CHAPTER 4

TISSUE ENGINEERING IN THE DEVELOPMENT OF REPLACEMENT TECHNOLOGIES

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Abstract: The field of tissue engineering is generating new scaffolds, bioreactors and methods for stimulating cells within complex cultures, with the aim of recreating the conditions under which cells form functional tissues. Hitherto, the primary focus of this field has been on clinical applications. However, there are many methods of in vitro tissue engineering that represent new opportunities in 3D cell culture and could be the basis for new replacement methods that either replace the use of a tissue isolated from an animal or the use of a living animal. This chapter presents an overview of tissue engineering and provides tissue-specific examples of recent advances.

INTRODUCTION

The tissues that make up the mammalian organism are remarkable 3D structures that carry out complex functions for many years by self-organizing billions of cells into defined architectures. Within these architectures, cells are held within niches that define their environmental cues for proliferation and differentiation. Over the lifetime of an organism, these tissues will spontaneously develop, renew and, occasionally, repair themselves.

The field of tissue engineering aims to replicate cell niches in vitro and thereby control complex cell behaviors to a level not currently achieved by cell culture methods. The major motivation for the investment in tissue engineering over the last decade has been to generate clinical products or procedures that restore tissue and function within a patient. The technical challenge faced in developing therapies based on tissue engineering has driven the development of new methods of handling cells in three dimensions. A considerable spill-over benefit of the advances in clinically-driven tissue engineering could be the development of

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replacement tissues that can be manufactured from small populations of expandable cells. Advances in stem cell technology can also be harnessed with tissue engineering, to generate differentiated models by using human cells, without immortalization.

This chapter provides an overview of the current state-of-the-art in tissue engineering and highlights the limited number of successes to date that have adopted tissue engineering principles in order to enhance replacement technologies.

OVERVIEW OF TISSUE ENGINEERING STRATEGIES

Scientific awareness of tissue engineering grew rapidly in the 1990s.[1-3] This awareness was fuelled by breakthroughs in the use of 3D polymer templates to host tissue formation and especially skin,[4] cartilage[5] and cardiovascular tissues.[3] In fact, most of the techniques required to engineer tissues had been under development for many decades before the term "tissue engineering" became widely recognized and used. The concepts of using cocultures, extracellular matrix (ECM) signals, bioreactors and 3D cell aggregates to enhance tissue functionality in cell populations were published in the 1970s.

However, the concept of combining such approaches in order to manufacture tissues of sufficient quality to implant into patients was realized by researchers in the 1990s and the potential of tissue engineering caught the imagination of scientists, clinicians and the general public.

An overarching principle of tissue engineering is the generation of in vitro environments for a cell population that mimic development, renewal or repair in in vivo environments. The key aspects of such environments are discussed below.

The ECM

Cell anchorage and motility require receptor-mediated interactions with the ECM.[6] Many tissue engineering and 3D cell culture methods employ natural ECM models to replicate integrin receptor-binding mechanisms. Alternatively, synthetic ECM mimics can be formed by the surface engineering of synthetic polymers to create surfaces that present short peptides that can bind to integrins.[7]

Soluble Growth Factors and Cytokines

The availability and concentration of growth factors represent a powerful switch in controlling cell behavior. Numerous growth factors and differentiation factors can be used to stimulate proliferation and differentiation. The simple addition of these factors to cell culture media is effective in many culture systems. However, the delivery of these factors is more sophisticated in vivo. For example, gradients and cascades of factors are used to pattern differentiation in stem cell populations. Furthermore, growth factor effects may be synergistic with ECM signalling.[8]

Biomechanical Conditioning

Many cell types show significant sensitivity to the local biomechanical environment.[9] The role of the biomechanical environment is clear for contractile or structural tissues. For many such tissue types, the restoration of appropriate physical forces has been demonstrated

to enhance tissue formation or maintenance in vitro. In other situations, such as in the liver, the direct role of mechanical forces in controlling cell niche is less clear. For these cases, it may be important to eliminate culture-induced forces (e.g., gravitational effects on cell suspensions). Biomechanical forces can be controlled through the use of bioreactors.

Cell-Cell Signaling

The functioning of many tissues is dependent on complex patterns of signaling between different cell types. The liver provides an excellent example, in which stellate cells and hepatocytes signal extensively through direct physical contacts.[10]

Architecture

The role of architecture in tissue functioning is observed at a number of different length scales. At the cell level, the simple organization of cells into 3D clusters can have a profound effect on the phenotype. This has been demonstrated by the work of Bissell et al.[11] Architectural effects are also evident in the structural components of tissues. For example, within the liver, the architecture of the liver lobule creates a unidirectional flow of blood from the periportal to the centrolobular regions. This flow pattern means that hepatocyte cells close to the periportal triads are exposed to blood that has just passed through the gut and has taken in any food or toxin-related molecules that have been absorbed. Hence, these cells bear the brunt of the liver's metabolic activity. In contrast, cells toward the centrolobular region have a phenotype that results in greater secretory activity. Thus, the architecture of a tissue can impose local fluid flow patterns, soluble molecule gradients, the polarity of cell-cell interactions and the location of cell-to-ECM binding.

REVIEW OF SPECIFIC TISSUE ENGINEERING ADVANCES BY TISSUE

In this section, examples of the application of the principles summarized in Overivew of Tissue Engineering Strategies will be provided.

Skin

The in vitro manufacture of skin is the most advanced area of tissue engineering and is the one example that has impacted on both clinical practice and replacement technologies. The success achieved in skin tissue engineering has been due to the following:

- The ability to expand primary cell cultures isolated from the dermal and epidermal layers.
- The limited thickness of the tissue, which permits good nutrition availability to all the cells without vascularization or sophisticated bioreactor design.
- The aspects of the skin required for some clinical and some replacement technologies are largely related to function as a barrier.
- There is a motivated user base that requires a replacement technology for product development.

Within the replacement field, two tissue engineered skin models have been fully validated and approved by regulators as functionally equivalent to the commonly performed in vivo methods.[12,13] The EpiDerm™ and the EpiSkin™ corrosivity tests can be used to determine the corrosive potential of a chemical by assessing the cytotoxic effect on reconstituted human epidermis, a test conventionally carried out in vivo by using the Draize rabbit skin test.[14] EpiDerm (MatTek, Ashland, MA, USA) comprises a reconstructed epidermis of human keratinocytes grown on inert polycarbonate filters, while EpiSkin (L'Oréal, Clichy, France) consists of a reconstructed epidermis grown on a Type I bovine collagen matrix, representing the dermis, surfaced with a film of Type IV human collagen.[15,16] Keratinocytes grown at the air-liquid interphase differentiate to form a stratified epithelium, which is similar to the stratum corneum of human skin and is suitable for use in an alternative assay system, effectively replacing the conventional in vivo model. Another reconstructed human epidermis product, Skin² ZK 1350™ (Advanced Tissue Sciences Inc., La Jolla, CA, USA), has also been discontinued, while a third, SkinEthic™ (SkinEthik, Nice, France), is of similar nature. The latter shows a similar behavior when subjected to the approved corrosivity protocols, but has not been fully validated nor been approved by regulators.[15,18,19] The European Center for the Validation of Alternative methods (ECVAM) International Validation Study on In Vitro Tests for Acute Skin Irritation has recently reported on the validity of the EpiSkin and EpiDerm assays and on the skin integrity function test.[46]

All of these reconstituted human skin models have similarity in terms of general structure, cellular composition and biochemistry and therefore provide a valuable tool in alternative toxicity testing, phototoxicity testing and drug transport studies. However, most of the current models are epidermis-only models. It is likely that a more-complex model, which includes an underlying dermis, will resemble the appearance of skin and its physiology more closely.

Other epithelia have also been reconstructed in vitro on artificial polymer matrices, as with human epidermis, as outlined above. MatTek has developed buccal (EpiOral™), corneal (EpiOcular™), ectocervico-vaginal (EpiVaginal™), gingival (EpiGingival™) and tracheal/bronchial (EpiAirway™) equivalents, while SkinEthik also offers alveolar, corneal, gingival, esophageal, oral and vaginal tissue equivalents. However, none of these reconstructed epithelia have thus far been used in the development of alternative assay systems for regulatory use.

Liver

There is a clear need for better in vitro models of the human liver. The challenge to engineer the liver is very significant and demonstrates a number of important tissue engineering principles. Within the body, the liver displays a remarkable regenerative capacity. For example, patients can spontaneously recover from major resections involving the removal of up to 80% of the liver mass. However, when hepatocytes are isolated in vitro, they display limited proliferative capacity and rapid changes in gene expression lead to dedifferentiation within hours and days. Hence, the use of human liver tissue in pharmacological and toxicological assays is inefficient and largely limited to very short-term studies involving acute metabolic events rather than chronic tissue-specific events. Therefore, if tissue engineering could restore the in vivo cues that permit liver regeneration and the long-term maintenance of liver functions, it would be possible to devise replacement technologies that increase the availability of in vitro tissue and permit chronic, repeat exposure studies.

A large body of literature, much of which predates the advent of tissue engineering as a defined field, involves investigations on the individual roles of the ECM, heterotypic cell-cell interactions and soluble factor effects. Applying each of these individual strategies can boost one or more liver-specific functions in vitro. However, in isolation, none of them can generate a liver tissue model that could profoundly affect replacement technologies.

The role of tissue engineering is to combine the influences of coculture, ECM and soluble factors and to contribute new ideas in the design of flow systems that mimic blood flow and the resulting chemical gradients within the liver. Excellent examples of this approach have been published by the team of Gerlach et al,[20] who demonstrated that oxygenation via gas-permeable hollow fibers that run through the 3D tissue space enhanced the metabolic activity of hepatocytes. More recently, they have built on their early work on oxygenation systems to evolve a new bioreactor that promotes the reorganization of hepatocytes and nonparenchymal cells into histiotypic structures.[21] This bioreactor system enabled cell metabolic activity to remain constant for at least 20 days. Both bile canaliculi and sinusoid-like structures developed spontaneously and increased proliferative activity was detected within the cell population. Proliferation combined with sustained liver-specific functions offer the potential to increase tissue mass from a primary cell source and thereby to reduce the tissue requirement for each clinical procedure. This type of bioreactor also shows significant potential as an in vitro model for drug metabolism and toxicology studies.[22]

Cima-Griffiths et al have also combined innovative bioreactor design and 3D cell culture. They have described a microarray bioreactor for the maintenance of heterotypic multicellular liver models.[45] The microarray system provides a thin transparent structure, within which a silicon scaffold provides square ports that host the liver cells. Fluid flow within the bioreactor occurs through the ports and hence, partially mimics liver blood flow dynamics. With this system, preformed spheroids were found to out-perform single cell suspensions as the format for the seeding of the bioreactor. When hepatocytes and nonparenchymal cells were formed into spheroids before addition to the bioreactor, histiotypic structures formed and albumin secretion was better maintained that when the same cell types were added directly, without the preformation of the spheroids.

Finally, Bhatia's group have recreated zonal patterns of hepatocyte function by controlling oxygen gradients within cocultures of hepatocytes and nonparenchymal cells.[23] Mathematical models of the oxygen gradients within the liver provided targets to be recreated in vitro. This team used a syringe pump to control the introduction of oxygenated medium at one end of flat-bed bioreactor. By culturing hepatocytes within this bioreactor, the in vivo-like zonation of expression of two key drug metabolizing enzymes was created. Furthermore, drug toxicity matched the zonation of the metabolizing enzymes.

Nerves

Tissue engineering approaches to enhance nerve regeneration provide examples of how molecular gradients and micron-scale patterns can be used to guide 2D and 3D pattern formation in tissues. Control over cell position within a regenerating tissue can be exerted by using location-specific ECM interactions or by using gradients of growth factors or trophic factors.

Patel et al[24] have demonstrated the ability to guide neurite extension by using peptides that mimic key ECM molecules. This provides an example of the guidance of neurites using a peptide containing the laminin-specific sequence, IKVAV. This peptide

was patterned on to a biodegradable polymer substrate by using microfluidic patterns to form stripes with widths of 12-70 μm and lengths of many millimeters. The PC12 cell line can respond to laminin surface cues by extending neurite-like projections and these projections follow the biochemical pattern.

Moore et al[25] have used the concept of patterning with a 3D scaffold with macroporous scaffolds and gradients of nerve growth factor (NGF) and neurotrophin-3 (NT-3). Gradients can be easily manufactured via the diffusion-limited mixing of the NGF/NT-3 with the polymer components that make up the scaffold, immediately prior to scaffold solidification. It was found that a NGF gradient of 310 ng/mL/mm was required to stimulate the guidance of neurite extension of chick dorsal root ganglion explants. However, this minimum concentration gradient was lowered if a graft of both NGF (200 ng/mL/mm) and NT-3 (200 ng/mL/mm) was prepared. This study provides an elegant demonstration of the ability to use synergistic effects between factors to elicit tissue-specific responses.

Cardiovascular Tissue

There are two major clinical drivers for cardiovascular tissue engineering, the regeneration of cardiac muscle to provide tissue repair after an infarction and the manufacture of small vessel conduits for vascular surgery.

The ability to regenerate cardiac muscle presents a major challenge, due to the lack of spontaneous repair or cardiomyocyte proliferation within the heart following ischemic damage. Clinical trials are under way to assess the therapeutic potential of cell therapies in which a cell population is injected in the heart wall. However, an alternative approach, whereby cardiac muscle is engineered in vitro, then surgically attached to the heart, offers greater potential in the search for tissue engineered models that could be used in toxicology.

The work of Radisic et al[26] provides a recent example of an approach to combining mechanical and biochemical cues to enhance the formation of cardiac muscle within scaffolds. A coculture of myocytes and cardiac fibroblasts was formed within an elastic porous scaffold. The scaffold was designed with an array of parallel channels running through its length, to promote fluid flow. This cell/scaffold construct was then transferred into a bioreactor, in which a novel culture medium flowed. The culture medium contained a perfluorocarbon (PFC) emulsion that carried high concentrations of oxygen (mimicking the role of hemoglobulin in the blood). Both the presence of the channels within the scaffold and the PFC carrying oxygen significantly improved the levels of cardiac muscle-specific markers such as troponin I and connexin-43. If this approach is combined with the current rapid progress in the derivation of cardiomyocytes from embryonic stem cells, and in vitro models of cardiac muscle, in which scale-up of manufacturing and scale-down of the size of the tissue under investigation, can be achieved.

Tissue engineered blood vessels (TEBV) are under intensive investigation as antithrombogenic and mechanically stable vessels that could be implanted into patients. L'Hereux et al[27] have formed layered tubular constructs of living adventitia, a decellularized internal membrane and an endothelium. TEBVs with internal diameters of 4.2 mm and wall thicknesses of approximately 409 μm displayed broadly similar burst pressures and compliance to saphenous veins.

Skeletal Muscle

Recent progress in the engineering of skeletal muscle has addressed a major shortcoming in many in vitro tissue engineering approaches, that is, the need for vascularization in order to generate thick tissues. Levenberg et al[28] have achieved early stages of in vitro vascularization using a 3D multicell construct. They combined myoblasts, embryonic fibroblasts and endothelial cells within porous scaffolds. The embryonic fibroblasts increased the levels of expression of vascular endothelial growth factor (VEGF) within the system and promoted the formation and stabilization of vessel networks. The myoblasts formed muscle tissue around the developing network of tubular endothelial cell constructs.

Gastrointestinal Tissue

Intestinal tissue engineering has, as yet, not been extensively studied and currently is limited to in vivo strategies where the body is used as the bioreactor.[29] The intestinal epithelium is a complex tissue, which lines the gastrointestinal tract and provides an interface between the contents of the gut lumen and the internal regions of the body. The epithelium is folded, so that it has a well-defined architecture of cavities, known as the crypts of Lieberkühn. Adult stem cells reside at the bases of these crypts and divide and migrate both up and down (in the case of the small intestine) the crypt, whilst differentiating to replenish the supply of mature functional cells. The differentiated cells perform their specific functions, undergo apoptosis and slough off into the lumen, having a total life-span of 5-7 days. The intestinal epithelium is an example of a rapidly renewing tissue, but the in vitro tissue engineering of this organ has so far eluded researchers, due to the difficulties associated with establishing a suitable cell source for this purpose; it is very difficult to culture primary intestinal progenitors in vitro.

Despite this, encouraging reports on oesophageal,[30,31] small bowel,[32] colonic[33] and stomach[33,34] tissue engineering have been published. All of these regeneration programs have been based on two common themes: (1) a material scaffold that is placed into an animal model following anastomosis (the removal of a section of the intestinal tube); or (2) epithelial organoids (mesenchymal cell cores surrounded by a polarized epithelium) that are obtained from a section of intestinal tissue and are seeded into a material scaffold before being transplanted into the animal. Such material scaffolds have included biodegradable poly(glycolic acid) (PGA) meshes, coated either with collagen or with poly(lactic acid) (PLA) and porcine small intestinal submucosa (SIS). All the seeded scaffolds were implanted in vivo, either into a region of the gastrointestinal tract of athymic mice following anastomosis or into the latissimus dorsi muscle of athymic mice. In all cases, post-implantation analysis demonstrated the presence of a functional and structural gastrointestinal epithelium, but which lacked the muscle component of the bowel. However, this research does demonstrate the feasibility of engineering a complex organ such as the gastrointestinal tract.

The Cornea

Diseases affecting the cornea and corneal trauma are a major cause of blindness worldwide.[35] For partial thickness defects, where only the surface of the cornea is affected, a novel treatment developed by the Okana group[36] involves “carrier free

cultivated corneal epithelial sheet transplantation". These bioengineered cell sheets are achieved by cultivating corneal limbal stem cells on temperature-sensitive culture dishes coated with the thermoresponsive polymer, poly-N-isopropylacrylamide (PIPAAm). Following culture, the stratified epithelial layers are simply released from the dish by the reduction of the temperature to the polymer's lower critical solution temperature (LCST) of 32°C.[37] Initial human trials revealed that the bio-engineered corneal epithelial cell sheet grafts remained intact and covered the entire cornea. Moreover, corneal transparency and visual acuity were restored. It is thought that by maintaining cell-cell interactions within the epithelial cell layer and between the epithelium and the underlying stroma, the long-term survival of the cultivated epithelial sheet is enhanced due to the maintenance of cell surface proteins and the ECM which is deposited. This technology has since been extended to the generation of corneal endothelial cell sheets, as a possible treatment for many pathological cases where the corneal endothelial layer is the only component requiring substitution.

The shortage of human corneal donors and the risk of immunorejection for the replacement of full thickness defects have been the driving force behind the generation of tissue-engineered corneal constructs for transplantation. Functional corneal reconstruction has largely focused on the culture of the three main layers of the cornea (epithelium, stroma and endothelium). The initial steps involve the isolation, culture and expansion of each cell type in vitro. The stroma is produced by mixing corneal keratocytes with a scaffolding material, usually collagen Type I and/or Type III, or a composite with glycosaminoglycans, which is subsequently remodeled by the keratocytes in the culture. The final stage involves culturing limbal epithelial cells (thought to include limbal stem cells) on the engineered stroma at an air-liquid interface.[38-41] These tissue engineered constructs have been shown to support neurite extension,[42] and to perform well when transplanted into an in vivo model.[43]

Airway Epithelium

A human airway epithelial wounding model have been developed by Wadsworth et al.[44] It has been used to study the pharmacological mechanism of the beneficial effects of glucocorticoids in the treatment of asthma. The model was constructed by using human bronchial epithelial cells (HBEC), which were cultured at an air-liquid interface and triggered to differentiate into the mucociliated phenotype. This model could be used to study repeated physical wounding and, hence, to study chronic mechanisms of drug action.

CONCLUSION

Tissue engineering has been a priority area for research across the world for the last decade and it is likely that it will remain a priority for many years to come. Scientifically, the field has advanced at a rapid rate and this chapter has highlighted the fact that many studies now involve the combined use of a number of engineering tools to recreate complex regenerative or repair environments.

Whilst the clinical and commercial impact of tissue engineering has been significant, there have been few examples of contributions toward the design of replacement technologies. The scientific case for using tissue engineering in replacement science is compelling since both fields require in vitro systems that accurately represent key

aspects of the structures, functions and reactions of tissues in vivo. One area of success has been the use of skin models, where the progress made in the use of skin models as replacement alternatives is possibly due to the relatively simple conditions required to create models that mimic the barrier properties in this relatively thin organ.

Looking to the future, there are a number of reasons for optimism about collaboration between tissue engineers and replacement alternative scientists. In the vast majority of cases, the clinical and commercial uptake of tissue-engineered products has been impeded by the complexity of the products and their manufacturing processes. For example, the first generation of tissue-engineered skin products has been clinically successful in terms of trial results, but disappointing in terms of commercial returns. Applying tissue engineering to tackle replacement offers a route to the early uptake of the new technology as the critical path to launching a successful replacement technology appears to be less arduous, although by no means simpler, than that involved in launching a living clinical product.

However, there are major barriers to the application of tissue engineering in the search for replacements. One barrier is the lack of communication between tissue engineers and replacement scientists. The clinical focus of tissue engineers does not provide many spontaneous opportunities for cross-talk between the communities. Organizations such as the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs), the Fund for Replacement of Animals in Medical Experiments (FRAME) and the Dr. Hadwen Trust, are promoting collaboration and discussion. Related to this problem, it can be difficult for tissue engineers, who lack a long background in replacement science, to identify the important replacement problems that their ideas can address.

Finally, there are at least two grounds for confidence that tissue engineering can make a major contribution to the future of replacement. Firstly, as shown in this chapter, the past few years have seen major advances in the engineering of complex tissues with embedded, albeit primitive, vascular networks and gradients of growth factors and oxygen, that begin to mimic a degree of the complexity of regenerating and developing tissues. Secondly, the rapid pace of development in stem cell technologies is generating populations of human cells that can be expanded to permit the scaling-up of in vitro models and that can be induced to differentiate and form most, if not all, the tissue types within the body.

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