19 Tissue Engineering

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Patients suffering from diseased or injured genitourinary organs are often treated with reconstructive surgery or transplants, but there is a severe shortage of donor tissue and organs. This shortage worsens yearly as modern medicine increases the human lifespan. The aging population grows, and the need for organs grows with it. Physicians and scientists have begun to look to the fields of regenerative medicine and tissue engineering to provide new options for these patients. These fields apply the principles of cell transplantation, material science, and bioengineering to construct biological substitutes that can significantly improve the quality of life of the urologic patient by eliminating the need for intensive grafting procedures or transplant surgery.

Tissue engineering, one of the major components of regenerative medicine, follows the principles of cell transplantation, materials science, and engineering to develop biological substitutes that can restore and maintain normal organ function. Tissue engineering strategies generally fall into two categories: the use of acellular matrices designed to direct the body's natural ability to use its own cells to regenerate damaged tissue, and the use of matrices seeded with cells in the laboratory to produce novel tissues and organs. Acellular tissue matrices are usually prepared by manufacturing artificial scaffolds, or by removing cellular components from donor tissues via mechanical and chemical manipulation to produce collagen-rich matrices (1–4). These matrices slowly degrade after implantation and are replaced by the extracellular matrix (ECM) proteins secreted by the in growing cells. Cells themselves can also be used for therapy via injection, either with carriers such as hydrogels, or alone.

The most common way to use cells in tissue engineering is to obtain a small piece of donor tissue and dissociate it into individual cells in the laboratory. These cells are either implanted directly into the host, or are expanded in culture and attached to a support matrix. The cell/matrix construct is then reimplanted into the host. The source of the donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous. Ideally, autologous cells are used, because in this case both structural and functional tissue replacement will usually occur with minimal complications. To accomplish this, a biopsy of tissue is obtained from a host, the cells are dissociated and expanded in culture, and the expanded cells are implanted back into the same host (2, 5–12). The use of autologous cells, although it may cause an inflammatory response, avoids rejection and thus, the deleterious side effects of lifelong immunosuppression can be avoided.

However, for many patients with extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous cells cannot be expanded from a particular organ, such as the pancreas. In these situations, stem cells are envisioned as an alternative source of cells from which the desired tissue can be derived. Stem cells can be derived from discarded human embryos (human embryonic stem cells), from fetal tissue, or from adult sources (bone marrow, fat, skin). However, there are ethical issues involved in the use of embryonic stem cells. Most human applications are currently banned in the United States, a policy that may be dramatically changed with the inauguration of a new President in January 2009. Despite this, the field of stem cell biology is advancing rapidly, and cutting-edge techniques such as therapeutic cloning and somatic cell reprogramming circumvent some of the ethical questions and offer potentially limitless sources of these cells for tissue engineering applications.

This chapter will review the major components of most tissue engineering techniques, and will describe how these techniques are being applied to the reconstruction and regeneration of the genitourinary system.

The Basic Components of Tissue Engineering

Cells

Native Cells

In the past, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement was the inherent difficulty of growing certain cell types in

large quantities. Even when some organs, such as the liver, have a high regenerative capacity in vivo, cell growth and expansion in vitro can be difficult. By studying the privileged sites for committed precursor cells in these organs, as well as by exploring the conditions that promote differentiation and/or self-renewal of these cells, it has been possible to overcome some of the obstacles that limit cell expansion in vitro. One example is the urothelial cell. Urothelial cells could be grown in the laboratory setting in the past, but only with limited success. Several protocols were developed over the past two decades that identify the undifferentiated cells in a mixed culture of cells isolated from the urinary tract, and keep them undifferentiated during their growth phase (11, 13–16). Using these methods of cell culture, it is now possible to expand a urothelial culture that initially covered a surface area of 1 cm^2 to one covering a surface area of 4,202 m^2 (the equivalent of one football field) within 8 weeks (11). These studies indicated that it should be possible to collect autologous bladder cells from human patients, expand them in culture, and return them to the donor in sufficient quantities for reconstructive purposes (11, 14–19). Major advances in cell culture techniques have been made within the past decade, and these techniques make the use of autologous cells possible for clinical application.

Embryonic Stem Cells

In 1981, pluripotent cells were found in the inner cell mass of the human embryo, and the term "human embryonic stem cell" was coined (20). These cells are able to differentiate into all cells of the human body, excluding placental cells (only cells from the morula are totipotent; that is, able to develop into all cells of the human body). These cells have great therapeutic potential, but their use is limited by both biological and ethical factors.

The political controversy surrounding stem cells began in 1998 with the creation of human embryonic stem (hES) cells derived from discarded embryos. hES were isolated from the inner cell mass of a blastocyst (an embryo 5 days post-fertilization) using an immunosurgical technique. Given that some cells cannot be expanded ex vivo, ES cells could be the ideal resource for tissue engineering because of their fundamental properties: the ability to self-renew indefinitely and the ability to differentiate into cells from all three embryonic germ layers. Skin and neurons have been formed, indicating ectodermal differentiation (21–23) (24). Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation (25–27). Pancreatic cells have been formed, indicating endodermal differentiation (28). In addition, as further evidence of their pluripotency, embryonic stem cells can form embryoid bodies, which are cell aggregations that contain all three embryonic germ layers while in culture, and can form teratomas in vivo (29). These cells have demonstrated longevity in culture and can maintain their undifferentiated state for at least 80 passages when grown using currently published protocols (30, 31).

However, in addition to the ethical issues surrounding the use of embryonic stem cells, their clinical application is limited because they represent an allogenic resource and thus have the potential to evoke an immune response. New stem cell technologies (such as somatic cell nuclear transfer and reprogramming) promise to overcome this limitation.

Therapeutic Cloning (Somatic Cell Nuclear Transfer)

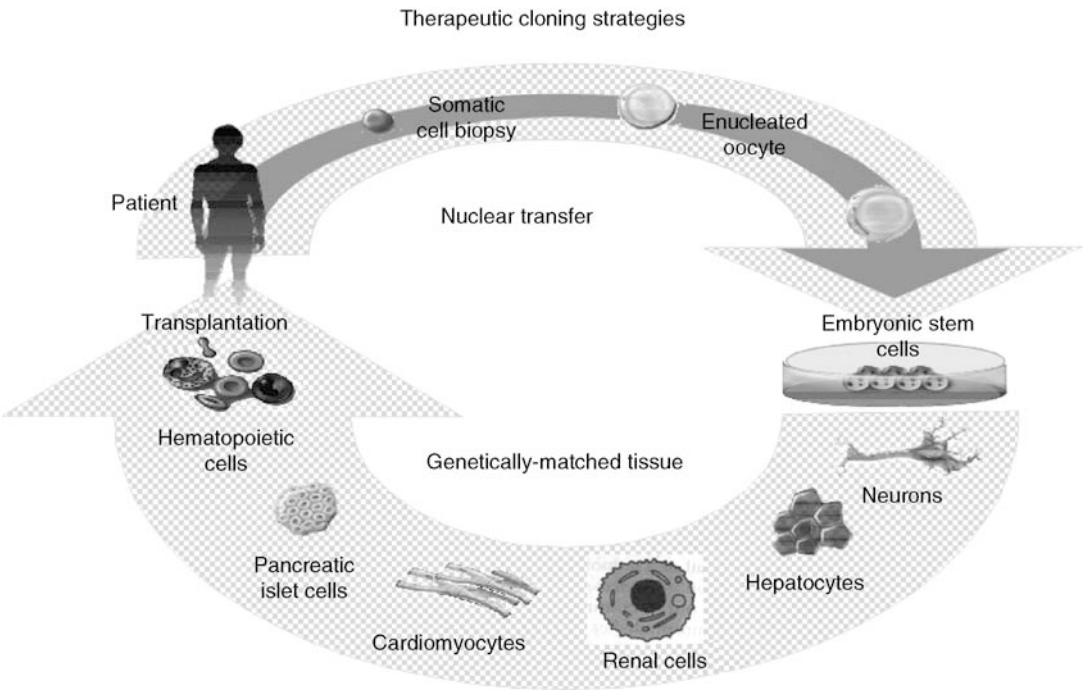
Somatic cell nuclear transfer (SCNT), or therapeutic cloning, entails the removal of an oocyte nucleus in culture, followed by its replacement with a nucleus derived from a somatic cell obtained from a patient. Activation with chemicals or electricity stimulates cell division up to the blastocyst stage.

At this point, it is extremely important to differentiate between the two types of cloning that exist – reproductive cloning and therapeutic cloning. Both involve the insertion of donor DNA into an enucleated oocyte to generate an embryo that has identical genetic material to its DNA source. However, the similarities end there. In reproductive cloning, the embryo is then implanted into the uterus of a pseudopregnant female to produce an infant that is a clone of the donor. A world-famous example of this type of cloning resulted in the birth of a sheep named Dolly in 1997 (32). However, there are many ethical concerns surrounding such practices, and as a result, reproductive cloning has been banned in most countries.

While therapeutic cloning also produces an embryo that is genetically identical to the donor, this process is used to generate blastocysts that are explanted and grown in culture, rather than in utero. Embryonic stem cell lines can then be derived from these blastocysts, which are only allowed to grow up to a 100-cell stage. At this time the inner cell mass is isolated and cultured, resulting in ES cells that are genetically identical to the patient. This process is detailed in *Fig. 19-1*. It has been shown that nuclear transferred ES cells derived from fibroblasts, lymphocytes, and olfactory neurons are pluripotetent and can generate live pups after injection into blastocysts.

Figure 19-1

Strategies for therapeutic cloning in regenerative medicine.



This shows that cells generated by SCNT have the same developmental potential as blastocysts that are fertilized and produced naturally (33–36). In addition, the ES cells generated by SCNT are perfectly matched to the patient's immune system and no immunosuppressive medications would be required to prevent rejection should these cells be used in tissue engineering applications.

Although ES cells derived from SCNT contain the nuclear genome of the donor cells, mitochondrial DNA (mtDNA) contained in the oocyte could lead to immunogenicity after transplantation. To assess the histocompatibilty of tissue generated using SCNT, Lanza et al. microinjected the nucleus of a bovine skin fibroblast into an enucleated oocyte (37). Although the blastocyst was implanted (reproductive cloning), the purpose was to generate renal, cardiac and skeletal muscle cells, which were then harvested, expanded in vitro, and seeded onto biodegradable scaffolds. These scaffolds were then implanted into the donor steer from whom the cells were cloned to determine if cells were histocompatible. Analysis revealed that cloned renal cells showed no evidence of T-cell response, suggesting that rejection will not necessarily occur in the presence of oocyte-derived mtDNA. This finding represents an important step in overcoming histocompatibility problems of stem cell therapy.

Although promising, SCNT has certain technical limitations that must be overcome prior to clinical application. There are also obvious etical concerns which must be resolved re: the potential abuses of therapeutic cloning. In addition, this technique has not yet been successful in humans. The initial failures and fraudulent reports of nuclear transfer in humans have reduced enthusiasm for human applications (38–40). However, it was recently reported that non-human primate ES cell lines were generated by SCNT of nuclei from adult skin fibroblasts (41, 42).

Before SCNT-derived ES cells can be used as clinical therapy, careful assessment of quality of the lines must be determined. For example, some cell lines generated by SCNT have contained chromosomal translocations and it is not known whether these abnormalities originated from aneuploid embryos or if they occurred during ES cell isolation and culture. In addition, the low efficiency of SNCT (0.7%) and the inadequate supply of human oocytes further hinder the therapeutic potential of this technique. Still, these studies provide promise that ES cell lines could one day be generated from human cells to produce patient-specific stem cells. Such cells would have the potential to cure many human diseases that are currently untreatable.

Reprogrammed Somatic Cells

Recently, exciting reports of the successful transformation of adult cells into pluripotent stem cells through specific genetic "reprogramming" has been published. Reprogramming is a technique that involves de-differentiation of adult somatic cells to produce patient-specific pluripotent stem cells, eliminating the need to create embryos. Cells generated by reprogramming would be genetically identical to the somatic cells (and thus, the patient who donated these cells) and would not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an "induced pluripotent state (iPS)" (43). These iPS cells possessed the immortal growth characteristics of self-renewing ES cells, expressed genes specific for ES cells, and generated embryoid bodies in vitro and teratomas in vivo. When iPS cells were injected into mouse blastocysts, they contributed to a variety of cell types. However, although iPS cells selected in this way were pluripotent, they were not identical to ES cells. Unlike ES cells, chimeras made from iPS cells did not result in full-term pregnancies. Gene expression profiles of the iPS cells showed that they possessed a distinct gene expression signature that was different from that of ES cells. In addition, the epigenetic state of the iPS cells was intermediate between that found in somatic cells and that found in ES cells, suggesting that the reprogramming was incomplete.

These results were improved significantly by Wernig and Jaenisch in July 2007 (44). In their study, DNA methylation, gene expression profiles, and the chromatin state of the reprogrammed cells were similar to those of ES cells. Teratomas induced by these cells contained differentiated cell types representing all three embryonic germ layers. Most importantly, the reprogrammed cells from this experiment were able to form viable chimeras and contribute to the germ line like ES cells, suggesting that these iPS cells were completely reprogrammed.

It has recently been shown that reprogramming of human cells is possible (45, 46). Yamanaka generated human iPS cells that are similar to hES cells in terms of morphology, proliferation, gene expression, surface markers, and teratoma formation. Thompson's group showed that retroviral transduction of the stem cell markers *OCT4, SOX2, NANOG, and LIN28* could generate pluripotent stem cells. However, in both studies, the human iPS cells were similar but not identical to hES cells. Although reprogramming is an exciting phenomenon, our limited understanding of the mechanism currently limits the clinical applicability of the technique. The future potential of reprogramming is quite exciting.

Placental and Amniotic Fluid Stem Cells

Recently, it has been shown that pluripotent cells may be derived from the amniotic fluid and placenta. Both amniotic fluid and placenta are known to contain multiple partially differentiated cell types derived from the developing fetus. Stem cell populations have been isolated from these sources. Called amniotic fluid and placental stem cells (AFPSC), they express embryonic and adult stem cell markers (47). The undifferentiated stem cells expand extensively without a feeder cell layer and double every 36 h. Unlike human embryonic stem cells, the AFPSC do not form tumors in vivo. Lines maintained for over 250 doublings retained long telomeres and a normal complement of chromosomes. AFPSC are broadly multipotent, and human lines can be induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. Examples of differentiated cells derived from AFPSC and displaying specialized functions include neuronal lineage secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium (GIRK) channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue engineered bone. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue. The cells could be obtained either from amniocentesis or chorionic villous sampling in the developing fetus, or from the placenta at the time of birth. They could be preserved for self use, and used without rejection, or they could be banked. A bank of 100,000 specimens could potentially supply 99% of the US population with a perfect genetic match for transplantation. Such a bank may be easier to create than with other cell sources, since there are approximately 4.5 million births per year in the USA (47).

Biomaterials

In the most common tissue engineering procedures, isolated cells are seeded onto a scaffold composed of an appropriate biomaterial. These biomaterials replicate the biologic and mechanical function of the native extracellular matrix (ECM) found in tissues in the body by serving as an artificial ECM. Biomaterials provide a three-dimensional space for the cells to develop into new tissues with appropriate structure and function. They can also allow delivery of appropriate bioactive factors (e.g., cell adhesion peptides, growth factors) to the developing tissue (48) to help regulate cellular function. As the majority of mammalian

cell types are anchorage-dependent and will die if no cell adhesion substrate is available, biomaterials provide this substrate that can deliver cells to specific sites in the body with high loading efficiency. Biomaterials can also provide mechanical support against in vivo forces so that the predefined three-dimensional structure of the engineered implant is maintained during tissue development.

The ideal biomaterial should be biodegradable, bioresorbable, and support the replacement of normal tissue without inducing inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection and/or necrosis. Degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate so that the concentration of these degradation products in the tissues remains at a tolerable level (49). The biomaterial should also provide an environment in which appropriate regulation of cell behavior (adhesion, proliferation, migration, and differentiation) can occur. Cell behavior in the newly formed tissue has been shown to be regulated by multiple interactions of the cells with their microenvironment, including interactions with cell-adhesion ligands (50) and with soluble growth factors. Since biomaterials provide temporary mechanical support while the cells undergo spatial reorganization into tissue, the properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, while in late development, it should begin to degrade so that it does not hinder further tissue growth (48).

Generally, three classes of biomaterials have been utilized for engineering tissues: naturally derived materials (e.g., collagen and alginate), acellular tissue matrices (e.g., bladder submucosa and small intestinal submucosa), and synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA). These classes of biomaterials have been tested for biocompatibility (51, 52). Naturally derived materials and acellular tissue matrices have the potential advantage of biological recognition. However, synthetic polymers can be produced reliably on a large scale with controlled properties such as strength, degradation rate, and microstructure, which would aid in the preparation of easily used, "off-the-shelf" scaffold material.

Naturally Derived Materials

Collagen is the most abundant and ubiquitous structural protein in the body, and may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction (53). Collagen implants, under normal conditions, are degraded through a process involving phagocytosis of collagen fibrils by fibroblasts (54). This is followed by sequential attack by lysosomal enzymes including cathepsins B1 and D. Under inflammatory conditions, the implants can be rapidly degraded largely by matrix metalloproteins (MMPs) and collagenases (54). However, the in vivo resorption rate of a collagen implant can be regulated by controlling the density of the implant and the extent of intermolecular cross-linking – the lower the density, the greater the space between collagen fibers and the larger the pores for cell infiltration, leading to a higher rate of implant degradation. Collagen contains cell adhesion domain sequences (e.g., RGD) that may help to retain the phenotype and activity of many types of cells, including fibroblasts (55)and chondrocytes (56).

Alginate, a polysaccharide isolated from seaweed, has been used as an injectable cell delivery vehicle (57) and a cell immobilization matrix (58) owing to its gentle gelling properties in the presence of divalent ions such as calcium. Alginate is relatively biocompatible and is approved by the Food and Drug Administration (FDA) for human use as wound dressing material. Alginate is a family of copolymers of D-mannuronate and L-guluronate. The physical and mechanical properties of alginate gel are strongly correlated with the proportion and length of polygluronic block in the alginate chains (57).

Acellular Tissue Matrices

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues. The matrices are often prepared by mechanical and chemical manipulation of a segment of tissue (1–4). These matrices slowly degrade upon implantation, and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or in growing cells.

Synthetic Polymers

Polyesters of naturally occurring *a*-hydroxy acids, including PGA, PLA, and PLGA, are widely used in tissue engineering. These polymers are FDA-approved for a variety of applications, including sutures (59). The ester bonds in these polymers are hydrolytically labile, and they degrade by nonenzymatic hydrolysis. The degradation products of PGA, PLA, and PLGA are nontoxic natural metabolites and are eventually eliminated from the body in the form of carbon dioxide and water (59). The degradation rate of

these polymers can be tailored to theapplication by altering crystallinity, initial molecular weight, and the copolymer ratio of lactic to glycolic acid. Generally, the optimal degradation time ranges from several weeks to several years. Since these polymers are thermoplastics, they can be easily formed into a three dimensional scaffold with a desired microstructure, gross shape, and dimension by various techniques, including molding, extrusion, solvent casting (60), phase separation techniques, and gas foaming techniques (61). Many applications intissue engineering often require a scaffold with high porosity and ratio of surface area to volume. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(orthoesters), can also be used to fabricate scaffolds for tissue engineering with controlled properties (62).

Engineering Specific Tissues and Organs

Investigators around the world, including our laboratory, have been working towards the development of several cell types, tissues, and organs for clinical application. The following sections will describe this research in detail.

Urethra

Various biomaterials without cells, such as PGA and acellular collagen-based matrices derived from decellularized small intestine and bladder, have been used in animal models for the regeneration of urethral tissue (4, 63–67). Some of these biomaterials, like acellular collagen matrices derived from bladder submucosa, have also been seeded with autologous cells for urethral reconstruction. Our laboratory has been able to replace tubularized urethral segments with cell-seeded collagen matrices (68, 69).

Acellular collagen matrices derived from bladder submucosa by our laboratory have been used experimentally and clinically. In animal studies, segments of the urethra were resected and replaced with acellular matrix grafts. Histological examination showed complete epithelialization and progressive vessel and muscle infiltration, and the animals were able to void through the neo-urethras (4). These results were confirmed in a clinical study of patients with hypospadias and urethral stricture disease (70) (*Fig. 19-2*). Decellularized cadaveric bladder submucosa was used as an underlying matrix for urethral repair in patients with stricture disease and hypospadias. Patent, functional neo-urethras were noted in these patients with up to a 7-year follow-up. The use of a readily available, "off-the-shelf" matrix appears to be beneficial for patients with abnormal urethral conditions and obviates the need for obtaining autologous grafts, thus decreasing operative time and eliminating donor site morbidity.

Unfortunately, the above techniques are not applicable for tubularized urethral repairs. The collagen matrices are able to replace urethral segments only when used in specific physical contact with existing tissue. However, if a tubularized repair is needed, the collagen matrices should be seeded with autologous cells to avoid the risk of stricture formation and poor tissue development (68). Therefore, tubularized collagen matrices seeded with autologous cells can be used successfully for total penile urethra replacement.

Bladder

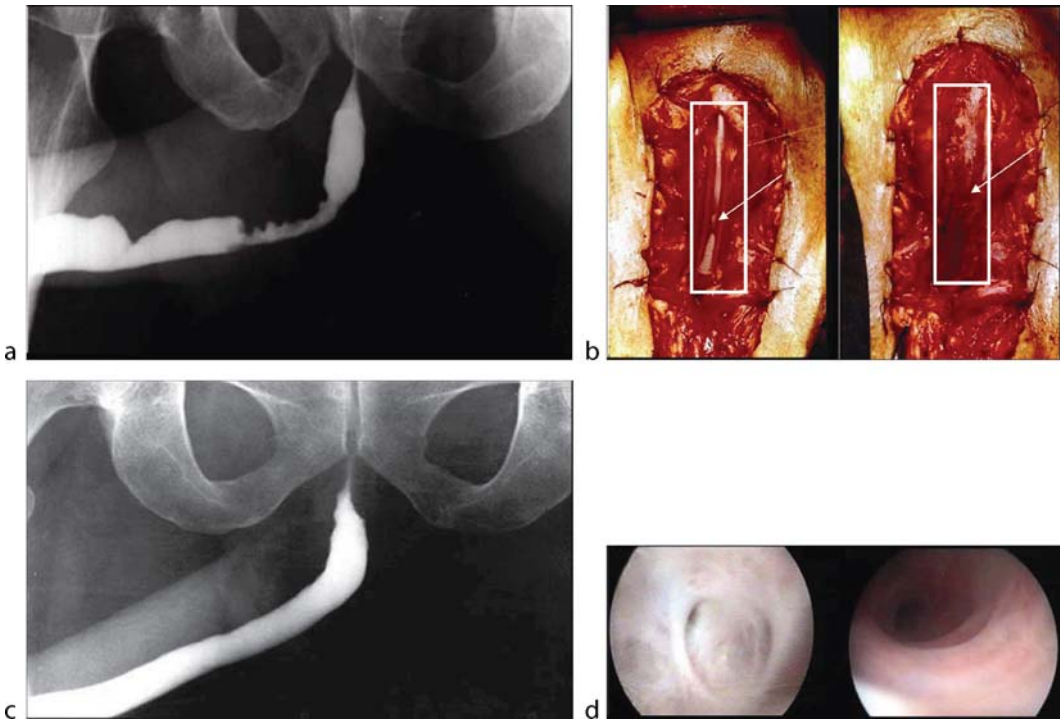
Currently, gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, gastrointestinal tissues are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of solutes. Due to the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative materials and tissues for bladder replacement or repair.

The success of cell transplantation strategies for bladder reconstruction depends on the ability to use donor tissue efficiently and to provide the right conditions for long term survival, differentiation, and growth. Urothelial and muscle cells can be expanded in vitro, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells (71). These principles were applied in the creation of tissue engineered bladders in an animal model that required a subtotal cystectomy with subsequent replacement with a tissue engineered organ in beagle dogs (12). Urothelial and muscle cells were separately expanded from an autologous bladder biopsy, and seeded onto a bladder-shaped biodegradable polymer scaffold. The results from this study showed that it is possible to tissue engineer bladders that are anatomically and functionally normal. Clinical trials for the application of this technology are currently being conducted.

A clinical experience involving engineered bladder tissue for cystoplasty reconstruction was conducted starting in 1999. A small pilot study of seven patients was reported, using a collagen scaffold seeded with cells either with or without omentum coverage, or a combined PGA-collagen scaffold seeded with cells and omental coverage (*Fig. 19-3*). The patients reconstructed with the engineered bladder tissue created with the PGA-collagen cell-seeded scaffolds showed increased compliance, decreased end-filling pressures, increased capacities and

Figure 19-2

Urethral repair using a collagen matrix. (a): Representative case of a patient with a bulbar stricture. (b): During surgery, strictured tissue is excised, preserving the urethral plate on the left side, and the matrix is anastamosed to the urethral plate in an onlay fashion on the right. The boxes in both photos indicate the area of interest, including the urethra, which appears white in the left photograph. In the left photograph, the arrow indicates the area of stricture in the urethra. On the right, the arrow indicates the repaired stricture. Note that the engineered tissue now obscures the native white urethral tissue in an onlay fashion in the right photograph. (c): Urethrogram 6 months after repair. (d): Cystoscopic view of urethra before surgery on the left side, and 4 months after repair on the right side. (See color plate 5)



longer dry periods (72) (*Fig. 19-4*). Although the experience is promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of engineering fully functional bladders. Further experimental and clinical work is being conducted.

Kidney

Renal tissue is arguably one of the most difficult tissues to replicate in the laboratory. The kidney is a complex organ and its unique structural and cellular heterogeneity creates many challenges. The system of nephrons and collecting ducts within the kidney is composed of multiple functionally and morphologically distinct segments, arranged in an elaborate architectonic pattern. For this reason, appropriate conditions must be provided to ensure the long-term survival, differentiation and growth of many types of cells. Recent efforts in kidney tissue regeneration have focused on the development of a reliable cell source (73, 74) (75, 76) (77, 78). Moreover, optimal growth conditions have been extensively investigated to provide adequate enrichment to achieve stable renal cell expansion systems (79, 80) (81–83).

Isolation of particular cell types that produce specific factors may be a good approach for selective cell therapies. For example, cells that produce erythropoietin could be isolated in culture, and these cells could eventually be used to treat anemia that results from end stage renal failure. However, total renal function would not be achieved using this approach. To create kidney tissue that would deliver full renal function, a culture containing all of the cell types comprising the functional nephron units should be used. Optimal culture conditions to nurture renal cells have been extensively studied and

Figure 19-3

Construction of engineered bladder. (a): Scaffold material seeded with cells for use in bladder repair. (b): The seeded scaffold is anastamosed to native bladder with running 4–0 polyglycolic sutures. (c): Implant covered with fibrin glue and omentum. (See color plate 6)

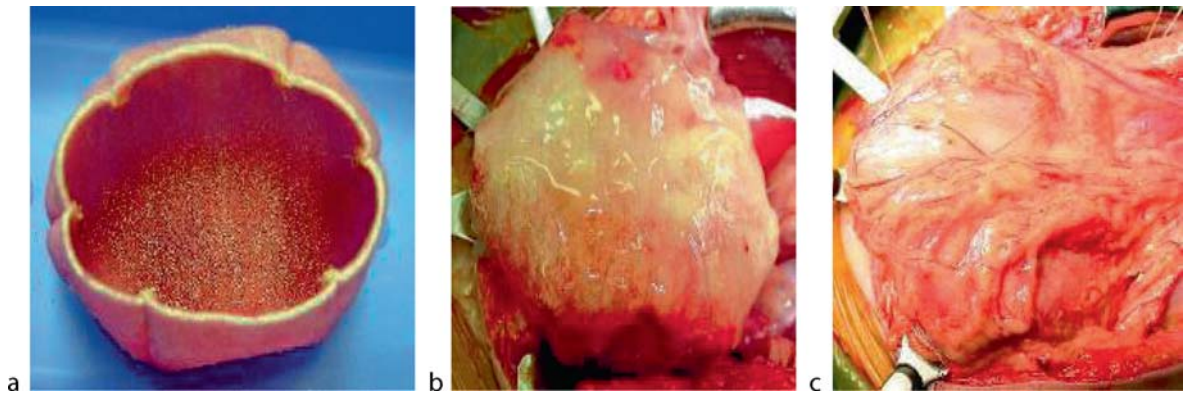
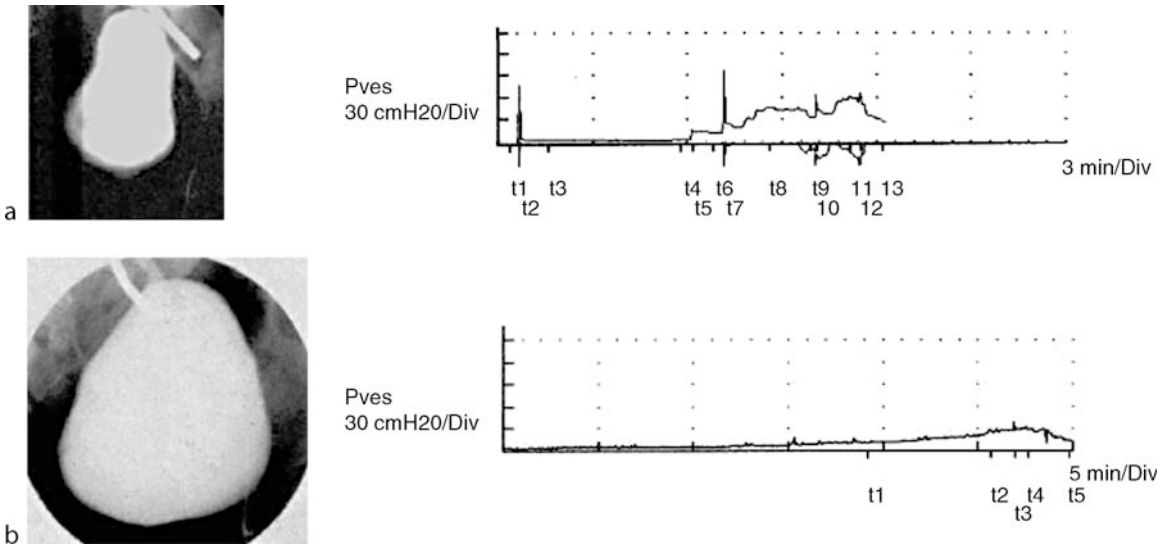


Figure 19-4

Cystograms and urodynamic studies of a patient before and after implantation of the tissue engineered bladder. (a): Preoperative results indicate an irregular bladder in the cystogram and abnormal bladder pressures as the bladder is filled via urodynamic study. (b): Postoperatively, findings are significantly improved.



cells grown under these conditions have been reported to maintain their cellular characteristics (84). Moreover, renal cells placed in a three-dimensional culture environment are able to reconstitute into renal structures.

Recent investigative efforts in the search for a reliable cell source have been expanded to stem and progenitor cells. Use of these cells for tissue regeneration is attractive due to their ability to differentiate and mature into the many specific cell types needed. This is particularly useful in instances where primary renal cells are unavailable due to extensive tissue damage. Bone marrow-derived human mesenchymal stem cells have been shown to be a potential source due to their ability to differentiate into several cell lineages (73, 74, 77). These cells have been shown to participate in the kidney development when they are placed in a rat embryonic niche that allows for continued exposure to a repertoire of nephrogenic signals (78). These cells, however, were found to contribute mainly to

regeneration of damaged glomerular endothelial cells after injury. In addition, the major cell source of kidney regeneration was found to originate from intrarenal cells in an ischemic renal injury model (73, 76). Another potential cell source for kidney regeneration is circulating stem cells, which have been shown to transform into tubular and glomerular epithelial cells, podocytes, mesangial cells, and interstitial cells after renal injury (74, 85–87) (75, 88, 89). These observations suggest that controlling stem and progenitor cell differentiation may lead to successful regeneration of kidney tissues.

Although isolated renal cells are able to retain their phenotypic and functional characteristics in culture, transplantation of these cells in vivo may not result in structural remodeling. In addition, cell or tissue components cannot be implanted in large volumes due to limited diffusion of oxygen and nutrients (90). Thus, a cell-support matrix, preferably one that encourages angiogenesis, is necessary to allow diffusion across the entire implant. A variety of synthetic and naturally derived materials has been examined in order to determine the ideal support structures for the regeneration (70, 72, 91–93). Biodegradable synthetic materials, such as poly-lactic and poly-glycolic acid polymers, have been used to provide structural support for cells. Synthetic materials can be easily fabricated and configured in a controlled manner, which make them attractive options for tissue engineering. However, naturally derived materials, such as collagen, laminin and fibronectin, are much more biocompatible and provide a similar extracellular matrix environment to normal tissue. For this reason, collagen based scaffolds have been used increasingly in many applications (94–97).

Developmental Approaches to Kidney Regeneration

Transplantation of a kidney precursor, such as the metanephros, into a diseased kidney has been proposed as a possible method for functional restoration. In an animal study, human embryonic metanephroi, transplanted into the kidneys of an immune deficient mouse model, has developed into mature kidneys (98). The transplanted metanephroi produced urine-like fluid, however, failed to develop ureters. This study suggests that development of an in vitro system in which metanephroi could be grown may lead to transplant techniques that could produce a small replacement kidney within the host. In another study, the metanephros was divided into mesenchymal tissue and ureteral buds, and each of the tissue segments was cultured in vitro (99). After eight days in culture, each portion of the mesenchymal tissues had grown to the original size. A similar method was used for ureteral buds, which also propagated. These studies indicate that if the mesenchyme and ureteral buds were placed together and cultured in vitro, a metanephros-like structure would develop and suggest that the metanephros could be propagated under optimal conditions.

In another study, transplantation of metanephroi into a non-immunosuppressed rat omentum showed that the implanted metanephroi are able to undergo differentiation and growth that is not confined by a tight organ capsule (100). When the metanephroi with an intact ureteric bud were implanted, the metanephroi are able to enlarge and become kidney-shaped tissue within 3 weeks. The metanephroi transplanted into the omentum were able to develop into kidney tissue structure with a well-defined cortex and medulla. Mature nephrons and collecting system structures are shown to be indistinguishable from those of normal kidneys by light or electron microscopy (101, 102). Moreover, these structures become vascularized via arteries that originate at the superior mesenteric artery of the host (101, 102). It has been demonstrated that the metanephroi transplanted into the omentum survive for up to 32 weeks post-implantation (103). These studies show that developmental approach may be a viable option for regenerating renal tissue for functional restoration.

Tissue Engineering Approaches to Kidney Regeneration

The ability to grow and expand renal cells is one of the essential requirements in engineering tissues. The feasibility of achieving renal cell growth, expansion and in vivo reconstitution using tissue engineering techniques was investigated (91). Donor rabbit kidneys were removed and perfused with a non-oxide solution which promoted iron particle entrapment in the glomeruli. Homogenization of the renal cortex and fractionation in 83 and 210 micron sieves with subsequent magnetic extraction yielded three separate purified suspensions of distal tubules, glomeruli, and proximal tubules. The cells were plated separately in vitro and after expansion, were seeded onto biodegradable polyglycolic acid scaffolds and implanted subcutaneously into host athymic mice. This included implants of proximal tubular cells, glomeruli, distal tubular cells, and a mixture of all three cell types. Animals were sacrificed at one week, two weeks, and one month after implantation and the retrieved implants were analyzed. An acute inflammatory phase and a chronic foreign body

reaction were seen, accompanied by vascular in growth by 7 days after implantation. Histologic examination demonstrated progressive formation and organization of the nephron segments within the polymer fibers with time. Renal cell proliferation in the cell-polymer scaffolds was detected by in vivo labeling of replicating cells with the thymidine analog bromodeoxyuridine (76). BrdU incorporation into renal cell DNA was confirmed using monoclonal anti-BrdU antibodies. These results demonstrated that renal specific cells can be successfully harvested and cultured, and can subsequently attach to artificial biodegradable polymers. The renal cell-polymer scaffolds can be implanted into host animals where the cells replicate and organize into nephron segments, as the polymer, which serves as a cell delivery vehicle, undergoes biodegradation.

Initial experiments showed that implanted cell-polymer scaffolds gave rise to renal tubular structures. However, it was unclear whether the tubular structures reconstituted de novo from dispersed renal elements, or if they merely represented fragments of donor tubules which survived the original dissociation and culture processes intact. Further investigation was conducted in order to examine the process (104). Mouse renal cells were harvested and expanded in culture. Subsequently, single isolated cells were seeded on biodegradable polymers and implanted into immune competent syngeneic hosts. Renal epithelial cells were observed to reconstitute into tubular structures in vivo. Sequential analyses of the retrieved implants over time demonstrated that renal epithelial cells first organized into a cord-like structure with a solid center. Subsequent canalization into a hollow tube could be seen by two weeks. Histologic examination with nephron segment specific lactins showed successful reconstitution of proximal tubules, distal tubules, loop of Henle, collecting tubules and collecting ducts. These results showed that single suspended cells are capable of reconstituting into tubular structures, with homogeneous cell types within each tubule.

Although these studies demonstrated that renal cells seeded on biodegradable polymer scaffolds are able to form some renal structures in vivo, complete renal function could not be achieved in these studies. In a subsequent study we sought to create a functional artificial renal unit which could produce urine (105). Mouse renal cells were harvested, expanded in culture, and seeded onto a tubular device constructed from polycarbonate (97). The tubular device was connected at one end to a silastic catheter which terminated into a reservoir. The device was implanted subcutaneously in athymic mice. The implanted devices were retrieved and examined histologically and immunocytochemically at 1, 2, 3, 4 and 8 weeks after implantation. Fluid was collected from inside the implant, and uric acid and creatinine levels were determined.

Histological examination of the implanted device demonstrated extensive vascularization as well as for mation of glomeruli and highly organized tubule-like structures. Immunocytochemical staining with anti-osteopontin antibody, which is secreted by proximal and distal tubular cells and the cells of the thin ascending loop of Henle, stained the tubular sections. Immunohistochemical staining for alkaline phosphatase stained proximal tubule-like structures. Uniform staining for fibronectin in the extracellular matrix of newly formed tubes was observed. The fluid collected from the reservoir was yellow and contained 66 mg/dl uric acid (as compared to 2 mg/dl in plasma) suggesting that these tubules are capable of unidirectional secretion and concentration of uric acid. The creatinine assay performed on the collected fluid showed an 8.2 fold increase in concentration, as compared to serum. These results demonstrated that single cells form multicellular structures can become organized into functional renal units that are able to excrete high levels of solutes through a urine-like fluid (105).

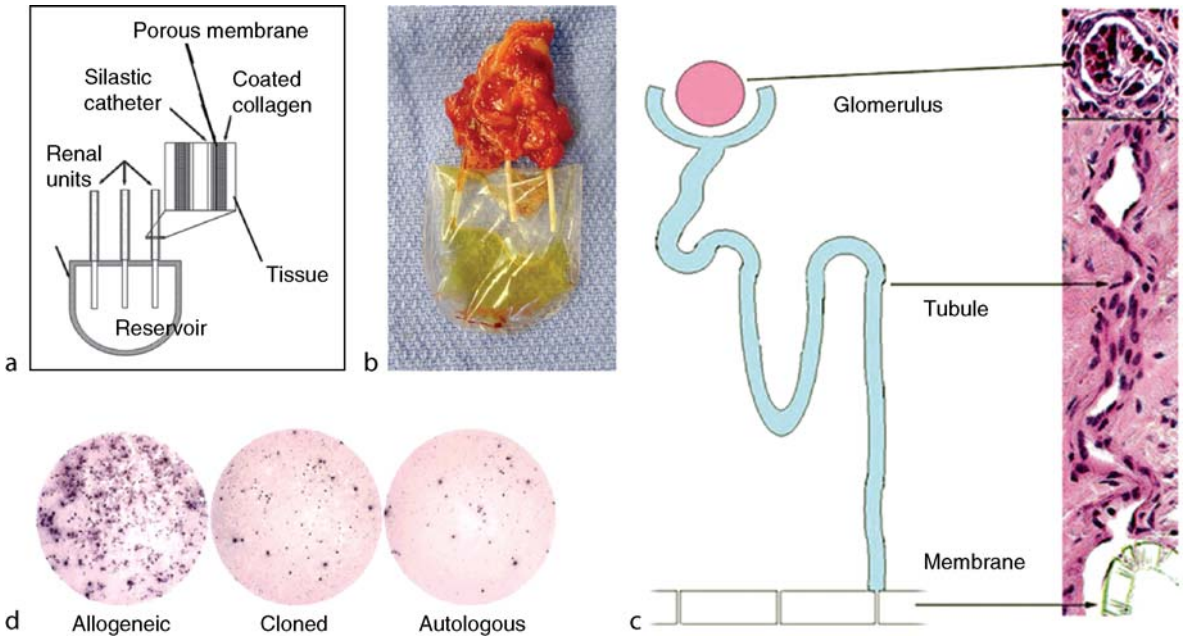
To determine whether renal tissue could be formed using an alternative cell source, nuclear transplantation (therapeutic cloning) was performed to generate histocompatible tissues, and the feasibility of engineering syngeneic renal tissues in vivo using these cloned cells was investigated (84). Nuclear material from bovine dermal fibroblasts was transferred into unfertilized enucleated donor bovine eggs. Renal cells from the cloned embryos were harvested, expanded in vitro, and seeded onto three-dimensional renal devices (*Fig. 19-5a*). The devices were implanted into the back of the same steer from which the cells were cloned, and were retrieved 12 weeks later.

This process produced functioning renal units. Urine production and viability were demonstrated after transplantation back into the nuclear donor animal (*Fig. 19-5b*). Chemical analysis suggested unidirectional secretion and concentration of urea nitrogen and creatinine. Microscopic analysis revealed formation of organized glomeruli and tubular structures (*Fig. 19-5c*). Immunohistochemical and RT-PCR analysis confirmed the expression of renal mRNA and proteins.

Since previous studies have shown that bovine clones harbor the oocyte mitochondrial DNA (106–108) the donor egg's mitochondrial DNA (mtDNA) was thought to be a potential source of immunologic incompatibility. Differences in mtDNA-encoded proteins expressed by cloned cells could stimulate a T-cell response specific for

Figure 19-5

Combining therapeutic cloning and tissue engineering to produce kidney tissue. (a): Illustration of the tissue-engineered renal unit. (b): Renal unit seeded with cloned cells, three months after implantation, showing the accumulation of urine-like fluid. (c): Clear unidirectional continuity between the mature glomeruli, their tubules, and silastic catheter. (d): Elispot analyses of the frequencies of T cells that secrete IFNγ after stimulation with allogeneic renal cells, cloned renal cells, or nuclear donor fibroblasts. Cloned renal cells produce fewer IFNγ spots than the allogeneic cells, indicating that the rejection response to cloned cells is diminished. The presented wells are single representatives of duplicate wells. (See color plate 7)



mtDNA-encoded minor histocompatibility antigens when the cloned cells are implanted back into the original nuclear donor (109). Maternally transmitted minor histocompatibility antigens in mice have been shown to stimulate both skin allograft rejection in vivo and cytotoxic T lymphocytes expansion in vitro (109) that could prevent the use of these cloned constructs in patients with chronic rejection of major histocompatibility matched human renal transplants (110, 111). We tested for a possible T-cell response to the cloned renal devices using delayed-type hypersensitivity testing in vivo and EliSPOT analysis of interferon-γ secreting T-cells in vitro. Both analyses revealed that the cloned renal cells showed no evidence of a T-cell response, suggesting that rejection will not necessarily occur in the presence of oocyte-derived mtDNA (*Fig. 19-5d*). This finding may represent a step forward in overcoming the histocompatibility problem of stem cell therapy (111).

These studies demonstrated that cells derived from nuclear transfer can be successfully harvested, expanded in culture, and transplanted in vivo with the use of biodegradable scaffolds on which the single suspended cells can organize into tissue structures that are genetically identical to that of the host. These studies were the first demonstration of the use of therapeutic cloning for regeneration of tissues in vivo.

However, a naturally derived tissue matrix with existing three-dimensional kidney architecture would be preferable to the artificial matrix used in these experiments, because it would allow for transplantation of a larger number of cells, resulting in greater renal tissue volumes. Thus, we developed an acellular collagen-based kidney matrix, which is identical to the native renal architecture. In a subsequent study we investigated whether these collagen-based matrices could accommodate large volumes of renal cells and form kidney structures in vivo (112).

Acellular collagen matrices, derived from porcine kidneys, were obtained through a multi-step decellularization process. During this process, serial evaluation of the matrix for cellular remnants was performed using histochemistry, scanning electron microscopy (SEM) and RT-PCR. Mouse renal cells were harvested, grown,

and seeded on 80 of the decellularized collagen matrices at a concentration of 30×10^6 cells/ml. Forty cell-matrix constructs grown in vitro were analyzed 3 days, 1, 2, 4 and 6 weeks after seeding. The remaining 40 cell-containing matrices were implanted in the subcutaneous space of 20 athymic mice. The animals were sacrificed 3 days, 1, 2, 4, 8 and 24 weeks after implantation for analyses. Gross, SEM, histochemical, immunocytochemical and biochemical analyses were performed.

Scanning electron microscopy and histologic examination confirmed the acellularity of the processed matrix. RT-PCR performed on the kidney matrices demonstrated the absence of any RNA residues. Renal cells seeded on the matrix adhered to the inner surface and proliferated to confluency 7 days after seeding, as demonstrated by SEM. Histochemical and immunocytochemical analyses performed using H & E, periodic acid schiff, alkaline phosphatase, anti-osteopontin and anti-CD-31 identified stromal, endothelial and tubular epithelial cell phenotypes within the matrix. Renal tubular and glomerulus-like structures were observed 8 weeks after implantation. MTT proliferation and titrated thymidine incorporation assays performed 6 weeks after cell seeding demonstrated a population increase of 116% and 92%, respectively, as compared to the 2 week time points. This study demonstrates that renal cells are able to adhere to and proliferate on the collagen-based kidney matrices. The renal cells reconstitute renal tubular and glomeruli-like structures in the kidney-shaped matrix. The collagen based kidney matrix system seeded with renal cells may be useful in the future for augmenting renal function.

We also investigated the feasibility of creating three-dimensional renal structures for in situ implantation within the native kidney tissue. Primary renal cells from 4 week old mice were grown and expanded in culture. These renal cells were labeled with fluorescent markers and injected into mouse kidneys in a collagen gel for in vivo formation of renal tissues. Collagen injection without cells and sham operated animals served as controls. In vitro reconstituted renal structures and in vivo implanted cells were retrieved and analyzed.

The implanted renal cells formed tubular and glomerular structures within the kidney tissue, as confirmed by the fluorescent markers. There was no evidence of renal tissue formation in the control and the sham operated groups. These results demonstrate that single renal cells are able to reconstitute kidney structures when placed in a collagen-based scaffolding system. The implanted renal cells are able to self assemble into tubular and glomerular structures within the kidney tissue. These findings suggest that this system may be the preferred approach to engineer functional kidney tissues for the treatment of end stage renal disease.

Genital Tissues

Reconstructive surgery is required for a wide variety of pathologic penile conditions, such as penile carcinoma, trauma, severe erectile dysfunction, and congenital conditions such as ambiguous genitalia, hypospadias, and epispadias. One of the major limitations of phallic reconstructive surgery is the scarcity of sufficient autologous tissue.

The major components of the phallus are corporal smooth muscle and endothelial cells. The creation of autologous functional and structural corporal tissue de novo would be beneficial. Autologous cavernosal smooth muscle and endothelial cells were harvested, expanded, and seeded on acellular collagen matrices and implanted in a rabbit model (113, 114). Histologic examination confirmed the appropriate organization of penile tissue phenotypes, and structural and functional studies, including cavernosography, cavernosometry, and mating studies, demonstrated that it is possible to engineer autologous functional penile tissue. Our laboratory is currently working on increasing the size of the engineered constructs.

Congenital malformations of the uterus may have profound implications clinically. Patients with cloacal exstrophy and intersex disorders may not have sufficient uterine tissue present for future reproduction. We investigated the possibility of engineering functional uterine tissue using autologous cells (115). Autologous rabbit uterine smooth muscle and epithelial cells were harvested, then grown and expanded in culture. These cells were seeded onto preconfigured uterine-shaped biodegradable polymer scaffolds, which were then used for subtotal uterine tissue replacement in the corresponding autologous animals. Upon retrieval 6 months after implantation, histological, immunocytochemical, and Western blot analyses confirmed the presence of normal uterine tissue components. Biomechanical analyses and organ bath studies showed that the functional characteristics of these tissues were similar to those of normal uterine tissue. Breeding studies using these engineered uteri are currently being performed.

Similarly, several pathologic conditions, including congenital malformations and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging due to the paucity of available native tissue. The feasibility of

engineering vaginal tissue in vivo was investigated (116). Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown, and expanded in culture. These cells were seeded onto biodegradable polymer scaffolds, and the cell-seeded constructs were then implanted into nude mice for up to 6 weeks. Immunocytochemical, histological, and Western blot analyses confirmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue-engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, while the non-cell-seeded structures were noted to be stenotic (116).

Other Emerging Technologies

Injectable Therapies

Both urinary incontinence and vesicoureteral reflux are common conditions affecting the genitourinary system. Both conditions are usually the result of damage to or malformation of a specific sphincter muscle. Currently, injection of bulking agents around the defective sphincter can be used clinically for these conditions, but biocompatibility of current synthetic bulking agents is a concern. The ideal substance for endoscopic treatment of reflux and incontinence should be injectable, nonantigenic, nonmigratory, volume stable, and safe for human use. Animal studies have shown that chondrocytes (cartilage cells) can be easily harvested and combined with alginate in vitro and the resulting suspension can be easily injected cystoscopically. A similar technique using muscle and muscle precursor cells with the hope of repairing the defective sphincter muscle has been studied. These technologies have been applied in humans for the correction of vesicoureteral reflux in children and for urinary incontinence in adults (117, 118).

Autologous Chondrocytes as Bulking Agents

Injectable bulking agents can be endoscopically used in the treatment of both urinary incontinence and vesicoureteral reflux. The advantages in treating urinary incontinence and vesicoureteral reflux with this minimally invasive approach include the simplicity of this quick outpatient procedure and the low morbidity associated with it. Several investigators are seeking alternative implant materials that would be safe for human use (119).

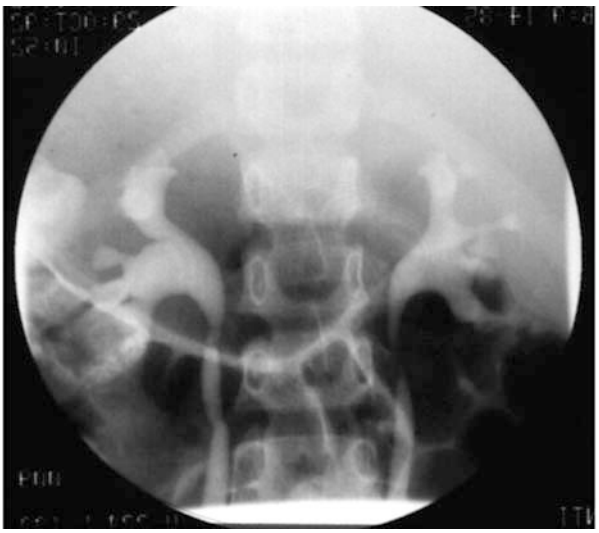
The ideal substance for the endoscopic treatment of reflux and incontinence should be injectable, nonantigenic, nonmigratory, volume stable, and safe for human use. Toward this goal long term studies were conducted to determine the effect of injectable chondrocytes in vivo (120). It was initially determined that alginate, a liquid solution of gluronic and mannuronic acid, embedded with chondrocytes, could serve as a synthetic substrate for the injectable delivery and maintenance of cartilage architecture in vivo. Alginate undergoes hydrolytic biodegradation and its degradation time can be varied depending on the concentration of each of the polysaccharides. The use of autologous cartilage for the treatment of vesicoureteral reflux in humans would satisfy all the requirements for an ideal injectable substance.

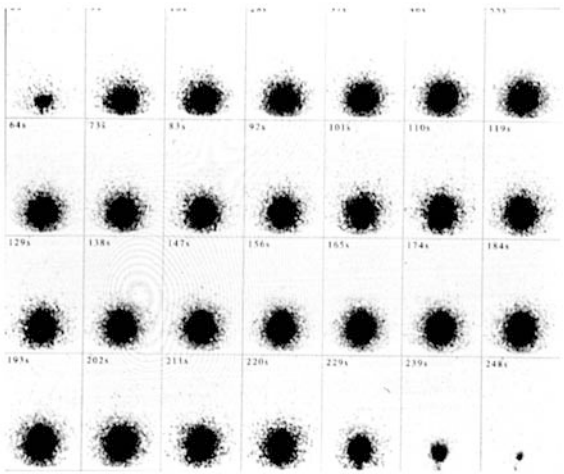
Chondrocytes derived from an ear biopsy can be readily grown and expanded in culture. Neocartilage formation can be achieved in vitro and in vivo using chondrocytes cultured on synthetic biodegradable polymers. In these experiments, the cartilage matrix replaced the alginate as the polysaccharide polymer underwent biodegradation. This system was adapted for the treatment of vesicoureteral reflux in a porcine model (121). These studies showed that chondrocytes can be easily harvested and combined with alginate in vitro, the suspension can be easily injected cystoscopically, and the elastic cartilage tissue formed is able to correct vesicoureteral reflux without any evidence of obstruction.

Two multicenter clinical trials were conducted using this engineered chondrocyte technology. Patients with vesicoureteral reflux were treated at ten centers throughout the US. The patients had a similar success rate as with other injectable substances in terms of cure (*Fig. 19-6*). Chondrocyte formation was not noted in patients who had treatment failure. It is supposed that the patients who were cured have a biocompatible region of engineered autologous tissue present, rather than a foreign material (117). Patients with urinary incontinence were also treated endoscopically with injected chondrocytes at three different medical centers. Phase 1 trials showed an approximate success rate of 80% at follow-up 3 and 12 months postoperatively (118). Several of the clinical trials involving bioengineered products have been placed on hold because of the costs involved with the specific technology. With a bioengineered product, costs are usually high because of the biological nature of the therapies involved. As with any therapy, the cost that the medical health care system can allow for a specific technology is limited. Therefore, the costs of bioengineered products have to be lowered for them to have an impact clinically. This is currently being addressed for multiple tissue-engineered technologies.

Figure 19-6

Autologous chondrocytes for the treatment of vesicoureteral reflux. (a): Preoperative voiding cystourethrogram of a patient with bilateral reflux. A catheter was inserted into the bladder via the urethra, and contrast material was instilled intravesically. Here, contrast material can be seen within both ureters and within the kidneys, indicating reflux is present. (b): Postoperative radionuclide cystogram of the same patient 6 months after injection of autologous chondrocytes. A catheter was inserted into the bladder via the urethra, and a radioactive solution was inserted into the bladder. The bladder was scanned during filling and emptying phases. This panel includes sequential images of the bladder as it was filled and emptied. This shows a normal, round bladder that fills and empties properly. If reflux had been present, the ureters would have been visible in the scan above the round bladder.





As the technologies advance over time, and the volume of the application is considered, costs will naturally decrease.

Injectable Muscle Cells

The potential use of injectable cultured myoblasts for the treatment of stress urinary incontinence has been investigated (122, 123). Myoblasts were labeled with fluorescent latex microspheres (FLM) in order to track them after injection. Labeled myoblasts were directly injected into the proximal urethra and lateral bladder walls of nude mice with a micro-syringe in an open surgical procedure. Tissue harvested up to 35 days post-injection contained the labeled myoblasts, as well as evidence of differentiation of the labeled myoblasts into regenerative myofibers. The authors reported that a significant portion of the injected myoblast population persisted in vivo. Similar techniques of sphincteric derived muscle cells have been used for the treatment of urinary incontinence in a pig model (124). The fact that myoblasts can be labeled and survive after injection and begin the process of myogenic differentiation further supports the feasibility of using cultured cells of muscular origin as an injectable bioimplant.

The use of injectable muscle precursor cells has also been investigated for use in the treatment of urinary incontinence due to irreversible urethral sphincter injury or maldevelopment. Muscle precursor cells are the quiescent satellite cells found in each myofiber that proliferate to form myoblasts and eventually myotubes and new muscle tissue. Intrinsic muscle precursor cells have previously been shown to play an active role in the regeneration of injured striated urethral sphincter (125). In a subsequent study, autologous muscle precursor cells were injected into a rat model of urethral sphincter injury, and both replacement of mature myotubes as well as restoration of functional motor units was noted in the regenerating sphincteric muscle tissue (126). This is the first demonstration of the replacement of both sphincter muscle tissue and its innervation by the injection of muscle precursor cells. As a result, muscle precursor cells may be a minimally invasive solution for urinary incontinence in patients with irreversible urinary sphincter muscle insufficiency.

Fetal Tissue Engineering

The prenatal diagnosis of fetal abnormalities is now more common and more accurate. Improvements in prenatal diagnosis have led to demand for novel interventions designed to reverse potentially life-threatening processes before birth. Having a ready supply of urologic-associated tissue for immediate surgical reconstruction of congenital malformations at birth may be advantageous. Theoretically, once the diagnosis of the pathologic condition is confirmed prenatally, a small tissue biopsy could then be obtained under ultrasound guidance. These biopsy materials could then be processed expanded in vitro. Using tissue engineering techniques, in vitro-reconstituted structures could then be readily available at the time of birth for reconstruction.

Summary and Conclusions

Regenerative medicine efforts are currently underway experimentally for virtually every type of tissue and organ within the human body. As regenerative medicine incorporates the fields of tissue engineering, cell biology, nuclear transfer, and materials science, personnel who have mastered the techniques of cell harvest, culture, expansion, transplantation, as well as polymer design and biomedical engineering are essential for the successful application of these technologies for patients. Various tissues are at different stages of development, with some already being used clinically, a few in preclinical trials, and some in the discovery stage. Recent progress suggests that engineered tissues may have an expanded clinical applicability in the future and may represent a viable therapeutic option for those who would benefit from the life-extending benefits of tissue replacement or repair.

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