



Regenerative biology and engineering: strategies for tissue restoration

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Over the past 50 years, we have made remarkable advances in the use of bionic devices and solid organ transplants as replacement parts for failing tissues and organs. These approaches to tissue restoration, however, have a number of drawbacks. Thus, a new approach, regenerative biology and engineering, has been developed, consisting of the strategies of cell transplantation, bioartificial tissue constructs, and stimulation of regeneration in vivo. Cell transplants have been successfully used to restore articular cartilage and to treat Parkinson's disease in humans. In rats, transplanted fetal and embryonic stem cell line-derived cardiomyocytes have been shown to differentiate and integrate well with the ventricular myocardium, suggesting the feasibility of using such transplants to restore damaged cardiac muscle. Diabetic symptoms in humans have been alleviated by implanting a bioartificial pancreas consisting of islet cells microencapsulated in alginate. Hydroxyapatite matrixes can stimulate the regeneration of bone across large gaps. Collagenous artificial matrixes can stimulate the regeneration of dermis, and peripheral nerve grafts embedded in a fibrin clot containing fibroblast growth factor-1 stimulate some regeneration of spinal cord axons in adult rats. Future research in regenerative biology will focus on several issues: (1) providing adequate sources of cells for transplantation and bioartificial tissue construction and determining ways to prevent these cells from coming under attack by the immune system, (2) developing new and better materials to build better bionic devices and bioartificial constructs and to stimulate regeneration in vivo, (3) determining how many tissues of the body might contain reserve cells for regeneration in vivo, (4) analyzing the molecular differences between cells and environments of regenerating versus nonregenerating tissues, and (5) understanding the factors and mechanisms involved in the proliferation and patterning of regenerating tissues. (WOUND REP REG 1998;6:276-290)

A number of tissues are regenerated throughout the human life cycle, either continuously to maintain tissue integrity in the face of normal cell turnover or in response to acute or chronic damage caused by trauma, disease states, or both. Blood, epithelia of skin and tubular organs, hair and nails, and bone marrow are examples of human tissues that regener-

BMP	Bone morphogenetic protein
CDK	Cyclin-dependent kinase
CNTF	Ciliary neurotrophic factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
IGF	Insulin-like growth factor
MHC	Major histocompatibility
MMP	Matrix metalloproteinase
MSCs	Multipotential mesenchymal stem cells
QA	Quinolinic acid
Rb	Retinoblastoma
SIS	Small intestine submucosa
TGF	Transforming growth factor

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ate continuously, as well as in response to damage. Bone, muscle, adrenal cortex, and kidney epithelium also regenerate in response to damage, and bone is continually remodeled in response to stress vectors.¹⁻⁴

The response of many other tissues to damage, however, is not regeneration, but repair by the formation of collagenous scar tissue.⁵ Scar tissue interrupts the normal tissue architecture, compromising its function to varying degrees, depending on the severity of the injury or disease process. Some examples of diseases and injuries that cause serious impairment by scarring are third-degree burns, spinal cord injuries, diabetes, macular degeneration of the neural retina, and myocardial infarction. These injuries and diseases are costly in terms of healthcare dollars and potential decline in quality of life. Thus, a major goal of biomedical science is to be able to restore the structure and function of damaged or dysfunctional tissues that do not regenerate naturally.

Over the past 50 years, we have made remarkable progress in our ability to replace body parts with bionic devices, solid organ and tissue transplants, or both. Such replacement parts, however, still pose significant biological problems, and they are not useful in all situations. Now, basic and applied research in the rapidly emerging field of regenerative biology and engineering offers several new strategies for restoring tissue structure and function. These strategies include transplantation of cells to form new tissue in the transplant site, the implantation of bioartificial tissues constructed *in vitro*, and the stimulation of regeneration from healthy residual tissues *in vivo*.^{6,7} All involve the partial recapitulation of embryonic development; thus, regenerative biology and engineering draw heavily on basic research in cell and developmental biology. In this article, these strategies are described, and some contemporary research issues associated with each are discussed.

CELL TRANSPLANTATION

In humans, cell transplants have been used clinically to successfully repair the articular cartilage of the knee and as therapy for Parkinson's disease. Cells have been transplanted in experimental animals as potential treatments for Alzheimer's disease and for the replacement of damaged cardiac muscle.

Brittberg et al.⁸ have described the autoplasmic transplantation of chondrocytes to fill in limited full-thickness defects of knee-joint articular cartilage. In their technique, a small biopsy of normal cartilage is taken by arthroscopy from a healthy area of the joint surface and is cultured, and chondrocyte clones are expanded *in vitro*. Dissociated chondrocytes from these clones are then implanted under a flap of periosteum sutured over the lesion site. Within the de-

fect, new cartilage is formed that stains with metachromatic dyes and with fluorescent antibodies to type II collagen. Although the new cartilage is presumably derived from the transplanted cells, its origin from chondrocytes at the edges of the defect or from osteogenic cells of the periosteal flap has not been ruled out. A major question about the regenerated cartilage is how long its integrity is maintained.

Parkinson's disease results from the death of dopaminergic neurons in the substantia nigra that project to the putamen and caudate (basal ganglia) that, in turn, project to the motor cortex and control motor function. Neurons of the putamen and caudate require dopamine from the substantia nigra to fire properly, so death of substantia nigra neurons results in loss of motor control and the muscle rigidity and tremor characteristic of the disease. Suspensions of dopaminergic ventral mesencephalic cells from 8- to 9-week human fetuses have been injected into the putamen of patients to treat severe Parkinson's disease.⁹ Dopamine production by the transplanted cells was assessed by positron emission tomography scan following the injection of 6L-18F-fluorodopa. Five months after transplantation, dopamine levels in the caudate and putamen had increased, and symptoms of the disease were dramatically alleviated. Cholinergic tissue from a variety of sources (fetal basal forebrain, nodose ganglion, intracortical adrenal chromaffin cells, and differentiated neuroblastoma cells) survived when injected into the terminal areas of the forebrain cholinergic projection system of rat models of Alzheimer's disease. The transplanted neurons exhibited axonal outgrowth, formed extensive synapses with host neurons, and significantly reversed deficits in cognitive behavior.¹⁰⁻¹²

To test the feasibility of using cell transplants to replace damaged cardiac muscle, suspensions of fetal cardiomyocytes from mice bearing a lac-Z reporter transgene driven by the cardiac heavy myosin promoter were injected into the uninjured ventricular myocardium of genetically identical hosts.¹³ The transplanted cells proliferated and differentiated into mature cardiac muscle, as indicated by expression of the lac-Z reporter. Electron microscopic analysis showed that the differentiated cells formed intercalated discs, suggesting their probable electrical coupling with host cardiac myofibers. Similar results have been obtained after transplanting fetal cardiomyocytes into the ventricular myocardium of dogs.¹⁴

None of the transplanted cells described earlier here elicited an immune response because none were "seen" by the immune system. The donor cartilage

cells were autogeneic (from the same individual). The donor cardiac cells were syngeneic (from a genetically identical individual), and the brain is an immunoprivileged site.

Stem cells have also been used as sources of cells for transplantation. Mouse embryonic stem cells that differentiate into cardiomyocytes have been selected in vitro and transplanted into the ventricular myocardium of *mdx* dystrophic mice.^{15,16} The transplanted cells were stably integrated into the host cardiac muscle, as judged by antidystrophin immunostaining. Development of a human embryonic stem cell line as a source of cardiomyocytes would avoid the ethical controversies associated with transplantation of fetal cardiomyocytes, but such a cell line is not yet available.

Another promising strategy is the transplantation of multipotential mesenchymal stem cells (MSCs), which reside in the bone marrow.¹⁷ In vitro, these cells are capable of differentiating into chondrocytes, osteocytes, adipocytes, fibroblasts, and skeletal muscle in response to dexamethasone, 1,25 dihydroxyvitamin D₃, or growth factors such as bone morphogenetic protein (BMP)-2.^{18,19} In vivo, MSCs would be expected to differentiate according to the microenvironment in which they find themselves. The use of MSCs for tissue restoration has several potential advantages. They are easy to isolate from small aspirates of bone marrow and are easy to expand in vitro, and because they can be obtained from the patient's own marrow, they elicit no immune rejection. A further advantage is their ease of transfection with exogenous genes, making them potentially useful in gene therapy protocols involving either the infusion of genetically engineered cells or their implantation in an encapsulating material allowing the diffusion of secreted proteins.¹⁹

Bone marrow–derived MSCs have been tested for their ability to restore cartilage, bone, and muscle in experimental animals and human beings. Implanted MSCs restored the normal pattern of bone and cartilage in full-thickness defects made in the articular surface of rabbit femoral condyles, but the long-term integrity of the cartilage in this repair has not been followed.¹⁷ A clinical trial is underway in which human leucocyte antigen (HLA)-matched donor bone marrow is transplanted into children with osteogenesis imperfecta caused by mutations in the genes for type I collagen.¹⁹ The idea is that the transplanted marrow will provide MSCs that will become osteoblasts, then osteocytes secreting normal collagen, thus lessening the severity of the defect. Bone marrow–derived MSCs injected into the tibialis anterior mus-

cle of *mdx* dystrophic mice resulted in a small but significant increase in muscle mass and dystrophin-positive myofibers.²⁰

Stem and progenitor cells capable of differentiation into the same phenotypes as bone marrow MSCs when treated with dexamethasone, BMP-2 and 4, insulin-like growth factor I and II, or muscle morphogenetic protein have been isolated from the connective tissue compartments of virtually all mammalian organs.^{21–24} The relation between these cells, bone marrow MSCs, and known reserve cells such as osteogenic cells of the periosteum, satellite cells of muscle, and pericytes of capillaries is unclear, as is their function in vivo. Bone marrow MSCs might serve as a continuing source of any or all of these peripheral stem and progenitor cells. Marked marrow MSCs infused into x-irradiated isogenic mice or into nonirradiated mice with severe combined immunodeficiency disease have been detected months later in marrow, spleen, bone, cartilage, lung, liver, and thymus.¹⁹

BIOARTIFICIAL TISSUES

A variation of the cell transplantation strategy is to construct bioartificial tissues in vitro from cells and a support matrix and to implant the construct into the body in place of the original.²⁵ The cells can be autogeneic, allogeneic, or xenogeneic, depending on the type of construct. They can be differentiated cells or stem/progenitor cells, and they can be either normal or genetically modified. The advantage of allogeneic or xenogeneic cells is that they can be cultured and banked in advance for use in cases in which there is not enough time to expand a patient's own cells to construct the tissue, but allogeneic and xenogeneic cells will elicit rejection if seen by the immune system. The support matrix must be able to maintain the functions of differentiated cells or to promote the differentiation of stem or progenitor cells. The ideal support matrix is biodegradable so that it is replaced over weeks and months with the natural matrix made by the cells. Collagens are the predominant proteins of support matrixes in the body. Collagen I is plentiful, is easy to extract and mold into a variety of shapes, and is biodegradable. It is thus the most widely used matrix biomaterial for both bioartificial tissues and stimulation of regeneration in vivo, either by itself or in combination with various glycosaminoglycans such as chondroitin 6-sulfate. Other frequently used matrix materials are alginate, polydioxanone, poly-ε-caprolactone, polyglycolic acid, and polylactic acid. All except alginate (in cross-linked form) are biodegradable.

Some matrix materials, such as polylactic acid fibers, can be molded into the shape of the tissue or organ to be constructed.

A number of investigators have devised cultured skin substitutes.²⁶ The basic strategy is to seed allogeneic dermal fibroblasts into an artificial matrix of polylactic or polyglycolic acid mesh or a matrix of collagen supplemented with other ECM molecules such as chondroitin sulfate and/or elastin and to overlay this artificial dermis with a meshed split-thickness skin graft or cultured allogeneic keratinocytes. The construct is then applied to an excisional wound. The cells are only slowly rejected as host fibroblasts and keratinocytes replace those of the construct. In essence, these skin equivalents act as biological dressings until host skin has regenerated (see later here). The quality of the regenerated skin is not perfect, however, and it lacks completely normal histological structure, hair, and glands.

Bioartificial tissue implants made of allogeneic or xenogeneic cells can avoid immune rejection if they are constructed as a closed system, that is, if the cells are encapsulated in immunoprotective devices or matrix materials that allow nutrient and gas exchange. A closed bioartificial pancreas using a nonbiodegradable matrix has been clinically tested in a 38-year-old male patient who had been diabetic for 30 years.²⁷ This construct consisted of high-guluronic acid alginate microcapsules containing islets harvested by collagenase digestion from eight cadavers. The patient received a total dose of 15,000 encapsulated islets per kg body weight implanted into the intraperitoneal cavity in two doses of 10,000 and 5,000 islets 6 months apart. Three months after the second implantation, diabetic symptoms were markedly abated, and the patient was able to discontinue insulin injections. Proinsulin levels, however, were high, suggesting that the dose of islets per kg body weight should be increased. How long the structural integrity of the microcapsules can be maintained and how long the islets can survive within the capsules are two important questions that have not yet been answered. Because of the shortage of human donor tissue, xenogeneic cells may be useful in making such microcapsules.

A closed xenogeneic bioartificial pancreatic device has been tested in dogs.²⁸ The device consists of a disc-shaped acrylic housing containing a single-coiled membranous tube attached to a vascular graft, which forms an arteriovenous shunt between the iliac artery and vein. Within the housing of the device, pig islets are distributed around the coiled tube in an agar or alginate matrix. The islets are nourished by blood

circulating through the tube. The device has remained patent for up to 3 1/2 years in normal dogs, demonstrating its excellent biocompatibility. It controlled the hyperglycemia of two pancreatectomized dogs for more than 8 months, as judged by normal levels of blood glucose and porcine C-peptide, low exogenous insulin requirement, and normal body weight. However, late vascular thrombosis was encountered in the majority of dogs used in the study.

Huntington's disease is a genetic disorder in humans that results from the degeneration of striatal neurons, particularly those containing the neurotransmitter γ -aminobutyric acid, and results in a marked retrograde degeneration of cortical neurons in a region that projects to the striatum. The result is progressive and irreversible motor, cognitive, and psychiatric deterioration. The neuropathology of the disease can be mimicked in monkeys by injecting quinolinic acid (QA) into the striatum. Ciliary neurotrophic factor (CNTF) is able to provide striatal neurons some protection from QA. Baby hamster kidney cells genetically engineered to secrete human CNTF have been encapsulated in hollow fibers of polyacrylonitrile-covinyl chloride, and the fibers have been implanted into the caudate and putamen of monkeys. One week after implantation, the monkeys received injections of QA into the implant sites. As judged by histology, immunocytochemical visualization of choline acetyltransferase, glutamic acid decarboxylase, and dopamine and cyclic-AMP-regulated phosphoprotein, QA injection resulted in a significant reduction of immunoreactive neurons in control animals, which was moderately attenuated in experimental animals by implant-secreted CNTF.²⁹

Autogeneic stem cells have been used to make bioartificial cartilage and bone. Bone marrow-derived MSCs were seeded into ceramic matrixes, and the construct was implanted to bridge gaps made in adult rat bones.^{17,30} The MSCs differentiated into osteoblasts, probably through the action of growth factors in the marrow or secreted by osteoblasts migrating into the ceramic from the periosteum and endosteum, and then differentiated into osteocytes, which secreted new bone matrix as the ceramic was degraded by osteoclasts. Multipotential cells isolated from rabbit skeletal muscle have been used to repair defects in the articular cartilage of rabbit knee joints.³¹ The cells were seeded into porous polyglycolic acid matrixes, and the constructs were implanted into the defects. By 12-weeks postimplantation, the matrix had dissolved, and the cells had formed a well-integrated surface layer of cartilage in the defect that

was approximately the same thickness as the normal articular cartilage. How durable the articular cartilage formed in this way might be is unknown.

REGENERATION IN VIVO FROM RESIDUAL TISSUES

The advantage of regeneration in vivo from residual tissues is that the new tissue would be autogeneic and regrow with the same histological and morphological architecture as the original.

There are three prerequisites for regeneration. First, regeneration-competent cells must be available. Second, local and systemic environments must be able to guide these cells into regenerative, as opposed to scar tissue, pathways, and third, factors inhibitory to regeneration must be absent. Depending on the tissue and species, vertebrate regeneration is achieved by four different mechanisms: compensatory hyperplasia, activation of reserve stem or progenitor cells set aside in tissues during embryogenesis, formation of injury-induced progenitor cells from differentiated cells, and reversible epithelial-mesenchymal transformation.

The mammalian liver regenerates by compensatory hyperplasia. The process involves the partial dedifferentiation of hepatocytes and their proliferation while maintaining their critical differentiated functions. Compensatory hyperplasia increases the mass of the liver, but does not restore its original lobular architecture. Hepatocyte growth factor/scatter factor appears to be the primary stimulator of hepatocyte mitosis, but epidermal growth factor (EGF) and transforming growth factor (TGF)- α are also important mitogens in hepatocyte proliferation.³² Newt cardiomyocytes also undergo partial dedifferentiation and proliferation to restore ventricular muscle mass following amputation of the tip of the ventricle, but it is not known whether this process is simply compensatory hyperplasia, as in the liver, or involves re-establishment of the original ventricular morphology as well.^{33,34}

The most common regenerative mechanism in vertebrates is regeneration by reserve stem or progenitor cells, such as the osteogenic cells of the periosteum and endosteum, which regenerate bone, the satellite cells, which regenerate muscle, the pericytes of capillaries, which regenerate smooth muscle, and the epithelial stem cells and hematopoietic stem cells, which maintain tissue integrity in the face of continual cell turnover. Recently, stem cell populations have also been found in the mammalian brain, liver, and

pancreas,³⁵⁻³⁷ although their function in vivo is largely unknown. Liver stem cells are known to be activated when hepatocyte destruction is so extensive (e.g., by carbon tetrachloride poisoning) that the liver cannot regenerate by compensatory hyperplasia of hepatocytes. As mentioned earlier, bone marrow MSCs are potential precursors for satellite and osteogenic reserve cells and perhaps for other stem and progenitor cells in connective tissue compartments.

Larval and adult urodele amphibians and larval anuran amphibians can regenerate a broader spectrum of tissues and complex structures than other vertebrates because of their unique ability to form progenitor cells by injury-induced histolysis and cell dedifferentiation. The tissues of these animals that are able to regenerate include the neural retina, lens, spinal cord, upper and lower jaws, limbs, and tails.³⁸⁻⁴⁰ Following injury or amputation, the extracellular matrix (ECM) is degraded at the site of damage, liberating the cells of the tissue. The liberated cells lose their phenotypic characteristics and revert to embryonic-like mesenchymal cells, which re-enter the cell cycle and proliferate to form a growing blastema. As the blastema grows, it autonomously patterns itself to redifferentiate precisely the missing structures.⁶

Histolysis and dedifferentiation have been most extensively studied in the regenerating limbs of salamanders and newts. After amputation of a limb, the tissue matrix at the amputation site, which is predominantly composed of collagens, elastins, and sulfated proteoglycans, is degraded and replaced with a more embryoniclike matrix that is much richer in fibronectin, hyaluronic acid, and tenascin.⁶ The degradation is accomplished by lysosomal acid hydrolases, particularly acid phosphatase and members of the matrix metalloproteinase (MMP) family, of which 11 have been identified in mammals and chickens. MMP-9 (gelatinase b; hydrolyzes gelatin, proteoglycan, and collagen types IV, V, VII, and X) is upregulated within a few hours of amputation in regenerating axolotl limbs⁴¹ and as early as 2 days in the adult newt limb.⁴² In both axolotl and newt, its expression declines after the formation of a conical blastema. MMP3/10a and MMP3/10b (stromelysins 1/2; degrade fibronectin, laminin, proteoglycan, and collagen types II, IV, V, IX, and X) are also strongly upregulated as early as 2 days in the regenerating newt limb. MMP3/10a is weakly transcribed up to 8-days postamputation then disappears by 15 days, which is when blastema cells are just beginning to accumulate, whereas expression of MMP3/10b con-

tinues through the palette stage.⁴² The different expression patterns of these three MMP genes suggest that they may have different roles in degradation of the limb ECM. Acid phosphatase is most strongly expressed during the period of dedifferentiation and blastema formation.⁴³ The cell types producing these proteases, however, have not yet been identified.

The cells of healing mammalian wounds also employ proteases to remodel ECM. Like the regeneration blastema, the early wound matrix is dominated by hyaluronate and fibronectin, but unlike the blastema, it is replaced not by normal matrix but by a matrix enriched in cross-linked collagen I. With the exception of the minimal dedifferentiation exhibited by hepatocytes in regenerating liver, mammalian cells are not known to dedifferentiate *in vivo* after injury, although some cell types, such as chondrocytes, will dedifferentiate under certain *in vitro* conditions.

How injury or amputation triggers dedifferentiation and re-entry into the cell cycle and the mechanism by which phenotypic-specific structure is dismantled is unclear. The act of liberation from the mature ECM itself, which likely involves the breaking of ECM ligand-cell surface receptor contacts, may play a role in starting the process. Tenascin and two other antiadhesive proteins, SPARC (osteonectin) and thrombospondin, promote reorganization of the actin cytoskeleton in bovine arterial endothelial cells by modulating adhesive contacts.⁴⁴ Whether these proteins are expressed in amputated urodele limbs is not known. Neither is it clear whether, or how, dedifferentiation and re-entry into the cell cycle are mechanistically linked. Factors in serum can drive cultured newt myotube nuclei into S phase in the absence of any overt dedifferentiation, but they do not progress further in the cell cycle, as is discussed later.^{45,46}

Interestingly, although the bone and muscle of urodele limbs contain osteogenic and satellite progenitor cells that are the source of regenerated bone and muscle following a nonamputational injury, there is no direct evidence that these cells participate in the regeneration of appendages following amputation.

The fourth mechanism, reversible epithelial-mesenchymal transformation, is used by urodeles and larval anurans to regenerate the spinal cord.^{47,48} In the injured mammalian spinal cord, a number of cytotoxic and inhibitory molecules are released, and fibrous astrocytes are activated that secrete proteoglycans and form scar tissue.^{49,50} In the injured urodele spinal cord, these inhibitors either are not present in sufficient amounts to prevent axon regeneration or are neutralized. Fibrous astrocytes do not

enter the lesion, and the damaged or severed axons regenerate. The ependymal cells (also called radiogial or ependymogial cells) lining the spinal canal play an important role in urodele spinal cord regeneration. In the intact cord, these epithelial cells send out processes through the gray and white matter to the pia mater. The processes branch within the white matter and terminate in expanded end-feet to form the glia limitans on the inner surface of the pia mater. This structural network distinguishes the mature urodele cord from that of birds and mammals. Urodele ependymal cells may be structurally more akin to the radiogial cells found in the mammalian embryonic and fetal central nervous system than to the ependymal cells in the adult mammalian central nervous system.⁴⁸

Following transection of the urodele spinal cord, the ependymal cells on the proximal and distal edges of the transection appear to transform to mesenchymal cells, with characteristic shifts in intermediate filament profile from cytokeratins to vimentin.⁴⁷ The cells proliferate to form a blastema that bridges the lesion. EGF promotes the migration and proliferation of ependymal mesenchyme cells *in vitro*, but it is not known whether this is also true *in vivo*. The mesenchyme cells *in vivo* secrete MMPs that remodel the ECM in the vicinity of the lesion and probably secrete neurotrophins, which promote axon survival *in vitro*.⁴⁷ They may also secrete factors that neutralize inhibitory agents and block the entry of fibrous astrocytes into the lesion. After bridging the lesion, the ependymal cells reform an epithelium and re-extend processes to the pia mater, forming channels that provide an environment favorable to the survival and regrowth of damaged axons and the establishment of functional synapses on either side of the lesion.

The molecular analysis of regenerating urodele spinal cord has only recently gotten underway.^{47,48} In addition to changes in intermediate filament expression, tenascin, fibronectin, and polysialated neuronal-cell adhesion molecule, which are prevalent in embryonic spinal cord and downregulated in adult cord, are upregulated during cord regeneration.

Stimulation of regeneration where it does not occur naturally

There are two reasons, not mutually exclusive, that most human tissues fail to regenerate. First, these tissues may have cells or axons capable of engaging in regeneration, but their environment may not contain the signaling molecules that stimulate regeneration, and/or there may be inhibitory signals that

divert the cells into a repair pathway. This includes situations in which regeneration may occur under one set of physical circumstances but not another, such as a bone fracture versus regeneration of bone across a large gap. Alternatively, nonregenerating tissues may have the appropriate molecular environment for regeneration but may lack regeneration-competent cells capable of responding to this environment. In either case, the tissues cannot recapitulate their embryogenesis. Thus, achieving regeneration of damaged tissues *in vivo* requires recreation of the embryonic environment in terms of cells, signaling molecules, or both.

A current strategy to stimulate the regeneration of nonregenerating tissues *in vivo* is to bridge lesions with natural or artificial matrixes that promote the migration, proliferation, and differentiation of local regeneration-competent cells or that promote the regrowth of severed axons. The ultimate goal of this strategy is to stimulate regeneration with easily manufacturable artificial matrixes that neutralize inhibitory factors and reproduce the molecular environment that guided the initial development of the tissue. There has been some success in applying this strategy to the regeneration of bone, dermis, peripheral nerve, and spinal cord.

Bone

Although bone will regenerate to restore continuity after a fracture, it is unable to regenerate across a gap, such as that created by surgical removal of a bone tumor, because the sources of osteogenic cells, the periosteum and endosteum, do not have the proper substratum on which to migrate and fill the gap. Calcium phosphate-based hydroxyapatite matrixes promote bone regeneration.^{51,52} The freshly made matrixes are malleable pastes and can be pressed into a defect in which they harden into a scaffold capable of temporarily substituting for bone. These scaffolds are osteoconducting as opposed to osteoinducing (i.e., the matrix supports the migration of osteoblasts from the injured host periosteum and endosteum rather than inducing osteogenic cells in these tissues to become osteoblasts). The migrating osteoblasts secrete growth factors that promote their differentiation into osteocytes, which in turn secrete natural bone matrix that gradually replaces the hydroxyapatite scaffold.

Hydroxyapatites have a high affinity for BMPs, which are members of the TGF- β family. BMPs are osteoinductive (i.e., they act as a signal to commit

osteogenic precursors, such as bone marrow MSCs, to become osteoblasts).⁵³ An osteoinductive ceramic matrix has been prepared by adsorbing BMP fractions onto porous hydroxyapatites. This BMP-hydroxyapatite composite induces rapid bone differentiation in skull defects of adult baboons.⁵² Only porous hydroxyapatite in block form, as opposed to particulate hydroxyapatite, is effective, showing the importance of matrix geometry in facilitating osteoblast induction.

Ceramic scaffolds must be degraded by osteoclasts as new bone forms, but some do not degrade adequately enough to be completely replaced with bone. Experiments have therefore been made with various biodegradable polymer matrixes, with or without incorporating growth factors of the TGF- β family or fibroblast growth factor (FGF) into the matrix. These matrixes have successfully promoted bone regeneration in several animal models.⁵⁴⁻⁵⁸ The optimum matrix for bone regeneration has not yet been devised, however.

Dermis

Biodegradable artificial matrixes can induce dermal regeneration in excisional skin wounds, although the regeneration is imperfect. These matrixes typically consist of collagen or gelatin sponges to which other ECM components, such as chondroitin 6-sulfate or elastin, are added.⁵⁹⁻⁶² The matrix is overlaid with either a meshed split-thickness epidermal graft or a suspension of dissociated keratinocytes. Fibroblasts from the surrounding dermis migrate into the artificial matrix and replace it with a matrix that resembles, but is not identical to, normal dermal matrix. These results show clearly that, given the appropriate environment, the adult dermis has the capacity for regeneration. However, it is not known whether the responding cells are the same dermal fibroblasts that normally form scar tissue or a subpopulation of regeneration-competent reserve cells that are normally suppressed in favor of repair. There is some evidence, based on differences in thymidine incorporation and pattern of cell association *in vitro*, that hypodermal fibroblasts form the granulation tissue of repair, whereas dermal fibroblasts might be the cells capable of regeneration.⁶³ The presence of an artificial dermal matrix in a wound might suppress wound contraction and proliferation of hypodermal fibroblasts and promote proliferation of dermal fibroblasts, leading to regeneration. The nature of the factors that suppress either dermal fibroblast proliferation after wounding

or hypodermal fibroblast proliferation in the presence of an artificial dermal matrix is unknown but could be investigated in vitro with an artificial matrix system.

Fetal skin regenerates perfectly. Several studies suggest that differences between fetal and adult skin in the proportions of ECM molecules and the relative concentrations of TGF- β isoforms are important in determining whether a wound scars or regenerates.^{64–66} Changing the relative concentrations of TGF- β isoforms of mouse fetal skin toward those of the adult and vice versa increases and decreases scarring, respectively.^{67,68} Thus, fetal skin is an important model for the design of an artificial dermal matrix to promote the perfect regeneration of adult skin.

Peripheral and central axons

Regeneration of cut peripheral nerve axons in mammals is promoted by factors resident in the Schwann sheath.^{69,70} However, if a segment of peripheral nerve is removed, the axons proximal to the cut sprout randomly to form a neuroma, and the gap is invaded by fibroblasts that form scar tissue. Nerve guides have been designed that promote regeneration across such a gap and into the distal Schwann sheath. Tubes made of silicon, porous or nonporous collagen, polylactic acid, and polyglycolic acid, with and without incorporation of various ECM molecules and growth factors, have been used with some success.^{7,71}

Mammalian spinal cord axons do not normally regenerate, although they have the intrinsic capacity to do so. Their failure to regenerate is due to inhibitory factors such as toxic proteins in degenerating myelin, oligodendrites, and axonal surfaces; calcium influx and associated toxic reactions; glutamate excitotoxicity; cholesterol depletion (arachidonic acid pathway); and astroglial scar formation.^{47,49,72} Toxic axonal damage can be prevented by cell membrane-protective agents such as methylprednisolone and lazarooids, which increase the chances for functional recovery in humans if administered within the first 8 hours after injury to the cord.^{73,74}

A number of investigations have shown that the mammalian spinal cord has extensive powers of regeneration under the appropriate environmental circumstances.⁵⁰ Extensive sprouting of severed axons following transection of the corticospinal tract in young adult rats has been observed after intracerebral administration of a monoclonal antibody to two myelin proteins, although the ultimate number of axons

that regenerate successfully is small. Combining antimyelin antibody treatment with injection of neurotrophin-3 enhanced corticospinal axon regeneration so that 5%–10% of the corticospinal fibers were regenerated in successful cases.^{75,76} The cord was only partially transected in these experiments, however, and the regenerated axons went through the intact part of the cord, not through the lesion itself.

Functional axonal regeneration across spinal cord lesions in neonatal rats occurs after transplanting fetal cord to bridge the lesion, as demonstrated by anterograde and retrograde labeling of axons with fluorescent dyes and by the recovery of righting reflexes and walking ability.⁷⁷ Fetal spinal cord implants, however, do not induce the functional regeneration of adult cord. Partial functional recovery has been achieved in adult rats by bridging cord lesions with grafts of intercostal nerves stabilized by a fibrin matrix impregnated with FGF-1.⁷⁸ The rats could support their weight on the previously paralyzed hind limbs and displayed movement in all leg joints but did not recover coordinated locomotion. The inclusion of FGF-1 in the matrix was essential; no functional regeneration took place in rats implanted with intercostal nerve/fibrin alone.

Other tissues

An interesting resorbable biomaterial for the promotion of regeneration is pig small intestine submucosa (SIS) processed to eliminate antigenicity. This material has been shown to promote the regeneration of blood vessels, urinary bladder, ligament, and tendon.^{7,79} SIS grafts consist entirely of extracellular matrix, composed mainly of collagen, but also containing hyaluronic acid, heparin, heparan sulfate, chondroitin sulfate A, and dermatan sulfate.⁸⁰ Further studies on the range of tissues in which SIS can promote regeneration and further qualitative and quantitative analysis of its composition may give valuable clues for the optimum molecular design of artificial matrixes that can promote regeneration. Partial regeneration of rabbit Achilles tendon across a gap has also been achieved by bridging the gap with a porous collagen-glycosaminoglycan matrix.⁸¹

RESEARCH ISSUES IN REGENERATIVE BIOLOGY AND ENGINEERING

Contemporary research issues in regenerative biology and engineering center around sources of cells, regulation of cell proliferation and differentiation, biom-

aterials, evasion of the immune system, and molecular differences in the cells and environments that characterize regenerating versus nonregenerating tissues. These issues overlap among the three strategies used in regenerative biology and engineering. Here, the major issues for each strategy are discussed.

Cell transplantation and bioartificial tissue construction

Providing reliable sources of cells for cell transplants and bioartificial tissue construction is a crucial issue that requires establishing culture banks of proliferating stem, progenitor, or differentiated cells that can be drawn on as required, as well as culture media that support the proliferation and differentiation of these cells. The cells can be in their natural condition or be genetically engineered to produce desired molecules. Many types of differentiated cells, such as liver hepatocytes and pancreatic islet cells, are difficult to expand *in vitro*. Stem and progenitor cells are often difficult to identify and purify. Furthermore, not all the factors essential for their proliferation and differentiation are understood. Thus, research on the factors that control the entry of stem, progenitor, and differentiated cells into and progression through the cell cycle and the factors that control the differentiation of specific cell phenotypes will be of prime importance for the design of media that allow cell expansion and differentiation. A defined medium containing hepatocyte growth factor, EGF, and TGF- β 1 has been found to support the proliferation of mature rat and human hepatocytes.⁸² The stem cells harbored by the adult rat brain were identified as stem cells because they proliferate *in vitro* in response to EGF to form neurospheres.^{35,83} Single cells of these neurospheres plated in medium without EGF will differentiate into neurons, astrocytes, or oligodendrocytes.^{83,84} The ability to culture these cells and differentiate them into the three major cell types of the central nervous system makes them a potential source for both neural cell transplantation and for inducing the regeneration of new central nervous system tissue *in vivo*.

Biomaterials science

Advances in biomaterials science are essential for advances in the construction of bioartificial tissues and for stimulation of regeneration *in vivo*.^{85,86} Such materials must meet several criteria. They must be "smart" (i.e., contain and/or be able to release appropriate biological signaling information to promote and maintain cell adhesion, differentiation, and tissue or-

ganization). In essence, we want to develop polymers that mimic the functions of the embryonic or adult ECM. Thus, research on polymer chemistry must be coupled with research on the molecular biology of ECM signaling systems that regulate cell morphology and function and how to incorporate biological molecules into synthetic polymer matrixes. For example, selective cell adhesion to substrates could be achieved by linking specific oligopeptide or carbohydrate recognition sequences to nonadhesive artificial polymers. Such adhesive selectivity would be desirable, for example, in artificial blood vessels, where the materials used should support the adhesion and migration of endothelial cells but not the adhesion of platelets, which could trigger thrombosis. Growth factors might also be bound to polymer matrixes and released as the polymers degrade.⁸⁷ If the matrix materials form a closed bioartificial system, they must allow the ready exchange of oxygen, nutrients, waste products, and molecules that regulate the metabolic and synthetic activities of the cells, while immunoprotecting cells.

Directional alignment is another matrix property that would be useful in bioartificial tissue construction or regeneration *in vivo*. Controlled alignment of the elements of the biomaterial would allow it to orient cells, by contact guidance, in particular directions essential for the function of the tissue. For example, circumferential orientation of smooth muscle cells (which would be important for construction or regeneration of tubular tissues such as blood vessels) entrapped in a forming collagen matrix has been achieved *in vitro* by using a strong magnetic field to orient the collagen fibrils as they form.⁸⁸ Copolymers of collagen I and chondroitin 6-sulfate in which the pore channels were aligned longitudinally produced superior regeneration of peripheral nerves within guide tubes compared with other directions of alignment.⁶⁹

One of the most important properties of matrix materials is controlled biodegradability. The most widely used matrix material, collagen I, is susceptible to rapid degradation *in vivo* and therefore is thermally or chemically cross-linked to increase its stability before use. Glutaraldehyde is the most commonly used chemical cross-linking agent because it provides the highest stability, but biomaterials cross-linked with glutaraldehyde also induce cytotoxicity. Recent experiments suggest that collagen I cross-linked with carbodiimide or acyl azide is more biocompatible than collagen I cross-linked by glutaraldehyde.⁸⁹ Only a few other biodegradable polymers, such as poly(gly-

colic acid), poly(lactic acid), polydioxanone, and their copolymers, are available today to make artificial matrices. These polymers have the disadvantages of being relatively stiff and lacking chemically active side chains for easy attachment of biologically active molecules, cross-linkers, or drugs. New matrix biomaterials based on tyrosine-derived polycarbonates and polyarylates have been devised in which the backbone and side-chain composition and length can be varied to change thermal behavior, mechanical properties, hydrophobicity, and cell-polymer interactions.⁹⁰ These materials are hydrophobic and degrade slowly, over months to years. Currently, they appear to be most useful in situations in which a long-term degradation profile is desired, such as in bone regeneration.

Experimental biomaterials are also being investigated that will undergo phase transformation at body temperature.⁹¹ For example, outside the body, such a material might be a liquid that occupies only a small volume, but when injected into the body, it would transform at the higher temperature into a semisolid or solid. If the transformation involved expansion in volume, only small apertures would be required to implant large constructs.

Evasion of the immune system

Wider use of cell transplants and bioartificial tissue implants, particularly open constructs, will require the ability to avoid immune rejection. Several innovative and promising methods have been explored for evasion of the immune system without compromising its infection-fighting function. Donor transgenic animals have been created that express immune regulatory molecules that inhibit the activation or synthesis of key proteins involved in rejection. One of these is human decay acceleration factor, which inhibits the hyperacute rejection caused by the endothelial cell damage and vascular ischemia triggered by activation of complement.⁹² Immunosuppressed monkeys, which would normally hyperreject donor pig hearts within a matter of minutes, survived for a median of 40 days after receiving pig hearts transgenic for human decay acceleration factor.⁹³

Another strategy has been to mimic immunoprivileged sites, such as the anterior chamber of the eye. Transplants are not rejected at these sites because cytotoxic T cells entering them are killed. T-cell apoptosis is mediated by a protein, FasL, produced by cells at the immunoprivileged site, which binds to the Fas receptor expressed on T-cell surfaces.⁹⁴ Syngeneic myoblasts transfected with the FasL gene were cotrans-

planted with allogeneic pancreatic islet cells under the kidney capsule of mice rendered diabetic with streptozotocin.⁹⁵ These fused to form myotubes expressing FasL, which killed infiltrating T cells, thus preventing rejection of the islets. The production of animals carrying transgenes that regulate or inhibit the immune system is also a potential way to solve the problem of renewable sources of donor cells, tissues, and organs.

Finally, perhaps the most intriguing strategy to pursue would be the production of antibodies directed at T-cell receptors that recognize foreign cell surface major histocompatibility antigens (MHCs) and the genetic engineering of "stealth" cells that do not express surface MHC antigens. In either case, T cells would be blind to foreign cells transplanted alone or as part of a bioartificial construct.

Regeneration in vivo

A number of questions about reserve stem or progenitor cells need to be answered, in addition to the question of what specific sets of factors control their differentiation into different phenotypes. How ubiquitous are such cells in mammalian tissues? Do they exist in every tissue? What are the molecular markers and/or morphological characteristics that identify them? What is their normal function in tissues that do not regenerate in response to injury? Do they take part in the formation of scar tissue? Do the tissues in which they are located turn over slowly, and do these cells function to replace the cells that turn over?

Factors stimulatory and inhibitory to regeneration

If stem or progenitor cells actually exist in every body tissue but are inhibited from participating in regeneration in most tissues because of the presence of inhibitory molecules and/or the absence of the appropriate stimulating molecules, the question of stimulating tissue regeneration becomes largely one of determining how to identify and neutralize inhibitors and/or identify and apply stimulators of regeneration to nonregenerating tissue.

One way to identify stimulatory and inhibitory molecules might be to construct cDNA libraries from regenerating tissue mRNA and subtract them with mRNA from uninjured tissue or tissue that responds to injury by repair. The subtracted libraries would then be transfected into cells, such as Chinese hamster ovary cells, that would express and secrete the proteins encoded by the cDNAs. The conditioned medium from the culture of aliquots of these cells in

multiple wells could then be tested on bioassay systems for their regeneration-promoting ability. Positive samples would be sequenced and their human counterparts identified for use as regenerative agents.

Physical factors are equally important for adequate regeneration to take place, and their roles in regeneration need to be carefully analyzed. For example, mechanical load (tension, compression) is an important factor that triggers the synthesis and/or release of molecular signals important to regeneration, differentiation, and hypertrophy, particularly in musculoskeletal tissues. In some vertebrates, such as the chicken or rat, muscle can regenerate from a remaining short muscle stump. In the rat, for example, regeneration from the stump of the gastrocnemius muscle is correlated with the regeneration of an Achilles tendon, which connects to the regenerating muscle. The tension exerted on the muscle stump by the Achilles tendon is thought to be an important factor in regeneration of the muscle.⁹⁶ Mechanical stress causes hypertrophy of cardiac and skeletal muscle and increases collagen synthesis in cardiac fibroblasts. In human skeletal muscle, hypertrophy is associated with increased release of FGF-2.⁹⁷ In cultured fetal rat cardiac fibroblasts, the increase in collagen synthesis is correlated with an enhanced response to TGF- β and IGF-1.⁹⁸ A transition from tendon to fibrocartilage in bovine deep flexor tendon is associated with increased expression and accumulation of two proteoglycans, aggrecan and biglycan. The levels of mRNAs for these proteoglycans and of TGF- β are increased by compression of fetal tendon, suggesting that fetal tendon cells respond to compression by increasing TGF- β synthesis, which, in turn, stimulates proteoglycans characteristic of fibrocartilage.⁹⁹

Knowledge of the factors that inhibit or stimulate regeneration is equally essential for the design of artificial matrixes that can induce regeneration of tissues *in vivo*.

Differences between regeneration and repair

Achieving a wider range and greater degree of regeneration in mammals requires that we acquire a deeper understanding than we currently have of the cellular and environmental differences between tissues that undergo repair and those that undergo regeneration. The best way to analyze such differences is to compare and contrast tissues that exhibit a difference in their ability to regenerate at different stages of their life cycle or take advantage of mutations that affect either regeneration or wound repair or to compare and con-

trast the basic biology of tissues in high-regeneration animals such as the urodeles with the same tissues in lower regeneration animals such as the mammals.

Such studies are just getting underway at the cellular and molecular level. Current evidence indicates that there are two important properties of urodele limb muscle cells not exhibited by mammalian muscle cells that are potentially correlated with the ability to regenerate.^{45,46} The first is the ability of cultured urodele myotube nuclei to re-enter the cell cycle in response to high (10%–20%) concentrations of serum, something that normal mammalian myotube nuclei cannot do.¹⁰⁰ Several lines of evidence indicate that the target for the serum factor(s) is the retinoblastoma (Rb) protein. First, the newt Rb protein is expressed and is hyperphosphorylated by cyclin-dependent kinase (CDK) 4/6. This would cause Rb to dissociate from E2F transcription factors, allowing these factors to activate genes that drive the nuclei into S phase.¹⁰¹ Second, newt myotubes transfected with a plasmid expressing a mutant Rb lacking sites of CDK4/6 phosphorylation cannot re-enter the cell cycle in response to serum factors. Third, transfection of newt myotubes with a plasmid expressing p16 (which inhibits CDK4/6) also blocks cell cycle re-entry in response to serum.¹⁰⁰ The response of newt myotubes to serum is dependent on the degree of contact of the myotubes with other cells. High-density cultures of myoblasts surrounding the myotubes prevent them from synthesizing DNA, whereas low-density cultures do not.¹⁰⁰ This suggests that contact inhibition normally inhibits newt myotubes from seeing the stimulatory factors in serum and that amputation and matrix remodeling relieves this inhibition. The nature of the serum factor(s) is unknown. The factor(s) is not FGF-2, EGF, or other standard growth factors such as platelet-derived growth factor and IGF, because none of these growth factors elicited DNA synthesis in cultured newt myotubes.¹⁰⁰

The second difference is the apparent immortality of urodele limb cells. Mammalian cells can be immortalized with oncogenes such as that encoding the SV40 T-antigen, but newt limb cells appear to be naturally immortal. They can be propagated in culture for at least 200 generations without undergoing senescence or crisis.¹⁰² Blastema cells cultured for 100 generations and implanted back into an amputated limb stump are incorporated into different tissue types of the regenerate.⁴⁶ The property that confers immortality may be linked to the ability of myotubes to enter S phase in response to serum factors and thus is worth pursuing in the context of inducing mammalian cells to do likewise when they ordinarily would not. Rodent embryo

fibroblasts were transfected with a newt cDNA library carrying a drug-resistance marker and drug selected for stably transfected colonies retaining newt sequences. Two such colonies containing the same sequence (designated h4) were isolated. The h4 sequence complemented the activity of a mutant T-antigen sequence that lacks immortalizing activity when transfected into rodent embryo cells by itself. Interestingly, the h4 sequence does not encode a protein but resides in the 3' untranslated region of a newt α glucosidase-related mRNA.⁴⁶ Transcripts of such regions have been shown to mediate a variety of regulatory processes, including myogenic differentiation.¹⁰³

We also need to understand how limb cells reorganize their internal architecture to assume an embryonic-like mesenchymal morphology, how the dedifferentiated state is maintained throughout growth of the blastema, and what factors are involved in the progression of dedifferentiated cells through the cell cycle beyond S phase. The serum factors that cause newt myotube nuclei to synthesize DNA are not sufficient to drive them through the G2/M transition.¹⁰⁰ Other factors provided by the wound epidermis and nerves, not yet unequivocally identified, are essential for complete cell cycling and blastema growth.^{104,105} The ability to respond to these factors and progress through the cell cycle might require the breakdown of myotubes into single cells and their dedifferentiation. The embryonic-like ECM is thought to be instrumental in maintaining blastema cells in an undifferentiated state,¹⁰⁶ probably by the nature of the contacts between cells and matrix. The homeobox gene *Msx-1* is expressed in blastema cells¹⁰⁷ and may be important in repressing genes responsible for differentiated function. The forced expression of *Msx-1* in mouse myoblasts downregulates *MyoD*, which maintains terminal muscle differentiation and prevents them from differentiating.¹⁰⁸

Finally, we need to understand the mechanisms by which the differentiation patterns of stem or progenitor cells are specified during regeneration. Patterning of regenerate tissues has been most intensively studied in regenerating urodele limbs.^{7,109} It is clear that many of the same genes involved in patterning of the structure of the embryonic chick limb bud are also expressed in the limb regeneration blastema,¹⁰⁹⁻¹¹² giving reason to believe that regenerate pattern may be controlled by interdependent proximodistal, anteroposterior, and dorsoventral organizing regions, as in the chick limb bud.¹¹³ These organizing centers may act as boundaries specifying the perimeter of the regenerate, within which the positional values of each axis are intercalated in each

axis.¹¹⁴⁻¹¹⁶ It will be interesting to know whether the genes expressed in association with these organizing regions are expressed in all cases of tissue regeneration or even during wound repair by scar tissue, as opposed to being expressed only during the regeneration of a complex structure such as a limb.

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