New Horizon Workshop: Scaffolds, Bioreactors and Biologics in Orthopaedic Research

(Organized by the Tissue Engineering and Regenerative Medicine International Society (TERMIS) and ORS)

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Bioreactors for musculoskeletal research

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Abstract

Musculoskeletal research has made great advances over a number of years. The advent of molecular biological techniques has allowed for detailed analysis and understanding of complex pathways. Within the musculoskeletal system it is clear that the mechanical forces experienced by cells contained within the tissue have a major influence on tissue homeostasis, pathogenesis and regeneration. These forces are often lacking during in vitro or ex vivo culture and yet the interplay between mechanical forces and other exogenous signals is likely to result in novel or synergistic effects. This has led to the development of various bioreactors that are able to apply one or more stimuli to the cultured tissue in order to further investigate these combined effects. Within this presentation the influence of mechanical stimulation on the differentiation and maturation of musculoskeletal tissues will be highlighted. The focus will be on cartilage and the behaviour of chondroprogenitors and chondrocytes, however, bone and intervertebral disc will also be discussed.

Introduction

During kinematic motion cells experience a number of different mechanical stimuli, hydrostatic pressure, tension, shear, compression or a combination of these. Maintenance of tissue homeostasis requires a basal level of stimulation to maintain the health of the tissue, with long term unloaded conditions such as bed rest known to have a detrimental effect. Regenerative medicine studies are frequently performed in the absence of these stimuli potentially making clinically relevant conclusions difficult. Incorporation of a bioreactor system into the study allows the composite effect of physical and soluble stimuli to be established. In vitro studies frequently rely on the application of exogenous growth factors. While this is a highly successful approach for mechanistic studies, it does not investigate the endogenous source of these factors during normal healing.

Bone marrow derived mesenchymal stem cells (BMSCs) are frequently used as a source material for cell based cartilage repair strategies. Whereas the articulating joint provides a unique, multiaxial load environment, in vitro studies are classically performed under static conditions, or using uniaxial load alone. Using a complex, multiaxial load bioreactor (Figure 1), we have demonstrated that superficial shear, superimposed over uniaxial load, can provide a chondrogenic signal in the absence of exogenous growth factors, namely TGF-β (Kupcsik et al., 2010; Li et al., 2010). This response is due to an increase in the production of endogenous TGF-β by the mechanically stimulated cells. Crucially, shear is major driver of the response observed (Schatti et al., 2011).

Using this device, we have also demonstrated that asymmetrical seeding of the construct, with a greater percent of the total cells being deposited in the superficial zone, leads to increased cartilage matrix deposition when using the same number of total cells (Gardner et al., 2016c). Deposition of both glycosaminoglycan and collagen II are increased in asymmetrically seeded scaffolds when compared to homogenously seeded scaffolds. This induced anisotropy is an interesting example of naturally induced changes induced by physical loads. The increase in endogenous TGFβ leads to an
increase in the latent form of the protein. Multiaxial load alone is capable of activating latent TGFβ by removing the non-covalently bound latency associated peptide, a critical step in the functional activity of endogenous TGFβ (Gardner et al., 2016b). This is possible even in the absence of cells. Such results are clinically relevant, and yet they could not be obtained under static conditions. This provides a new insight into mechanically induced chondrogenesis and offers an experimental test bed for clinical therapies. It allows for the identification of novel markers and clinically relevant targets that are only modified during articulation (Gardner et al., 2016a). In addition, the therapy in its entirety, including the effect of the rehabilitation protocol, can be investigated using human derived cells. This is leading to a new field of regenerative rehabilitation (Perez-Terzic and Childers, 2014).

The use of human cells and a more physiologically relevant loading environment, leads to more clinically relevant studies being performed which should increase the potential translation into the clinic.

Figure 1. Articular motion is a combination of complex multiaxial load (Left). This can be mimicked using a ceramic hip ball bioreactor system (middle) that is able to recapitulate the articulating motion (right).

References


Tissue engineering tools for orthopedic applications: Gene Therapy

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Abstract

Gene transfer offers unique capabilities to regenerative medicine by delivering morphogenetic stimuli to sites of tissue damage in a local and sustained fashion. In its simplest form, the gene (usually a cDNA) encodes a product that stimulates cell division, cell migration, differentiation or matrix production. Unlike their recombinant counterparts, proteins synthesized under these conditions are likely to have undergone authentic post-translational modification and, being nascent proteins, may have higher specific activity. Gene transfer is also an advantage when delivering gene products with intra-cellular sites of action, such as species of non-coding RNA and transcription factors. The use of inducible promoters or other molecular control mechanisms offers opportunities for regulated gene expression. Genes can be delivered by *ex vivo* or *in vivo* means using viral or non-viral vectors. These principles have been applied successfully in animal models of cartilage repair, the healing of bone, tendon, ligament, muscle and intervertebral disk degeneration. Clinical translation is complicated by issues of safety, cost and long time lines. However, one cartilage repair protocol has proceeded to a human clinical trial and a related product for the gene therapy of osteoarthritis is under review for a biologics license application filed with the Korean FDA.

Introduction

Among the challenges confronting the field of tissue engineering and regenerative medicine (TERM) is the need to deliver morphogens and other such factors locally, in a sustained fashion, to sites of healing. The use of scaffolds for this purpose has been widely explored, with varying success; gene transfer offers an alternative approach\(^1\). The concept is quite straightforward. Genes of interest or, more usually, their cDNA equivalents are delivered to cells within the defect. Genes are transferred to cells by means of vectors, which may be derived from viruses or be of non-viral origin. Unlike the treatment of genetic disease that require life-time transgene expression, or periodic readministration, TERM will probably only need transgene expression for a limited time, which is less demanding in terms of vector properties. Vectors may be introduced directly into sites of tissue damage (*in vivo* delivery), or introduced into cells outside the body for later implantation of the genetically modified cells (*ex vivo* delivery). Both strategies have been explored experimentally in orthopaedic contexts. In some *in vivo* protocols, vectors are associated with matrices to form a “gene activated matrix” (GAM) prior to implantation. Although most experimental studies use strong constitutive promoters to drive transgene expression, the use of inducible promoters offers possibilities for regulating the level of gene expression and its timing. It is also possible to deliver multiple genes and express them at different times, although this has not been explored in the present context. *Ex vivo* gene delivery raises the question of which cells to use. Although differentiated cells of the target tissue can be used for this purpose, there is an increasing tendency to use progenitor cells. However, it remains unclear whether they remain at the defect site and form new tissue directly, or whether they serve as transient sources of gene product and then disappear.
Cartilage Repair
Two general strategies have emerged. One of these uses standard *ex vivo* gene delivery with genetically modified chondrocytes or, more commonly, chondroprogenitor cells that are implanted into defects in cartilage. A variety of transgenes have been explored, including those encoding FGF-2, IGF-1, BMP-7, TGF-β and the transcription factor Sox9. Adenovirus and, more recently, adeno-associated virus (AAV) have been the most commonly used vectors. A second approach seeks to improve the clinical outcome when using microfracture. To this end, Madry and Cucchiarini are developing technologies where AAV vectors are applied directly to marrow as it emerges after penetration of the sub-chondral plate. In a related strategy, marrow is recovered and mixed with vector as it clots. The resulting “gene plug” is then press fit into full-thickness defects. This approach has shown promise in rabbit models using adenovirus vectors encoding BMP-2 and Indian Hedgehog. It has also been evaluated in chondral defects in sheep. Genetically modified, allograft chondrocytes persist within defects in rabbit cartilage after implantation and a clinical trial using allograft human chondrocytes that express high levels of TGF-β has been successfully completed (ClinicalTrials.gov Identifier: NCT01825811).

Bone Healing
Using gene transfer to enhance bone healing has been the subject of considerable research (reviewed in reference) that has utilized most of the strategies available to the field. Considerable progress has been made with the approach of Lieberman’s group using the *ex vivo* viral transduction of cells with a BMP-2 cDNA. Initially they used adenovirus to transduce bone marrow stromal cells. This approach, however, is time-consuming and would be expensive clinically because of the need to cultivate autologous cells under GMP conditions. To expedite matters, their more recent work uses lentivirus to transduce freshly isolated marrow buffy coat cells. Because of safety concerns with lentivirus, a “suicide gene” can also be included to allow deletion of the genetically modified cells if necessary. Another expedited approach seeks to transduce freshly harvested biopsies of muscle, fat or marrow and then to immediately implant the genetically modified tissue into osseous lesions. Results in rat models are promising, but data from large animals are lacking. A different approach, which is an adaptation of the GAM strategy, coats allograft bone with AAV vectors encoding osteogenic products. This produces “allograft revitalization”, leading to remodeling of the implant and its replacement with newly synthesized host bone. So far, this has only been demonstrated in mice. There have, however, been a number of studies using a variety of other approaches in large animal models that show promising results.

Tendon, Ligament, Muscle and Intervertebral Disk
Repair of all of these tissues has been investigated from the point of view of gene therapy. Most of the studies are preliminary but encouraging. With tendons and ligaments there is much interest in using BMPs-12, -13, -14 and scleraxis, which have tenogenic properties. Strategies for healing skeletal muscle include the delivery of genes encoding growth factors such as IGF-1 in conjunction with an angiogenic stimulus, such as VEGF, while inhibiting scar formation by blocking TGF-β. The intervertebral disc is interesting because resident cells express transgenes for extended periods of time, even when the vector or transgene are highly antigenic. This reflects the avascular nature of the disk and its dense matrix.
surrounding cells in the nucleus pulposus. Kang and colleagues have been able to maintain disk height in rabbit models of intervertebral disk degeneration by the intra-diskal injection of AAV vectors.

Clinical Translation
Orthopaedic gene therapy has been discussed for over 20 years without generating a clinically approved product. In this it reflects the field of gene therapy as a whole, which is only now developing drugs approved for clinical use. Clinical translation is difficult in the TERM orthopaedic area because the target conditions are neither genetic nor lethal, leading to heightened concerns about safety. Industry funding is sparse, because companies see long-time lines and questionable return on investment. The field of gene therapy as a whole has had major mood swings, but is now again on an upward trajectory which should provide collateral benefit to TERM protocols in orthopaedics. One product (Invossa™) for treating osteoarthritis, a related area, comprises an allograft chondrocyte line transduced to express TGF-β. It has completed Phase III trials in Korea (ClinicalTrials.gov Identifier: NCT02072070), where an application for biologics license approval has been submitted to the Korean FDA. This product is about to start Phase III trials in the US.

References


Bioscaffolds for Musculoskeletal Indications
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A key unresolved element in existing musculoskeletal cell therapy approaches is the formation of the normal 3D structural organisation of cartilage and the integration with the surrounding cartilage and subchondral bone. This 3D structure involves hierarchies of collagen families organised and tailored to provide the cell niche. Mimicking these structures for regenerative medicine approaches has relied on creating polymer or natural scaffolds which can represent the in vivo characteristics. One example of our work is the use of collagen scaffolds which can be processed to provide complex structures using different formulations (Price et al 2015). These structures also include a complex cell community with maintained progenitor cell populations as well as intermediary and mature phenotypes present in cartilage and bone.

Tissue engineering can provide more physiologically relevant 3D models for simulating this structure by combining biology with material chemistry which has the potential to provide next generation therapeutic approaches to musculoskeletal repair. What is lacking is the maintenance of the complex cell community which can tailor a biologically relevant niche and capturing the biological signal processes occurring during development and adult growth in a controlled manner.

We have been using tethered biological molecules on substrates such as glass and polymers of varying degradation rates to guide cells in 3D models to generate complex structures. In this presentation, we demonstrate this principle with an example where we control the timing, location and level of Wnt signalling; a key component which is highly regulated during embryonic development and for the maintenance of adult tissues. The ability to provide a defined and directed source of Wnt proteins is crucial to fully understanding its role in tissue development and to mimic its activity in vivo. In addition, the Wnt signalling family is thought to be involved in joint pathology and the pathways may be involved in the progression of OA and bone degeneration (Lories et al 2013). In this workshop, our recent work as part of the UK MRC UK Regenerative Medicine hub in Engineering the Stem Cell Niche will be presented. We have investigated using the Wnt3A cue to maintain stem cell populations alongside establishing a zonal architecture of differentiating osteogenic cells within a 3D model (Lowndes et al 2016). This work ultimately aims to provide a complex hierarchy of bone and cartilage cells within an implant which can be used for improved treatment of regeneration of bone in multiple clinical applications.

Our findings suggest that Wnt3a surfaces can act as a stem cell niche, maintaining Stro1 expression at the base and increasing the number of migratory cells that switch expression towards an osteogenic lineage. In this way we can recapitulate the cascade of cell types prior to treatments and create a 3D structure tailored to the cell types present within. We are currently testing varying polymers based Wnt bandages for in ex vivo cranial repair models to test the
endogenous responses to implants and their integration.

Highlights:

- One step aldehyde based chemistry to covalently immobilize hydrophobic Wnt proteins on glass or polymer scaffold materials.
- Long-term storage and continued activation of Wnt signaling over days.
- Wnt-platform can enrich and maintain adult/embryonic stem cells in 2D cultures.
- Basal Wnt can direct 3D-multicellularity for engineering tissues.
- Immobilized Wnt3a surfaces can be adapted to 3D-culture to direct human mesenchymal stem cell differentiation

References


Taken from: Lowndes et al (2016) Stem Cell Reports 12;7(1):126-37 Immobilized Wnt3a surfaces can be adapted to 3D-culture to control human mesenchymal stem cell differentiation. (A) hMSCs were seeded onto immobilized Wnt3a ± DTT to form a confluent monolayer before overlaying a collagen gel. After 7 days in culture cells were fixed and stained with DAPI to mark individual cells. The percent of cells per layer of the collagen gel (lower level: up to 72µm from the gel base, middle: 72-132µm and upper: 132-179µm) normalized to number of cells at the base. A representative bottom-up max projection with DAPI (white) marking each nucleus; example cells in each layer marked with arrows. (n=3 biological replicates, mean ± SEM). The scale bar represents 10 µm. (B and C) hMSC in collagen gels were fixed after 7 days and immunostained for Stro1 (B) and Osteocalcin (C). Expression levels were compared between immobilized Wnt3a and DTT treated. Quantification of normalized (subtracted background) image pixel intensity relative to cell number was plotted. (n=3 biological replicates, mean ± SEM; statistical significance between groups determined by Post-hoc Mann-Whitney tests; p values correspond to *<0.05). (D) Representative histological staining of hMSC gels for calcium deposition (Alizarin red staining) after 7 days of culture. The scale bar represents 100 µm. (E) hMSCs were grown on immobilized Wnt3a ± DTT, BSA ± soluble Wnt3a (50ng/mL). Migrating cells reported as the percent of total cells in each defined layer of the gel (lower level: up to 80 µm from the gel base, middle: 80-140 µm and upper: 140-200 µm). (n=3 biological replicates, mean ± SEM) (F) The percent migratory cells in each layer when grown on immobilized Wnt3a surfaces ± IWR treatment. (n=3 biological replicates, mean ± SEM) (G) After 7 days hMSCs grown on BSA with soluble Wnt3a, immobilized Wnt3a or immobilized Wnt3a with IWR treatment were fixed and immunostained for Stro1. Staining across the middle of the well at the base layer (4x mag) was quantified. (n=3 biological replicates, mean ± SEM; statistical significance determined by a one-way ANOVA test; p values correspond to *<0.05, **<0.01 and ***<0.001 ) (for representative confocal images see Figure S4C). (H) After 7 days hMSCs grown on immobilized Wnt3a ± IWR treatment were fixed and immunostained for Osteocalcin. A representative image of an Osteocalcin expressing cell within the collagen gel (left) and the quantification of Osteocalcin in the layers of the collagen gel (right). (n=3 biological replicates, mean ± SEM; p values correspond to *<0.05) The scale bar represents 50 µM.
Lowndes et. al. Figure 4