being studied for other tissues such as the trachea, esophagus, intestine, and kidney. A diseased esophagus, for example, can be treated clinically with autografts from the colon, stomach, skin, or jejunal segments. However, such procedures may result in

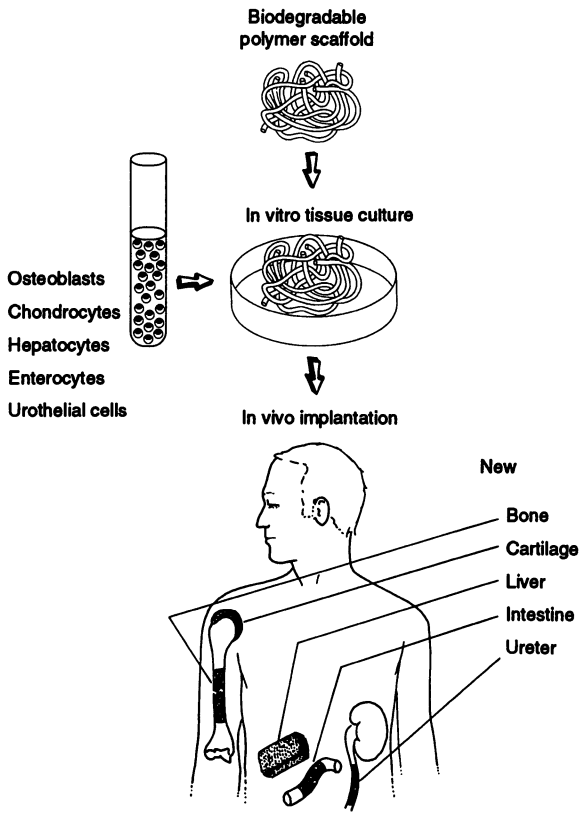

Fig. 2. In one approach to open-system implants, three-dimensional highly porous scaffolds composed of synthetic polymers serve as cell transplant devices. These devices may facilitate formation of structural and functional tissue units by the transplanted cells. This approach is based on the following observations: (i) Every tissue undergoes remodeling. (ii) Isolated cells tend to reform the appropriate tissue structure under appropriate experimental conditions. For example, when capillary endothelial cells are placed on the proper substrate in vitro, they form tubular structures. (iii) Although isolated cells have the capacity to form the appropriate tissue structure, they do so only to a limited degree when placed as a suspension into tissue. Such cells begin without any intrinsic organization and have no template to guide restructuring. (iv) Tissue cannot be implanted in large volumes—cells will not survive if they are located more than a few hundred micrometers from the nearest capillary. Thus, the open-system implants are designed so that the polymer scaffold guides cell organization and growth and allows diffusion of nutrients to the transplanted cells (*32*). Ideally, the cell-polymer matrix is prevascularized or would become vascularized as the cell mass expands after implantation. Vascularization could be a natural host response to the implant or could be artificially induced by slow release of angiogenic factors. The polymer could be degradable or nondegradable. Materials that disappear from the body after they perform their function obviate concerns about long-term biocompatibility.

graft necrosis, inadequate blood supply, and other complications. Copolymer tubes consisting of lactic and glycolic acid have been sutured into dogs after removal of 5-cm esophageal segments. Over time, connective tissue and epithelium covered the polymer graft (which had begun degrading) and the dogs were able to drink freely and eat semisolid food (*31*). In a similar approach, fetal intestinal cells have been placed onto these copolymer tubes and implanted in rats. Histologic examination several weeks later revealed that some animals had well-differentiated intestinal epithelium lining the tubes, and this epithelium appeared to secrete mucous (*32*). Tubular structures have also been used in kidney replacement studies. Current treatments for end-stage renal failure are based solely on nonphysiological driving forces and are not able to mimic active molecular transport accomplished by renal tubular cells. As a first step toward creating a bioartificial kidney, renal tubular cells have been grown on acrylonitrile–vinyl chloride copolymers or microporous cellulose nitrate membranes. In vitro, these cells transported insulin, glucose and tetraethylammonium in the presence of a hemofiltrate from a uremic patient (*33*). This approach has not yet, to our knowledge, been explored in vivo.

Mesoderm

Cartilage, bone, and muscle. Over 1 million surgical procedures in the United States each year involve cartilage replacement. Current therapies include transplantation (removing healthy cartilage, carving it into desired shapes, and reimplanting it where needed in the same patient) and implantation of artificial polymer or metal prostheses. Problems arise, however, in that donor tissue for transplantation is limited, and it is extremely difficult to form delicate three-dimensional implants from host cartilage. Artificial prostheses may result in infection and adhesive breakdown at the host-prosthesis interface. A prosthesis also cannot adapt in response to environmental stresses as does cartilage (*34*).

The need for improved treatments has motivated research aimed at creating new cartilage that is based on collagen-glycosaminoglycan templates (*35*), isolated chondrocytes (*36*), and chondrocytes attached to natural (*37*) or synthetic (*38, 39*) polymers (Fig. 3C). In mice chondrocytes grown for 1 to 6 months on highly porous scaffolds of polyglycolic or polylactic acid maintained the scaffold's original three-dimensional shape, appeared glistening white macroscopically, contained sulfated glycosaminoglycans and type II collagen, and closely resembled cartilage histologically (*39*). It is critical that cartilage transplants of appropriate thickness be mechanically functional. Recently, chondrocytes grown in agarose gel culture have been shown to produce tissue with stiffness and compressibility similar to articular cartilage (*40*). The use of well-stirred bioreactors for cultivating chondrocytes on polymer scaffolds in vitro may enable nutrients to penetrate the center of this nonvascularized tissue, leading to relatively strong and thick (up to 0.5 cm) implants (*41*).

Over 1 million operations annually involve bone repair. Conventionally, bone ingrowth is accelerated through the use of autogenous bone grafts or allogenic bone. The former can be a successful procedure but is often material limited and causes donor site morbidity and contour irregularities (*42*). The latter can also be a successful procedure, but cell-mediated immune responses to transplantation alloantigens and pathogens can be problematic.

Metals and ceramics are also used in several forms: bioinert (for example, alumina), resorbable (for example, tricalcium phosphate), porous (for example, hydroxyapatite-coated metals), and bioactive (for example, hydroxyapatites and certain glasses). Bioactive materials form a bond with the surrounding tissue upon implantation. Such materials are currently being used in middle ear surgery, vertebral surgery, and other applications. Alumina prostheses have been used in a variety of dental and orthopedic procedures because of their minimal interaction with surrounding tissue and low coefficients of friction and wear rates. Porous materials (pore size >100 μm) allow bone to grow into the pores, which strengthens the union between the implant and bone. In practice, it may be desirable for these materials to degrade over time because they lose strength as they age. A critical aspect of ceramic design is determining the appropriate composition, microstructure, pore size, porosity, and surface chemistry to match the specific biologic and metabolic requirements of tissues and disease states (*43*).

Synthetic and natural polymers have been explored for bone repair, but it has been difficult to create a polymer displaying optimal strength and degradation properties. Another approach involves implantation of demineralized bone powder (DBP), which is effective in stimulating bone growth in animals and humans; however, DBP cannot induce sufficient bone formation in most non-bony sites. Bone morphogenic proteins (BMP), now produced by genetic engineering but originally derived from DBP, or growth factors such as transforming growth factor–β (TGF-β), are other promising strategies. The former induce formation of both cartilage and bone (including marrow) and the latter augment bone growth (*44*). Such molecules have theoretically unlimited availability; effective delivery systems for such agents will be important. Bone growth can also be induced when cells are grown on synthetic polymers or ceramics. For example, when marrow cells are grown on porous calcium phosphates in mice, bone forms inside the pores within 3 weeks (*45*).

The ability to generate muscle fibers may be useful in the treatment of muscle injury, cardiac disease, disorders involving smooth muscle of the intestine or urinary tract, and in patients with muscular dystrophy. It has been difficult to find drug therapies to treat such diseases; however, gene therapy or

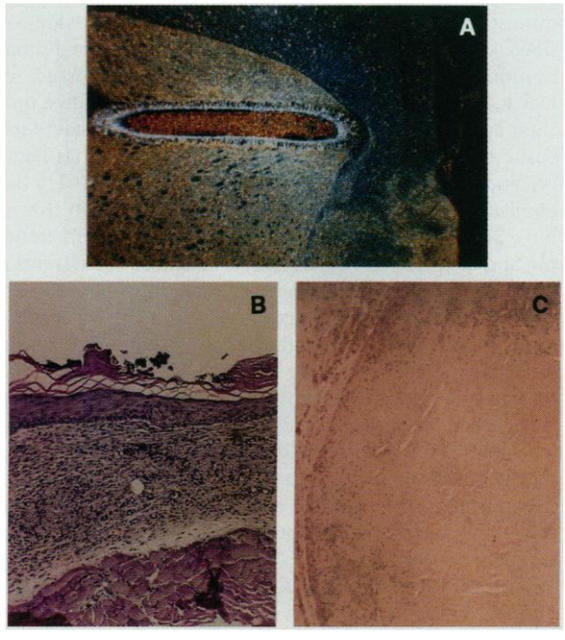

Fig. 3. Histologic sections of engineered tissues. (**A**) Dopamine-producing cells in a polymer capsule in the guinea pig striatum 12 weeks after transplant. Cells were visualized (orange color) by immunostaining for tyrosine hydroxylase [reprinted with permission from (*9*)]. (**B**) New dermis produced from neonatal fibroblasts that were placed on degradable polymers with a thin overlying skin graft and transplanted into a mouse. Photo was taken 10 days after the transplant. (**C**) New cartilage produced from chondrocytes seeded onto degradable polymers 7 weeks after transplant into a nude mouse. (B) and (C) are stained with hematoxylin and eosin.

cell-based therapies may provide a means of treating diseases such as Duchenne muscular dystrophy (DMD) (*46*). Normal myoblasts from unaffected relatives have been transplanted into patients with DMD and shown to produce dystrophin for 1 to 6 months after transplant, although the efficiency of myoblast transfer was low. Myoblasts can migrate from one healthy muscle fiber to another (*47*); thus, if the efficiency of transfer can be increased, cell-based therapies may be useful in treating DMD and other muscle atrophies.

Heart disease is the single greatest killer in the United States. Once patients become symptomatic, their life expectancy is usually markedly shortened. This decline is generally attributed to the inability of cardiac cells to regenerate after injury. In contrast, skeletal muscle has the capacity for tissue repair, presumably because of satellite cells that have regenerative capability. Recently, the feasibility of using autotransplanted skeletal muscle satellite cells multiplied in vitro and placed into damaged heart muscle was explored in a canine model. Preliminary results showed that muscle formation occurred at 8 weeks, but not at 14 weeks after transplant (*48*).

Blood vessels and cells. The design of artificial blood vessels and vascular grafts is an active research area. Although large-diameter [>5 mm internal diameter (i.d.)] vascular grafts have been successfully developed with polymers such as Dacron or expanded polytetrafluoroethylene, it has been difficult to develop vascular grafts of <5 mm i.d. because of biological reactions at the blood-material and tissue-material interface. These reactions lead to stricturing and total occlusion from clotting and scarring. To circumvent these problems, grafts have been made from relatively inert materials (with either heparin coatings or polyethylene oxide surfaces) or from materials that interact in a desirable way with blood cells (*49*). One idea has been to line polymers in vitro with endothelial cells to promote hemocompatibility (*50*). Such grafts have allowed these blood vessels to stay open in short-term clinical studies. Endothelialization in vivo can be induced by the healing responses of host tissue, which leads to coverage of grafts with endothelial and smooth muscle cells (*51*). Polymer surface modification by chemical means (for example, plasma discharge) or protein adsorption may also be desirable. The latter approach may be useful in designing materials that interact appropriately with cells, but it may be difficult to design materials that selectively support endothelial cell adhesion. Materials that promote endothelial cell attachment unfortunately often simultaneously promote attachment of platelets and smooth muscle cells, with the attendant adverse effects of clotting and pseudointimal thickening. Polymers have recently been designed that contain a cell adhesion ligand specific for endothelial cells (*52*).

There are 18 million human blood transfusions in the United States annually. Because donor blood suffers from problems of limited storage time, donor shortage, requirements for typing and cross-matching, and infectious disease transmission, there is a critical need for blood cell substitutes. Red blood cells provide a number of functions, one of which is oxygen transport. Oxygen-containing fluids or materials offer enormous applications for use in emergency resuscitation, angioplasty, shock, tumor therapy, exchange transfusion, and organ preservation. Several oxygen transporters are under development. A primary candidate is hemoglobin, which not only serves as the natural oxygen transporter in blood but also functions in carbon dioxide transport, as a buffer, and in regulating osmotic pressure. Early clinical trials of cell-free hemoglobin were complicated by its lack of purity, instability, and high oxygen affinity, but these problems have subsequently been addressed by various chemical modifications (*53*). One remaining problem is the limited source of hemoglobin. It is unlikely that there will be sufficient outdated human blood to prepare practical quantities of hemoglobin for widespread clinical use. Genetically engineered human hemoglobin or hemoglobin from bovine sources could be an alternative to human hemoglobin if no toxic effects are associated with their use.

Solutions of perfluorocarbons (PFCs; large organic molecules in which hydrogens are replaced by fluorines) dissolve 40 to 70% oxygen per unit volume, nearly three times the oxygen-carrying capacity of blood. PFCs are not metabolized and are immiscible with blood. They must be emulsified with dispersing agents such as nonionic detergents or phospholipids. Critical factors in the choice of PFCs include their emulsifying ability, emulsion stability, tissue retention time, vapor pressure, safety, and the effectiveness and safety of the emulsifying agent required. Although PFCs have advantages in terms of unlimited supply and oxygen-carrying capacity, they also have a number of disadvantages, including complement activation, toxicity, and retention by the reticuloendothelial system (RES), which then reduces the body's ability to clear waste products (*53*).

Clinical trials of both modified hemoglobins and PFCs have been conducted. The results of the hemoglobin trials have varied depending on the protocol and the specific hemoglobin preparation tested. Some studies have shown allergic or toxic responses, and others have shown good tolerance and clinical improvement (for example, in a study of patients with sickle cell anemia). No single preparation has demonstrated clear superiority, and further study is required. Clinical trials with PFCs have shown efficacy, but toxic effects have limited the current allowable dose in humans.

Research to create functional substitutes for platelets (by encapsulating platelet proteins in lipid vesicles) has also been conducted (*54*). The success of this research will depend on the identification of the membrane proteins critical to platelet function and the ability to minimize RES uptake and toxicity.

Finally, it is possible that bone marrow stem cells could be maintained in culture and induced to multiply and differentiate into the various cellular elements of blood. Several culture systems are under development to foster cell division (*55–57*). For example, a combination of rapid medium exchange and use of appropriate growth factors has allowed up to a sixfold expansion of stem cells in 2 weeks (*55*). Maintenance of low oxygen concentrations (*56*) and the addition of stem cell factor may also facilitate in vitro cell expansion (*58*).

Future Research

Numerous research areas are critical for the success of tissue engineering. Much must be learned from cell biology, such as what controls cellular differentiation and growth and how extracellular matrix components affect cell function (*59*). Immunology and molecular genetics will contribute to the design of cells (for example, by gene therapy) or cell transplant systems that are not rejected by the immune system (*60*).

Cell source and cell preservation are other important issues. The transplanted cells may come from cell lines or primary tissues—from the patients themselves, other human donors, animal sources, or fetal tissue. In choosing the cell source, a balance must be struck between ethical issues, safety issues, and efficacy. Cryopreservation has been used successfully for certain cells (*61*), but procedures need to be broadened so that cell banks can be created for many different tissues. Large-scale cell culture systems are also important to ensure proliferation of needed cells in vitro prior to transplantation and to solve nutrient transport issues (*62*). Sterilization of the transplants is also critical.

The materials used in tissue engineering represent a major area of study. Natural materials are advantageous in that they contain information (for example, particular amino acid sequences) that facilitates cell attachment or maintenance of differentiated function. Countering this advantage is the fact that many natural materials suffer batch-to-batch variations or scale-up diffi-

culties. Synthetic polymers, on the other hand, allow precise control over molecular weight, degradation time, hydrophobicity, and other attributes, yet they may not interact with cells in a desired manner. Recently, the advantages of both natural and synthetic polymers have been combined in strategies whereby critical amino acid sequences from natural polymers are grafted onto synthetic polymers (63). Polymer processing is another key issue. Many implants are made of composite materials or highly porous structures; methods of manufacturing such implants reproducibly may be crucial to their success (64). The development of controlled release systems, which deliver molecules over long time periods, will be important in administering numerous tissue-inducing factors, growth factors, and angiogenesis stimulators (65). Finally, it will be useful to develop methods of surface analysis for studying interfaces between cell and materials (66) and mathematical models (67) and in vitro systems (68) that can predict in vivo cellular events.

Current methods of transplantation and reconstruction are among the most costly therapies available today. Tissue engineering offers the possibility of substantial future savings by providing substitutes that are less expensive than donor organs and by providing a means of intervention before patients are critically ill. In addition, cell transplant systems may complement gene therapy approaches in facilitating transfer of large populations of cells expressing a desired phenotype. Few areas of technology will require more interdisciplinary research than tissue engineering or have the potential to affect more positively the quality and length of life.

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71. To address this problem, several approaches are under study: (i) purifying alginates [A. M. Sun, I. Vacek, I. Tai, in *Microcapsules and Nanoparticles in Medicine and Pharmacy*, M. Donbrow, Ed. (CRC Press, Boca Raton, FL, 1992), pp. 315–322]; (ii) synthesizing hydrogels with polyethylene oxide chains (which are relatively resistant to protein and cell adsorption) on their surface [A. S. Sawhney and J. A. Hubbell, *Biomaterials* **13**, 863 (1992)]; (iii) encapsulating cells with synthetic polymers that have good biocompatibility, such as polyacrylates—interestingly, procedures for using polyacrylates expose cells to organic solvents, yet viability and function of a number of mammalian cell types are retained [M. V. Sefton, L. Kharlip, V. Marvath, T. Roberts, *J. Controlled Release* **19**, 289 (1992); H. Uludag and M. V. Sefton, *Biotech. Bioeng.* **39**, 672 (1992)]; and (iv) synthesizing polymers that form gels upon exposure to ions in water (for example, certain polyphosphazenes) [S. Cohen *et al.*, *J. Am. Chem. Soc.* **119**, 7832 (1990)].
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The Basic Science of Gene Therapy

Richard C. Mulligan

The development over the past decade of methods for delivering genes to mammalian cells has stimulated great interest in the possibility of treating human disease by gene-based therapies. However, despite substantial progress, a number of key technical issues need to be resolved before gene therapy can be safely and effectively applied in the clinic. Future technological developments, particularly in the areas of gene delivery and cell transplantation, will be critical for the successful practice of gene therapy.

Ever since the development of recombinant DNA technology, the promise of the technology for dramatically improving the practice of medicine has been vigorously championed. Most of the advances affecting the clinical management of patients have involved either the development of new molecular techniques for the diagnosis of specific inherited and acquired diseases or the development of new therapeutic products made possible by the ability to engineer the overexpression of specific genes. Recombinant DNA technology has also produced the means for defining the roles of specific gene products in the pathogenesis of human disease. The ability to characterize disease in such molecular terms has already led to more precise and effective clinical interventions.

However, the idea underlying gene therapy—that human disease might be treated by transfer of genetic material into specific cells of a patient, rather than by conventional drugs—has yet to make its mark in medicine. Although the concept may appear to be elegantly straightforward and the most direct application of recombinant DNA technology, research has indicated that successful implementation of gene transfer in the clinic will require the coordinated development of a variety of new technologies and the establishment of unique interactions between investigators from divergent medical and basic science disciplines.

Although several reviews of gene therapy research have been published (*1*), few have focused on the technical issues that continue to impede the translation of preclinical studies of gene therapy into effective clinical protocols. In this review and commentary, I have attempted to define those issues and to suggest new areas of investigation that may help to resolve them.

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Development of Methods for Gene Delivery

The transduction of appropriate target cells represents the critical first step in gene therapy; consequently, the development of methods of gene transfer suitable for different forms of therapy has been a major focus of research (Table 1). The single common feature of these methods is the efficient delivery of genes into cells. In the case of retroviral vectors and adeno-associated virus vectors, the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell. These vectors have been considered most often for ex vivo gene therapy, which involves removal of the relevant target cells from the body, transduction of the cells in vitro, and subsequent reintroduction of the modified cells into the patient. All of the other methods of gene transfer result primarily in the introduction of DNA sequences into the nucleus in an unintegrated form. Those methods, which result in high, but transient, gene expression, have predominantly been considered for use in in vivo gene therapies, in which genetic material is directly transferred into cells and tissues of the patient. As discussed below, little is known about the fate and properties of DNA delivered to cells by these other methods.

Retroviral vectors. Interest in retrovirus-mediated gene transfer stems primarily from the ability of some vectors to stably transduce close to 100% of target cells.

Although retrovirally mediated gene transfer is ideal for many ex vivo applications of gene therapy, several features of the gene transfer method may limit its applicability, particularly with regard to in vivo therapies. First, retrovirus entry into cells is absolutely dependent on the existence of the appropriate viral receptor on the target cell. To provide a means of infecting most cells of interest, researchers have developed packaging cell lines capable of generating viruses of a variety of host ranges (*2*). Because the identities of most retroviral receptors are unknown, however, it has not been possible to determine the distribution of receptors in different cell types. Problems encountered in transducing specific cell types (such as hematopoietic stem cells) may be due, in part, to the lack of expression of appropriate viral receptors. Second, replication of the target cells is necessary for proviral integration to occur. Although previously it had been assumed that this requirement reflected the necessity for DNA synthesis, recent studies suggest that viral integration may depend on mitosis (*3*). Thus, successful gene transfer depends on the ability to induce proliferation of the target cell, at least for short periods of time.

Another problem is that the retroviral