Supplement Review

Tissue engineering: chondrocytes and cartilage

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Chapter summary

Tissue engineering offers new strategies for developing treatments for the repair and regeneration of damaged and diseased tissues. These treatments, using living cells, will exploit new developments in understanding the principles in cell biology that control and direct cell function. Arthritic diseases that affect so many people and have a major impact on the quality of life provide an important target for tissue engineering. Initial approaches are in cartilage repair; in our own programme we are elucidating the signals required by chondrocytes to promote new matrix assembly. These principles will extend to other tissues of the musculoskeletal system, including the repair of bone, ligament and tendon.

Keywords: extracellular matrix, joint disease, osteoarthritis, regenerative medicine

Introduction

Tissue engineering is a new development and in this review we set out some of the principles that underlie it. The potential applications of tissue engineering in medicine are diverse, and as a basis for new treatments for musculoskeletal diseases it is likely to have a major impact in rheumatology and orthopaedics. The progress in research in tissue engineering has been rapid, but it is acknowledged that more basic research is necessary to develop its full potential. This requires the formation and close collaboration of interdisciplinary research teams. One of the focus areas of tissue engineering in our research programme is in cartilage replacement and we summarise some of the strategies being pursued in this area in the newly formed UK Centre for Tissue Engineering in the Universities of Manchester and Liverpool [1].

The principles of tissue engineering

Tissue engineering has emerged through a combination of many developments in biology, material science, engineering, manufacturing and medicine. The strategies developed in tissue engineering involve a range of approaches, the key element of which is the use of biologically based mechanisms to achieve the repair and healing of damaged and diseased tissues. This application distinguishes it from the use of medical devices, and the delivery of an assembled 'tissue equivalent' distinguishes it from a pharmaceutical product. Tissue engineering addresses the problem caused by many injuries and disease processes that result in physically damaged tissues and organs in our bodies which, if left unattended, repair imperfectly or not at all. It is the intervention with an engineered tissue that presents the prospect of achieving successful repair where it would not otherwise occur.

Tissue engineering is part of new wave of developments in biomedicine in which our scientific understanding of how living cells function will enable us to gain control and direct their activity to promote the repair of damaged and diseased tissues. The potential for medical intervention with a tissue engineering solution is seen nowhere better than with the chronic, persistent leg ulcer, which, in a

A glossary of specialist terms used in this chapter appears at the end of the text section.

patient with diabetes, provides a constant source of discomfort and incapacity. The patient does not lack the inherent capacity to heal a skin wound, but healing is failing to occur naturally at the site of the ulcer. What are lacking are the biological signals, chemical messengers and physical cues that initiate the events of cell migration, blood vessel formation and tissue assembly for normal wound healing. If we can provide these biological signals in a 'tissue engineered' package, we can kick-start a repair process that can be completed by the patient's own tissues.

How can this be achieved? What forms the tissue engineered package? The precise form will vary with the medical application for which it is designed. There are, however, two typical elements: one or more type(s) of living cell with particular tissue functions; and a material support that forms a structure for both culturing the cells in the laboratory and the surgical delivery of the tissue equivalent to the patient. This support might be in the form of a lamella or tubular structure, or in a more complex three-dimensional structure, depending on the clinical application. The 'package' therefore contains several important, and quite different, material components and its assembly involves a manufacturing process that extends from the culture of living cells to the fabrication techniques for 3-dimensional structures, involving engineering and bioreactor design. It also has to be carried out in a regulatory framework that will ensure the monitoring and documenting of all stages of the process. Tissue engineering thus requires the coordination of a range of different disciplines and its development depends on bringing together broadly based research teams to form interdisciplinary collaborations, such as within our research programme at UK Centre for Tissue Engineering, Universities of Manchester and Liverpool [1]. Input is required from cell biologists, molecular biologists, biomaterial scientists, bioengineers and healthcare physicians.

Post genomic research and stem cells

A key to new developments in tissue engineering lies in the current progress in research on living cells. Sequencing the human genome is close to completion and it is one of the inevitable benefits of the post genomic age that a more complete knowledge of the biological signals and cues that trigger natural repair processes will be discovered. It is this knowledge that will be exploited in tissue engineering applications. So, the strategies behind current developments in tissue engineering depend heavily on living cells and tissues, and on our abilities to control their function. The theme that is particularly emphasised in our programmes is that, in many clinical applications, the planned long-term solution will engage the patient's own cells and tissues in the process to complete a biological repair. This is where it differs from the established use of medical devices that have been very successful in some applications, such as joint replacements, but in which the damaged tissues are replaced with inert nonbiological materials. The applications of tissue engineering will be more diverse than has been possible with the nonbiological devices. These will range from small blood vessel replacement, repair of bone, tendon, ligament and cartilage, healing of skin wounds, nerve regeneration and the repair of problems causing incontinence, to a range of applications in reconstructive surgery. It may also extend to cellular based therapies for degenerative problems in muscle, heart and brain.

To help fuel these aspirations, there are remarkable new developments in stem cell research, which show that we all contain sources of cells that retain the capacity to form the different tissues in our bodies [2]. In addition to embryonic stem cells, which in early embryonic development have all the potential to divide and form all the tissues of the body, it is now recognized that, even as adults, we all contain some cells that retain the ability to form different tissues. It has long been known that these cells can be found in bone marrow (stromal stem cells) but, more recently, evidence has suggested that they can be found in other sites in the body and can even, for example, be harvested from the fat removed at liposuction! There is great interest in these sources of cells for tissue engineering applications, although much research is needed before their full potential (and limitations) will be known.

Research on the biomaterials associated with tissue engineering was initiated ahead of the developments in cell biology. There is currently much research and innovation in the development of new biomaterials. These extend from well-tried polylactates and polyglycolates to novel ceramics, caprolactones and hydrogels. Techniques are being developed for their formation into scaffolds, felts and weaves. Matrix printing devices are being explored for the fabrication of three-dimensional structures with microarchitecture that might mimic complex living organs, such as liver or kidney tissues. Different applications require different material properties to cope both with the required tensile and compressive forces and with elastic deformation and compliance. There is also much innovation occurring in polymer chemistry, but each new polymer or biomaterial requires extensive evaluation to assess how it interacts with living tissue, how long it survives in the body, what wear-products it produces and how it degrades. The ability to generate new chemistry currently far outpaces the speed at which their biological advantages and disadvantages can be thoroughly assessed.

There is also considerable interest in the use of natural biopolymers for tissue engineering applications, such as the long chain polysaccharide hyaluronan and its chemically derivatised forms, and various preparations of natural and recombinant proteins, including collagens and even

silk. Many of these materials provide the opportunity for chemically linking biological signaling molecules such as peptides or small proteins, to provide sources of the signaling molecules that will trigger cell responses to help the healing process. For example, these 'smart' materials could be used to provide signals to encourage blood vessel development. When an engineered tissue is placed in the body, it requires the development of a blood supply from the patient for it to become integrated with surrounding tissues; this is essential for the completion of the healing process. The principle exceptions are cartilage, intervertebral disc and cornea, which are largely avascular tissues.

Strategies for articular cartilage repair

Articular cartilage provides its own particular challenges for tissue engineering. Its structure appears simple and it only contains one cell type; however it is has a complex highly organised extracellular matrix (ECM). Articular cartilage is frequently damaged as a result of trauma and degenerative joint diseases that may be driven by changes in biomechanics, cytokines, growth factors and cellular responses [3–6]. Cartilage has no blood vessels, it is not innervated and normal mechanisms of tissue repair, involving the recruitment of cells to the site of damage, do not occur. The challenge for cartilage tissue engineering is to produce cartilage tissue with suitable structure and properties *ex vivo*, which can be implanted into joints to provide a natural repair that with time will become integrated with the patient's tissues.

The physical properties of articular cartilage depend on the structure and organization of the macromolecules in the ECM. They can largely be understood in terms of the contribution made by fibrillar and nonfibrillar components [5]. The structure of collagen gives it impressive tensile properties, which are utilized in a special way in the predominant type II collagen, found in cartilage, to produce a tissue that is not only strong in tension but also resistant to compression. This is achieved by filling the interfibrillar matrix with a very high content of proteoglycan, primarily aggrecan [7,8], which draws water into the tissue as it creates a large osmotic swelling pressure. The osmotic pressure caused by the negatively charged groups on aggrecan create a large difference in the concentration of ions inside the cartilage compared with outside. Water is drawn into the tissue as a result of this osmotic imbalance and, because aggrecan is assembled into large supramolecular aggregates [9], it is too large and immobile to redistribute itself. The water thus swells and expands the aggrecan-rich matrix. This places the collagen network under tension and an equilibrium is achieved when tension in the collagen network balances the swelling pressure. This confers the tissue with compressive resilience and, as aggrecan offers great resistance to any fluid flow, the tissue behaves as a stiff elastic polymer to sudden impact loading, but shows some slow inelastic deformation with sustained loads. The articular cartilage thus forms a tough but compliant load-bearing surface and these characteristics depend on the integrity of the collagen network and on the retention within it of a high concentration of aggrecan. Part of the challenge of tissue engineering cartilage is thus to provide the essential cells and signals that will establish a cartilage ECM and recapitulate this molecular organisation that forms the basis for the essential mechanical properties of the tissue.

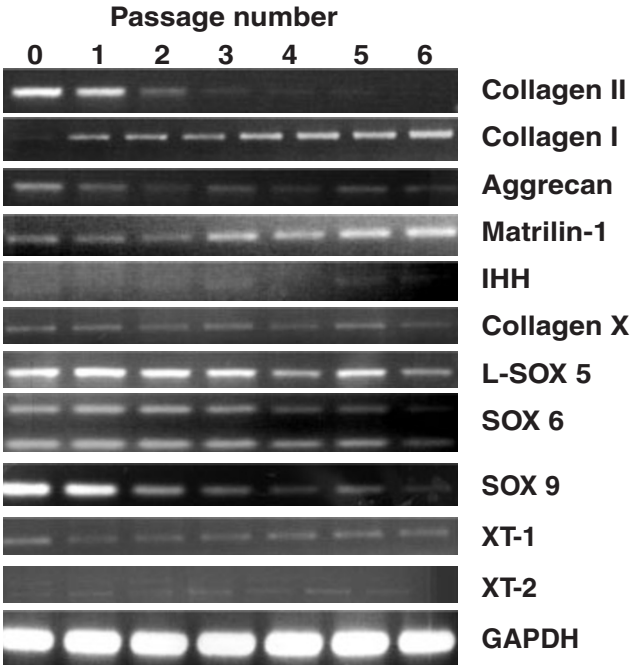
Our tissue engineering approaches to cartilage repair are focussing on developing efficient methods to form cartilage with chondrocytes in culture. This approach depends on the availability of suitably differentiated chondrocytes to produce and maintain the specialised ECM of the tissue. These cells may be obtained through culturing primary autologous/heterologous chondrocytes [10], mesenchymal stem cells [11] and embryonic stem cells [12]. An important factor is the production of chondrocytes in sufficient numbers to form tissue constructs of an appropriate size. In the case of autologous chondrocytes, the expansion of the cell population can be carried out in monolayer culture conditions, although during this process chondrocytes become fibroblastic and lose their characteristic pattern of matrix protein expression [13]. Our initial studies are focussing on human articular chondrocytes, their expansion in monolayer culture under differing conditions and their subsequent potential to re-express a full matrix-producing phenotype.

Gene expression in chondrocytes passaged in monolayer culture

Human articular cartilage was obtained with informed consent from knee replacement operations. The cartilage from regions of the joints with intact tissue were dissected from the underlying bone, chopped finely and digested, first in trypsin for one hour, and subsequently overnight, in 0.08% bacterial collagenase in medium containing 10% foetal bovine serum. Both digestions were at 37°C with constant agitation. Washed, filtered cells were cultured as monolayers in DMEM, containing penicillin/streptomycin and 10% foetal bovine serum. The cells were split at a 1:2 ratio at confluence.

RNA was isolated from chondrocytes at various passages, to build up an initial profile of the changes in gene expression during cell expansion. The level of mRNA expression of a number of ECM markers (including chondrocyte-specific ECM genes [collagen II and aggrecan] and a fibroblast ECM gene [collagen I]) was assessed using RT-PCR. In addition, the expression of transcription factors important to chondrogenesis (SOX9, L-SOX5 and SOX6 [13,14]), of developmentally regulated cartilage genes associated with the further differentiation and hypertrophy of chondrocytes (matrilin-1, indian hedgehog and collagen X) and of other genes (such as the recently cloned xylosyl-

Figure 1

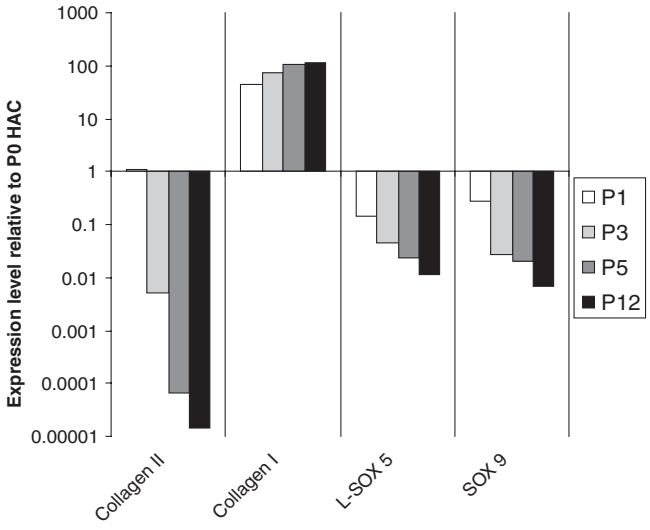


The changes in expression of genes in human articular cartilage chondrocytes with increasing passage in culture, detected using RT-PCR. Passage 0 refers to cells that are freshly isolated from the tissue and have not been cultured. GAPDH, glyceraldehyde phosphate dehydrogenase; IHH, indian hedgehog; XT, xylosyltransferase.

transferase isoforms [15]) was examined. Probes for these different genes have been designed and tested in the development of specific and sensitive methods to follow changes in gene expression. The changes in expression of these genes in chondrocytes with increasing passage in culture are shown in Fig. 1.

Expression of chondrocyte-specific genes, such as collagen II and aggrecan, decreased with time in culture, whilst collagen I expression increased, as previously reported [16]. Developmentally regulated gene transcripts characteristic of chondrocyte hypertrophy were not expected to be found in significant quantities in mature articular cartilage. Only very low expression of collagen X and indian hedgehog was detected at any stage of monolayer culture. Matrilin-1, however, was detected and its expression tended to increase with culture time. Matrilin-1 is expressed in other nonarticular cartilages and the significance of the expression in articular chondrocytes is currently unknown. Xylosyltransferase expression was examined as a possible indicator of the glycosaminoglycan synthesis of the cells. Two human isoforms have been cloned, but the expression level of both was low and remained the same throughout the culture period.

Figure 2



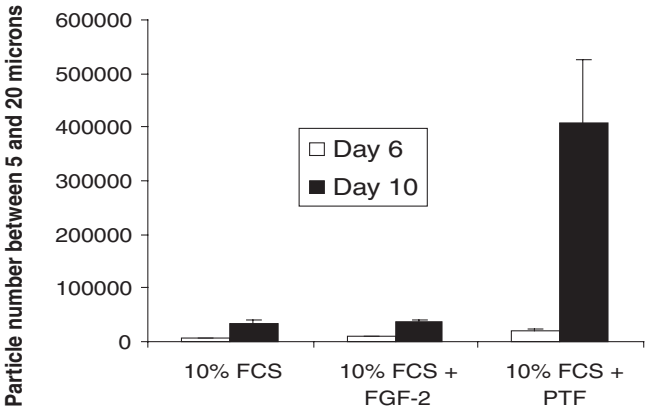
Real time RT-PCR analysis of RNA from human articular cartilage chondrocytes at different passage in culture. The values shown are expression levels of the indicated genes relative to the expression level found in passage (P) 0 cells that are freshly isolated from the tissue.

These initial studies have been extended by using real time, quantitative RT-PCR for the analysis of gene expression levels. This has major advantages over normal RT-PCR as it provides an accurate quantitative assay of gene expression. Polymerase chain reactions have been conducted using an Applied Biosystems 7700 and the amplified product was detected using the fluorescent DNA binding dye SYBR Green. This technique has enabled us to gain accurate data representing changing expression levels between cultured chondrocytes and those freshly isolated from the tissue. Results (Fig. 2) show that the upregulation of collagen I is rapid following the isolation of chondrocytes and their transfer into monolayer culture, and it precedes the downregulation of collagen II. Downregulation of the transcription factors SOX9 and L-SOX5 also precedes changes in collagen II expression. It is also clear that these changes in gene expression are largely complete by passage 5, with little difference between this stage and further culture up to passage 12.

Rates of chondrocyte proliferation

The effects of selected growth factors have also been investigated on the chondrocytes cultured in 10% serum. Published studies, screening an extensive number of growth factors and media supplements [16], have reported that human chondrocytes proliferate most rapidly in medium supplemented with 10% foetal calf serum, platelet-derived growth factor-BB, fibroblast growth factor-2 (FGF-2) and transforming growth factor β-1. When medium containing these supplements was added to human articular cartilage cells at passage 4, they began

Figure 3



Proliferation of passage 1 human articular cartilage cells grown on tissue culture plastic (24-well plate) in different culture media. FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; PTF, medium containing 10% serum, 5 ng/ml FGF-2, 10 ng/ml platelet-derived growth factor-BB and 1ng/ml transforming growth factor β-1.

to divide far more rapidly than parallel cultures in just 10% serum. The same was evident in these cells cultured in the supplemented medium from the beginning of the first passage. Cells seeded in 24-well plates at 2000 cells/well were counted in medium containing 10% serum, 10% serum + 5 ng/ml FGF-2 or 10% serum, 5 ng/ml fibroblast growth factor-2 (FGF-2), 10 ng/ml platelet derived growth factor-BB and 1ng/ml transforming growth factor β-1 ('PTF media') after 6 and 10 days in culture (Fig. 3). At 6 days, the number of cells in PTF-treated wells was twice that of those in either the control or FGF-2-treated wells, whilst by ten days the difference was ten-fold. This growth factor combination was extremely effective in achieving a rapid expansion of human chondrocytes in culture.

Re-expression of chondrogenic phenotype

A further aim of this project is to quantify the extent to which chondrocytes can re-express a chondrocyte phenotype once placed in a three-dimensional culture environment. The SOX transcription factors, particularly SOX9, appear to be sensitive indicators of the differentiation state of the cell and the expression levels of these genes, as well as those of collagen I, II and aggrecan, will be compared with those of freshly isolated cells. It will be important to determine if the rapid expansion of cell numbers in monolayer culture is detrimental or beneficial to their chondrogenic potential. We have developed sensitive methods of assessing the matrix assembly around chondrocytes, based on determining by confocal fluorescence recovery after photobleaching (confocal-FRAP) the translational diffusion of fluorescent tracer molecules of defined size [17–21]. This will be used to optimize conditions for matrix assembly and develop neocartilage constructs that can form the basis for a tissue engineered product.

Future prospects

Research in tissue engineering is expanding fast worldwide [22], and new UK [23], European [24], Japanese [25] and International [26] societies have begun to provide a forum for worldwide developments. Current advances in cell biology arising in post genomic research will have a major impact on tissue engineering programmes. This will be complemented by the development of novel biomaterials and fabrication methods that will aid the design and effective delivery of tissue engineering treatments to the patient. It is likely that tissue engineering products will become commonplace within 20 years, but it is important to perform a full cost/benefit analysis for such treatments if they are to be funded and fully exploited within the tough constraints of healthcare budgets, such as those in the UK.

Concluding remarks

The application of tissue engineering provides new possibilities for therapeutic intervention and will extend what can be done for individuals with many chronic conditions such as arthritis. A greater focus on strong basic research is now helping to convert some of the past hype in tissue engineering into practical applications.

Glossary of terms

Confocal-FRAP = a technique using the analysis of fluorescent recovery after photobleaching with a confocal microscope to measure self-diffusion and tracer diffusion in polymer networks, such as those found in tissue extracellular matrix; ECM = extracellular matrix; PTF medium = medium containing 10% serum, 5ng/ml FGF-2, 10ng/ml platelet-derived growth factor-BB and 1ng/ml transforming growth factor β-1; SOX genes = (from 'Sry-type high-mobility-group-box') a family of genes that encode for transcription factors important during tissue development; these genes all contain a Sry-type high-mobility-group box, which is a protein motif involved in DNA binding; SOX9, L-SOX5 and SOX6 are expressed in chondrocytes.

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