BioTime ad, perhaps?
SPECIAL ISSUE
DELIVERY, RETENTION AND ENGRAFTMENT OF PROGENITOR CELLS IN CELL THERAPY

Guest Editor, Glenn D. Prestwich
Department of Medicinal Chemistry; The University of Utah; Salt Lake City, UT USA

1 Introduction: Delivery, retention and engraftment of progenitor cells in cell therapy
Glenn D. Prestwich, Guest Editor
Citation: Prestwich GD. Introduction: Delivery, retention and engraftment of progenitor cells in cell therapy. Biomatter 2013; 3:e24549; http://dx.doi.org/10.4161/biom.24549

REVIEWS

4 Hyaluronan, neural stem cells and tissue reconstruction after acute ischemic stroke
Pouria Moshayedi and S. Thomas Carmichael
Citation: Moshayedi P, Carmichael ST. Hyaluronan, neural stem cells and tissue reconstruction after acute ischemic stroke. Biomatter 2013; 3:e23863.4-12; http://dx.doi.org/10.4161/biom.23863

13 Encapsulated stem cells for cancer therapy
Khalid Shah
Citation: Shah K. Encapsulated stem cells for cancer therapy. Biomatter 2013; 3: e24278; http://dx.doi.org/10.4161/biom.24278

20 Hyaluronic acid hydrogels for vocal fold wound healing
Joel Gaston and Susan L. Thibeault
Citation: Gaston J, Thibeault SL. Hyaluronic acid hydrogels for vocal fold wound healing. Biomatter 2013; 3:e23799; http://dx.doi.org/10.4161/biom.23799

27 Enhancing retention and efficacy of cardiosphere-derived cells administered after myocardial infarction using a hyaluronan-gelatin hydrogel
Rachel Ruckdeschel Smith, Eduardo Marbán and Linda Marbán
Citation: Smith RR, Marbán E, Marbán L. Enhancing retention and efficacy of cardiosphere-derived cells administered after myocardial infarction using a hyaluronan-gelatin hydrogel Biomatter 2013; 3:e24490; http://dx.doi.org/10.4161/biom.24490

33 Delivery of EPC embedded in HA-hydrogels for treatment of acute kidney injury
Brian B. Ratliff and Michael S. Goligorsky
Citation: Ratliff BB, Goligorsky MS. Delivery of EPC embedded in HA-hydrogels for treatment of acute kidney injury. Biomatter 2013; 3:e23284; http://dx.doi.org/10.4161/biom.23284

40 Immunotherapeutic organoids A new approach to cancer treatment
Marta Compte, Natalia Nuñez-Prado, Laura Sanz and Luis Álvarez-Vallina

48 Defining cell-matrix combination products in the era of pluripotency
Hal Sternberg, Jeffrey Janus and Michael D. West
Citation: Sternberg H, Janus J, West M. Defining cell-matrix combination products in the era of pluripotency. Biomatter 3: e24496; http://dx.doi.org/10.4161/biom.24496

Note about this issue.
With this issue—Volume 3, Issue 1; Jan/Feb/Mar 2013—Biomatter transitions to an online only journal. This issue has been printed specially for the 11th Annual Meeting of the ISSCR.

The full online citation for each article is included here on the Table of Contents. Please be sure to use this when citing articles contained herein.

About the cover.
An illustrative model on how changes in HA regulate NSPCs in normal state and in response to injury.
For more information see Moshayedi et al., pp. 3–11.
EDITORIAL POLICIES AND GUIDELINES

Our Editorial Policies and guidelines for submitting manuscripts may be found here:
http://www.landesbioscience.com/journals/biomatter/guidelines

PRESUBMISSION INQUIRIES
Presubmission inquiries should be addressed to Pedro L. Granja, Editor-in-Chief, pgranja@ineb.up.pt.
All other inquiries should be directed to Liz Gilmer, Managing Editor, liz@landesbioscience.com.

SUBMISSION OF MANUSCRIPTS
Manuscripts should be submitted online: http://biomatter.msubmit.net

COPYRIGHT AND COPYRIGHT CLEARANCE CENTER
The Copyright Revision Act (PL 94-553), which became effective January 1, 1978, states that the copyright of a work is vested in the author from the moment of creation. Therefore, all authors who wish to publish in Biomatter must grant an exclusive license to Landes Bioscience. It is understood that the authors grant Landes Bioscience an exclusive license to publish the work and also grant rights of reproduction, derivation, distribution, sale and display.

Authors who prepared their articles as part of their official duties as employees of the US Federal Government are not required to transfer copyright to Landes Bioscience, since these articles are considered to be in the public domain. However, it is necessary for these authors to sign the License to Publish form. In the case of articles supported by federal grants or contracts, a License to Publish is also required. The federal government may retain a nonexclusive license to publish or republish such material.
Delivery, retention and engraftment of progenitor cells in cell therapy

Glenn D. Prestwich, Guest Editor

Department of Medicinal Chemistry; The University of Utah; Salt Lake City, UT USA

In a recent issue of Science Translational Medicine, a multidisciplinary group of thought leaders in the field of biomaterial research and development contributed to a collection of opinion pieces highlighting the unique challenges and opportunities involved in translating biomaterials for use in humans. These “Insider Views” were provided by experts from the industry, nonprofit, academic, clinical, intellectual property, venture capital, and regulatory sectors. They highlighted the potential bottlenecks that can occur in the biomaterial product development path, from uncertainty about the studies needed for regulatory approval to the risk associated with developing and commercializing an innovative biomaterial. These insider insights conveyed the message that translating regenerative medicine and cell therapy technologies to the clinic resembles a disorienting rollercoaster ride. To keep biomaterial development and approval on track, innovators need to negotiate the twists and turns associated with seven requirements of all product stakeholders: clinical need, intellectual property protection, preclinical validation, regulatory pathway, business and financial strategies, product design, clinical trial, and reimbursement.

A recent TERMIS survey profiled the perceptions of 37 institutional investors regarding the numerous hurdles. Successfully translating biomaterial technology into a product that truly benefits patients requires a balance of innovation and practicality. Although innovative technology is the starting point, it is the execution by a company to create and market a simple and effective medical product that determines whether a novel biomaterial reaches the clinic.

We are bombarded weekly with press releases about how stem cell therapies will soon change our lives. For example, headlines in Genetic Engineering and Biotechnology News tout “Accelerating R&D of Cell-Based Therapies” (October 1, 2012), “Cellular Therapy Wave Finally Cresting” (November 1, 2012), “Regen Med Nears the Market” (November 15, 2012), “Technologies Evolving for Cellular Therapies” (January 15, 2013), and “10 Most Significant Events in Cell Therapy in 2012” (March 15, 2013). Indeed, real progress is being made as “Cardiac stem cell therapies inch toward clinical litmus test” (Nature Biotechnology, January 2013). Reviewing the past year, Fisher and Mauck recount significant events in 2012 in tissue engineering and regenerative medicine. In a State of the Art Review, Pashuck and Stevens summarize the tremendous potential for regenerative biomaterial therapies in light of the major scientific, regulatory and business hurdles that must be navigated to reach the market place.

This themed issue was conceived to place a human face on these difficult issues by focusing on the efforts of seven translational research groups to mature, deliver and retain therapeutic cells at sites in need of clinical repair or regeneration. These research teams have in common the use of a clinical-grade injectable hyaluronan (HA)-based semi-synthetic extracellular matrix known as HyStem® combined with progenitor cells, and the goal of each team is to realize the promise of treatment of human patients in the clinic. Let’s start at the top, figuratively and literally. The first two papers describe injection of therapeutic cells into the brain. Moshayedi and Carmichael (pp. 3–11) describe the use of HA hydrogels with neural stem cells for tissue reconstruction after acute ischemic stroke. Retention of cells in an anti-inflammatory matrix that supports cell growth and proliferation improves outcomes. In a very different context, Shah (pp. 13–19) presents the use of HA hydrogels for encapsulation of therapeutically engineered cells into a post-resection cavity in the brain following removal of malignant glioblastoma multiforme. The third paper by Gaston and Thibeault (pp. 21–28) summarizes the many uses of HA hydrogels for prevention and repair of injury to the human vocal folds.

In the fourth paper we get to the heart of the matter. Smith, Marban and Marban (pp. 29–34) describe the use of cardiosphere-derived cells (CDCs), which have already used successfully in two clinical trials by intracoronary infusion in buffer. They describe how injection of CDCs encapsulated in the HA-gelatin HyStem-C after a myocardial infarct in a preclinical model enhances cell retention and engraftment, increases angiogenesis, adds cardiac muscle mass, and improves cardiac outcome relative to infusion of the CDCs alone. In the fifth paper, delivery of endothelial progenitor cells (EPCs) embedded in HA-gelatin hydrogels also serves as a treatment for acute kidney injury. Ratliff and Goligorsky (pp. 35–41) summarize their preclinical studies in which gel-encapsulated EPCs can be delivered into the kidney capsule, or by slow release from EPC-gel constructs placed in the ear pinnae with a small amount of hyaluronidase. In both cases, increased kidney function, angiogenesis, and engraftment are observed.

The final two papers draw attention to other important uses of HA-gelatin matrices. Compte, Nuñez-Prado, Sanz and Vallina.
(pp. 43–50) draw attention to the concept of immunotherapeutic organoids as a new approach to cancer treatment. Echoing the use of engineered cells in the brain by Shah, this team highlights the practical importance of living cell factories capable of secretion of recombinant antibodies in vivo, an effect uniquely attributable to long-lived engineered mesenchymal stem cells delivered subcutaneously in an HA-gelatin hydrogel matrix. Finally, Sternberg, Janus and West (pp. 51–57) introduce the concept of monoclonal embryonic progenitor (hEP) cells, which are clonally expanded human embryonic stem cells at an intermediate stage of differentiation. Combining these PureCell lines with the HA-gelatin hydrogel leads to HyStem-4D bead arrays, in which the hydrogel serves in expansion and differentiation in the dimension of time as well as three spatial dimensions.

It has been a pleasure working with the authors and editors to develop this themed issue. These stories of translational research embody the translational imperative: embrace complexity, engineer versatility, but deliver simplicity.5

References
Hyaluronan, neural stem cells and tissue reconstruction after acute ischemic stroke

Pouria Moshayedi and S. Thomas Carmichael*
Department of Neurology; David Geffen School of Medicine at UCLA; Los Angeles, CA USA

Keywords: stroke, neural repair, regeneration, hyaluronan, brain

Introduction: Focal Ischemic Stroke

Acute ischemic stroke is one of the main causes of mortality and disability. It annually affects 93 new and recurrent cases per 100,000 population in the US, which imposes a direct economic cost of over $73 billion.1 There is only one approved medical therapy for stroke: tPA is a “clot busting” agent that acts to reperfuse damaged brain, and must be given within 4.5 h of the stroke onset. However, the affected patients are left with lifelong motor, sensory and cognitive disabilities,2 which indicates that despite reperfusion associated with opening of an occluded blood vessel there exists non-salvaged and damaged brain tissues. Therefore neural repair strategies are required to improve neurological dysfunction.

The most common stroke subtype is non-hemorrhagic stroke, caused by a sudden arterial occlusion that disrupts blood flow to brain tissue3 and leads to tissue necrosis in the center of the territory of occluded artery, known as the infarct core.3 Brain areas that are adjacent to the stroke and connected to the region that will become the infarct core survive the stroke. Evidence from human and animal studies indicates that plasticity in the regions of the brain adjacent to and connected with the stroke exhibit the most recovery of function.1-6 Mechanisms of recovery in these areas include neurogenesis, in which newly born immature neurons migrate into the peri-infarct cortex in large numbers and may provide a direct trophic support or cell replacement in this tissue. Also, axons in peri-infarct cortex sprout new connections by activating a specific “sprouting transcriptome.” Recovery is also seen in the remapping of motor and sensory maps in peri-infarct cortex.4-6

However, this recovery is limited, making stroke the leading cause of adult disability. Neuronal circuits in peri-infarct cortex are stunned or dysfunctional, exhibiting increased inhibition7 and altered responses to excitatory signals.8 Experimental studies have shown that the self-repair processes of axonal sprouting and neurogenesis could be enhanced by specific treatment directed at tissue reorganization, as opposed to neuroprotective strategies which maximize the extent of spared neurological functions by protecting the brain tissue against active cell death mechanisms.9

Because neural repair processes occur over weeks and months after the stroke they may be amenable to more delayed treatments that could be given to a wider array of patients than acute neuroprotective therapies.10

Ischemia, among many changes, induces the proliferation and migration of endogenous neural stem and progenitor cells (NSPCs) in the brain.5,11 These play a neuroprotective role by modulating intrinsic inflammatory responses to injury.12-15 Transplanted stem/progenitor cells may enhance these endogenous processes after stroke such as axonal sprouting, neurogenesis and angiogenesis.13,14 Stem/progenitor cells can also directly integrate into damaged neural networks by differentiating into neurons and glial cells,5,16 although this does not appear to be a substantial element in the tissue repair after NSPC transplantation.17 Stem cell transplantation after stroke can both enhance endogenous repair processes and potentially create new neuronal circuits or brain architecture in partially damaged areas next to the stroke core. Therefore, stem cell transplantation is a therapeutic option for post-stroke neural damage.

However, there are pre-clinical and clinical challenges that limit the application of neural stem/progenitor cells in stroke. Peri-infarct tissue is the area where the most significant post-stroke...
regeneration occurs and therefore transplanted cells need to be delivered as close as possible to that area for a maximum effect on the recovering circuits after stroke. However, direct injection of stem/progenitor cells into the peri-infarct cortex carries the theoretical risk of damaging the very tissue that recovers after stroke. The infarct cavity is a compartmentalized area of dead, necrotic core of infarcted tissue does not provide transplanted cells with a viable matrix and required growth factors to help them regenerate and reorganize the damaged tissue. As infarcted or damaged tissue is a target for neural repair in stroke and other CNS degenerative diseases the limited vascular and trophic support and increased inflammation in this target region can be an explanation as to why clinical stem cell therapy had limited success or variable outcome in patients suffering from disorders such as stroke and Parkinson.

A potential solution to stem cell targeting in stroke is the encapsulation of neural stem/precursor cells in a bio-compatible matrix to enhance their survival and/or differentiation in stroke. Such a matrix might provide signals through the structure of the hydrogel backbone, the incorporation of growth factors or the inclusion of protein motifs that provide pro-survival, pro-growth or pro-differentiation effects. There have been several studies applying biopolymers as carrying scaffolds for neural stem/progenitor cells transplanted to brain focal ischemic lesions (Table 1). In this review, we will focus on the use of Hyaluronan (HA) and discuss options for a bio-compatible hydrogel matrix with a potential to enhance the outcomes of neural cell transplantation for brain ischemic lesions. Tissue engineering is a multi-faceted approach, as there are several pathologies in damaged tissues that should be corrected. The main deficit in stroke is due to the lost or damaged neuronal circuits. In order to repair injured circuits and possibly restore neurons glial support is needed, as well as a vasculature to supply oxygen and nutrients. Inflammation with both its destructive and constructive roles actively contributes to the brain tissue response to the implanted hydrogel. An efficacious hydrogel design, therefore, should take these into consideration and analyze interactions of the hydrogel elements with the injured brain tissue. We explore in this review the interactions of HA with the processes involved in inflammation, foreign body response, angiogenesis, NPC differentiation and axon growth.

**Hyaluronan**

HA is a glycosaminoglycan composed of repeated disacharid D-glucuronic acid and N-acetyl-D-glucosamine. It is produced by a membrane-bound group of enzymes named hyaluronan synthases, which organize D-glucuronic acid and N-acetyl-D-glucosamine disaccharids into a non-branched linear polymer that can range between 1 MDa to 4 MDa in size; this is what generally known as high molecular weight (HMW) HA. HMW HA is very flexible and can readily form into coils, nets or fibers (see an electron micrograph in Fig. 1). HMW HA is strongly negatively charged and therefore absorbs up to 10–10,000 times its weight water. It is generally a bio-inert molecule that acts to maintain a hydrated and porous environment, absorbs mechanical shock and regulates osmotic balance. HMW HA can also sequester and gradually release growth factors and other bioactive molecules to communicate a local biological influence over cells. HMW HA is specifically interesting from a tissue

<table>
<thead>
<tr>
<th>Article</th>
<th>Stroke model/species</th>
<th>Cells transplanted/density per animal</th>
<th>Hydrogel</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bible et al., 2009</td>
<td>MCAO/rat</td>
<td>NSC/3.15 × 10⁷ cells in 30 µl</td>
<td>Fibronectin-coated PLGA particles</td>
<td>Descriptive analyses of cell survival, cell-scaffold-tissue integration, cell differentiation, angiogenesis and host inflammatory response</td>
</tr>
<tr>
<td>Jin et al., 2010</td>
<td>MCAO/rat</td>
<td>hES-NPC/6 × 10⁶ cells in 50 µl</td>
<td>Matrigel</td>
<td>Reduction in lesion volume, improving cell survival, differentiation and behavioral indices</td>
</tr>
<tr>
<td>Yu et al., 2010</td>
<td>MCAO/rat</td>
<td>NSC/1.5 × 10⁶ in 5 µl</td>
<td>Collagen</td>
<td>Cell survival, NSC synapse formation and neurological severity score improved</td>
</tr>
<tr>
<td>Zhong et al., 2010</td>
<td>PT/mice</td>
<td>ES-NPC/10³ cells in 7 µl</td>
<td>Hyaluronan /Heparin /Collagen</td>
<td>Improving transplant cell survival and host inflammatory response, angiogenesis and astrocytic reactivity</td>
</tr>
<tr>
<td>Matsuse et al., 2011</td>
<td>MCAO/rat</td>
<td>MS-NSC/2 × 10⁶ cells, 12 µl in striatum and 8 µl in cortex</td>
<td>Collagen with bFGF in gelatin microspheres</td>
<td>Infarct volume, cell survival and distribution, angiogenesis, number of host NSCs and motor behavior improved</td>
</tr>
<tr>
<td>Bible et al., 2012</td>
<td>MCAO/rat</td>
<td>hNSC/2.1–2.5 × 10⁶ cells in 25–40 µl</td>
<td>Acellular ECM</td>
<td>Descriptive analyses of cell imaging by MRI, cell migration, differentiation and cell-host tissue interaction</td>
</tr>
</tbody>
</table>

**Table 1. Application of biopolymers in neural stem/progenitor cell therapy of focal brain ischemic lesion**

ES, embryonic stem; h, human; MCAO, middle cerebral artery occlusion; MS, mesenchymal stem; NPC, neural precursor cell; NSC, neural stem cell; PLGA, poly(lactic-co-glycolic acid); PT, photothrombotic.
Hydrogel polymer engineering standpoint as it can diminish the interaction of encapsulated cells with other cells and growth factors and therefore “conceal” them from local harmful signals; this is mainly done by modulating inflammatory response of macrophages. 24 HMW HA can be degraded by enzymes, hyaluronidases, into oligomers that, unlike high molecular HA, are highly bioactive (see below). HA degradation is facilitated in inflammation and injury by the production of reactive oxygen and nitrogen species. 25

HA is a reasonable choice to encapsulate cells for transplantation into the brain because it is naturally and abundantly found in the brain. 26 HA has a role in the brain more than just space-filling, hydration and matrix provision. HA influences cell adhesion, migration, axon path-finding, brain regional specificity and therefore, it is actively involved in normal development of the brain. 26, 27 HA is increasingly deposited in the aged brain and diminishes oligodendrocyte precursor (OPC) maturation. 28 HA in the demyelinated plaques of multiple sclerosis prohibits OPC maturation and myelin repair. 31 HA is a component of the perineuronal net that modulates mature cerebral neurons. 29 HA is also involved in brain pathologies and diseases. HA promotes malignant glial cell adhesion, migration and metastasis in the brain. 24 It also contributes to mossy fiber sprouting in the hippocampus that will eventually lead to temporal lobe epilepsy. 27 HA is involved in immunomodulation, tissue injury and repair in the brain through the innate immune receptors toll-like receptors 2 and 4 (TLR2 and TLR4), signaling through the main inflammatory transcription factor NFκB, and tumor necrosis factor α secretion. 26, 30 These phenomena are mediated through HA receptors, mainly CD44, RHAMM and TLR4. Hence, HA affects a variety of physiologic and pathologic functions, which makes its application intriguing and challenging.

HA gives different biological signals depending on the molecular weight (see Table 2 for an overview). High molecular HA (> 500 kDa) that is normally found in the brain plays a structural role and silences inflammation, angiogenesis and neural differentiation. 32-34 But in pathologies, such as brain ischemic stroke, HA is fragmented into 6- to 40-mers through the action of hyaluronidases and reactive oxygen or nitrogen species. Fragmented HA has a distinct role in activating innate and adaptive immune response, as well as inducing proliferation, motility, and tubule formation of endothelial cells and angiogenesis (refs. 35 and 36 and personal communication with M. Slevin). This effect is mediated by HA receptor CD44 on the endothelial cells. 37 This allows tissue engineers to vary the molecular sizes of HA. By encapsulating cells in high molecular weight HA, cells can be transplanted and protected from inflammation upon transplantation in the acute phase of brain damage and through a controlled and gradual degradation, low molecular weight HA is released and can induce angiogenesis and differentiation of stem cell.

Every material implanted in the brain is at risk of being isolated by the brain foreign body response. 38 This starts with the brain’s attempt to heal the wound caused by implantation. Upon failure to recognize the implant as “self” or to degrade it, the brain mounts a chronic activation of inflammatory response and gliotic scar formation that will eventually seal-off the “stranger” from accessing normal brain tissue and will effectively cause implant malfunction. From the anti-inflammatory effects of HMW HA it might be predicted that HA will reduce scar formation in the brain and peripheral nerves. This is indeed true: 39, 40

Figure 2 shows how applying HMW HA can attenuate astrocytic reactivity and scar formation in spinal cord injury. These data render HA gel a bio-compatible choice for neural tissue engineering.

Hyaluronan and Angiogenesis

Nutrients and oxygen can functionally diffuse up to 150–250 μm from blood capillaries. 25 The size of infarcted tissue in the brain is several fold larger than this critical range. Therefore any tissue engineering solution for the brain after stroke should consider reconstructing a normal microenvironment with a proper accessibility to oxygen and nutrients, and envisage a solution for the formation of new vessels in tissue constructs. Angiogenesis requires activation of endothelial cells with pro-angiogenic factors (such as vascular endothelial growth factor or VEGF, platelet-derived growth factor or PDGF, fibroblast growth factor or PFG, hepatic
also enhances matrix metalloproteinase (MMP)-2 and -9 expression, promoting matrix degradation and progression of angiogenesis. MMPs also activate endogenous TGF-β that in turn contributes to angiogenesis. Interactions of short chain HA with RHAMM lead to cytoskeletal changes and subsequent migration of endothelial cells that further contribute to the formation of new vessels.26

Growing human umbilical vein endothelial cells (HUVECs) on HA gels improved their proliferation. This augmentation was proportional to the concentration of HA and by using 1% HA gel cells maximally increased their proliferation over 2-fold in a 48 h interval. HA also protects HUVECs against apoptosis induced by serum deprivation. An HA gel enhanced angiogenesis, arteriogenesis and improved ischemia in an experimental model of mice limb ischemia when it encapsulated HUVECs.43 Implanting HA hydrogels in the brain has improved angiogenesis. 44 HA is also important in maintaining vascular integrity. 45 These data show HA has the potential to support the formation of a vasculature to allow generation and survival of engineered constructs in sizes required to replace the infarcted brain tissue. Some studies have bound oligomeric HA to culture surfaces and thereby induced endothelial cell proliferation and tube formation.46 An alternative approach would be to engineer HMW HA gels to persistently degrade and provide host tissue endothelial cells with a continuous source of pro-angiogenic oligomeric HA.47

Table 2. A comparative summary of findings in high molecular weight HA vs. low molecular weight HA

<table>
<thead>
<tr>
<th>High molecular weight hyaluronan</th>
<th>Low molecular weight hyaluronan</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Provides hydration and porosity</td>
<td>- Activates immune response</td>
</tr>
<tr>
<td>- Sequesters and gradually releases bioactive molecules</td>
<td>- Induces angiogenesis</td>
</tr>
<tr>
<td>- Conceals encapsulated cells from other cells and humoral factors</td>
<td>- Promotes proliferation, differentiation and migration of stem/progenitor cells</td>
</tr>
<tr>
<td>- Keeps stem/progenitor cells in a quiescence state</td>
<td></td>
</tr>
<tr>
<td>- Silences inflammation</td>
<td></td>
</tr>
<tr>
<td>- Prohibits angiogenesis</td>
<td></td>
</tr>
<tr>
<td>- Inhibits astrocyte activation and scar formation</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Attenuation of astrocyte activation and deposition of chondroitin sulfate proteoglycan (CSPG) by HMW HA gel injection in vivo. Longitudinal images of spinal cord from control (“SCI”) and HA-implanted animals are shown 10 d after spinal cord injury (SCI). Reproduced with permission from reference 39. Scale bar = 100 μm.

growth factor or HGF, angiopoietin-1 and transforming growth factor-β or TGF-β). These factors induce a process of endothelial branching from existing capillaries into adjacent tissue and penetration into an implanted matrix. Endothelial cells need to survive and proliferate inside such a transplanted matrix, and be capable of remodeling it to migrate through and establish preliminary cord-like capillary structures; these will advance into mature capillaries and further to small arteries and veins.

HA has been widely studied and appreciated as a pro-angiogenic tool in tissue engineering because it can initiate and maintain angiogenesis. HMW HA promotes quiescence in endothelial cells, but oligomeric HA promotes their proliferation and migration; this effect is mediated directly through endothelial cells and also indirectly by inducing inflammatory cells to secrete pro-angiogenic factors. HA relays these messages through CD44, RHAMM and TLR-4 receptors.26,43,44 Long polymers of HA bind to CD44 and interfere with any biological signal transduction; this inhibits endothelial cell proliferation that leads to arrest of angiogenesis. Oligomeric HA, in contrast, attaches to CD44 and promote proliferation of endothelial cells. This molecule
Hyaluronan and Neural Stem/Progenitor Cells

There are many indications that HA influences neural stem/progenitor cells in neural tissue development and therefore HA is a suitable candidate to encapsulate NSPCs in neural tissue engineering. HA content is high in developing brain and it declines to 25% two weeks after birth.\textsuperscript{49} In vitro studies show that the addition of HA contributes to increasing water absorption and porosity of bio-matrices.\textsuperscript{50} Water absorption and resultant changes in the three dimensional HA structure are responsible for some of its biological properties. For example, HA is associated with neural crest cell distribution along the neural tube perhaps by creating a porous milieu for cell migration through the tight cell-cell junction of neuroepithelial cells. Ninety percent of HA is associated with water, suggesting HA contributes to cell migration by providing a loose matrix. HA is diffusely found in the developing brain while NSPCs are migrating in radial or tangential directions. Although HA decreases in postnatal brain, it is still found in cerebellum and corpus callosum, where HA is re-organized into dense meshworks; interestingly, those areas are home to an extensive migration of progenitor cells.\textsuperscript{26}

Besides being a permissive conduit for migration, HA actively interacts with cells to tune the cellular machinery for migration. Through RHAMM receptors HA induces calmodulin-mediated signaling that affects actin and microtubule.\textsuperscript{31} HA-rich fiber tracts may also function as physical cues guiding the migration of newly born cells in the CNS.\textsuperscript{26} It has been suggested that HA induces neural progenitor migration through RHAMM activation. CD44, another known receptor for the HA molecule, is found on NPCs and mediates their interaction with endothelial cells and their transmigration through vessels;\textsuperscript{52} these stem cell functions lead to establishing neurovascular stem cell niche that plays a crucial role in normal adult angiogenesis as well as integration of exogenous transplanted NSPCs.

HA is found in the niche surrounding stem cells and it affects stem cell proliferation and differentiation. It is specifically found in the brain ventricular regions with ongoing stem cell proliferation.\textsuperscript{26} HA interaction with CD44 receptor on stem cells influences mitosis spindle formation, which in turn affects symmetric vs. asymmetric division and therefore controls self-renewal vs. differentiation.\textsuperscript{53} HA is generally believed to slow the proliferation of stem cells in the brain. Enzymatic HA removal induces proliferation of oligodendrocyte-type 2 astrocyte progenitors,\textsuperscript{54} now classified as oligodendrocyte progenitor cells. HA is partly the reason for failed remyelination of demyelinated axons in multiple sclerosis by oligodendrocyte progenitor cells (see Fig. 3 for an illustration): While HMW HA secreted by astrocytes in demyelinated lesions prohibits OPC differentiation to myelin-producing cells, removing HMW HA promotes oligodendrocyte maturation.\textsuperscript{31} On the other hand, it has been suggested that degradation of HA promotes proliferation of cells.\textsuperscript{35} Moreover, LMW HA (as the degradation product of HMW HA) can induce proliferation, differentiation and migration of NSPCs.\textsuperscript{26} Degradation of HA gels encapsulating neural progenitor cells causes differentiation and maturation of those cells; this fits well with the in vivo finding showing a decline in the brain HA content in the postnatal period.\textsuperscript{14} It is therefore possible to utilize the dual effect of HA on stem cells by encapsulation of NSPCs in high molecular size HA to prohibit proliferation and maturation, and by a timely degradation of HA and provision of cells with LMW HA turn on their proliferation and differentiation machinery.

These data have inspired researchers to employ HA hydrogel to recapitulate the normal stem cell niche: by providing NSPCs with a 3-dimensional matrix close in composition to the ECM, and by further supplementing HA hydrogel with collagen-I, abundantly found in basal lamina of the subventricular zone.\textsuperscript{36} Cells growing in these gels have a lower proliferation rate compared with a 2-dimensional culture, and their neural differentiation improves by almost 5-fold. Other research groups have enriched HA gels with an optimized ratio of collagen\textsuperscript{57} or added carefully-tuned amounts of fibroin (produced by Bombyx mori silkworm)\textsuperscript{59}, heparin sulfate\textsuperscript{44} or IKVAV (a protein motif originally found in laminin) plus brain-derived neurotrophic factor\textsuperscript{29} to provide NSPCs with an optimally engineered microenvironment to improve their survival, proliferation and differentiation. By altering the hydrogel compliance instead of changing biochemical factors, differentiation of NSPCs to neurons was optimized in gels mimicking neonatal brain compliance vs. those gels similar to adult brain in elasticity.\textsuperscript{59} Neural progenitor cells (NPCs) encapsulated in a combined hydrogel made of HA, heparin sulfate and collagen transplanted to the stroke cavity were protected against the host inflammatory insult and their survival improved (Fig. 4).\textsuperscript{14} Applying this biopolymer matrix, however, did not influence NPCs differentiation.

Hyaluronan and Axon Growth

Attempts to study the role of HA in axonal local sprouting and long-distance regeneration started with simple histological observations in late 1980s.\textsuperscript{60} These found HA as a fine mesh around brain and spinal cord myelinated axons,\textsuperscript{61,62} as well as in endoneurial tubes in the peripheral nerves.\textsuperscript{63} HA was also found in the perineuronal nets, the specialized ECM structures around cell bodies in the CNS that among many roles also regulate synaptic connectivity.\textsuperscript{61,62}

These phenomenological studies had a clear conclusion: HA is likely to affect a neuron’s physiological functions in the developing and adult nervous system, and also could determine the regenerative response to nervous system injuries. These were the foci for further investigations. HA was found to fine-tune axon pathfinding and contribute to architecturally specific areas in the brain: layer-specific termination of entorhinal fibers to the dentate gyrus, an area responsible for formation of new memories, was abolished by degrading HA molecules.\textsuperscript{64} Another region that HA serves as a guidance molecule is the optic chiasm, where those axons of the optic nerve coming from the nasal halves of retina will cross and give rise to the very specific optic pathway. HA localizes in the medial part of the optic chiasm, the point where axon crossing takes place.\textsuperscript{65} Data suggests the HA receptor CD44 is involved in mediating the HA role in axon crossing.
Further experiments revealed HA removal deters axon crossing in the optic chiasm.66

HA is also involved in the nervous system response to injuries and pathological conditions. The presence of HA was correlated with the axon growth in regenerating limbs of larval ambystoma.60 This finding was echoed in a murine model of peripheral nerve injury: HA was associated with successful axon regeneration and it was co-localized in Schwann cells proliferation areas and the bands of Büngner, two heralds of endogenous peripheral nerve repair.63 Mossy fiber sprouting in hippocampus is responsible for the formation of a recurrent aberrant excitatory network and the consequent temporal lobe epilepsy. Increases in HA expression in the hippocampus are associated with temporal lobe epilepsy and removing HA in the dentate gyrus protects the brain from epileptogenic chemicals (e.g., kainic acid) that induce mossy fiber sprouting.27

These findings prompted researchers to apply HA to promote regeneration in the peripheral and the central nervous systems. Their initial concept considered HA as a hydrated open lattice to allow axon growth as well as free diffusion of nutrients and...
growth factors. Early attempts applied an injectable form of HA to amplify axon growth, myelination and conduction velocity in regenerating peripheral nerve. 47,48 HA also diminished the perineurial scar formation, a known impediment to the regeneration, in re-opposed stamps of peripheral nerves. 49

HA promoted regeneration of dorsal root ganglia axons in vitro, but it failed in vivo to induce spinal cord regeneration. 50 These findings revisit the established concept of compromised axon regeneration in the CNS. CNS axons are facing multiple barriers in their internal machinery as well as their surrounding environment in order to mount an effective regenerative response and therefore additional molecules are required to persuade severed axons to re-grow. Hence plain HA gels were supplemented with elements of the ECM or membrane receptor ligands to promote axon growth into the implanted HA matrix in the lesioned brain 51 or spinal cord. 52

Perspectives and Caveats

Tissue engineering will fail if it does not address the many pathologies present in the damaged brain tissue. In early stages of neural repair a focus was on neuronal replacement. However, controlling inflammation, securely delivering stem/progenitor cells, promoting angiogenesis, inducing axon regeneration and evading the foreign body reaction are examples of demands from the local environment in a more biologically active tissue repair approach. Further, these are not distinct processes but are inter-related. As discussed in this review, HA is present in the developing and adult brain with a functional consequence for interaction with angiogenesis, inflammation, axonal and cell migration guidance and with effects on cell proliferation and differentiation. This implies applying HA in tissue constructs will modulate inflammation, angiogenesis, stem cell proliferation/differentiation and axon regeneration and therefore improve brain ischemic lesions. In addition to those, tissue engineering capabilities of HA for protein motif incorporation indicate a future in which HA can serve to influence not just the individual elements of tissue repair, but multiple aspects of tissue repair at the same time. However there exists a dual functional role of HA by size (see Table 2) and therefore a successful tissue engineering approach requires an intelligent construction, maintenance and degradation of the HA hydrogel.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Supported by California Institute of Regenerative Medicine Grant RT2-01881.


19. Lin TN, Sun SW, Cheung WM, Li F, Chang C. Dynamic changes in cerebral blood flow and angiogenesis after transient focal cerebral ischemia in rats. Evaluation with serial magnetic resonance imaging. Stroke 2002; 33:2985-91; PMID:12468801; http://dx.doi.org/10.1161/01.STR.01.003575.97888.9D.


30. Macovski A. Volume 3 Issue 1


Encapsulated stem cells for cancer therapy

Khalid Shah1-4

1Molecular Neurotherapy and Imaging Laboratory; Massachusetts General Hospital; Boston, MA USA; 2Department of Radiology; Massachusetts General Hospital; Harvard Medical School; Boston, MA USA; 3Department of Neurology; Massachusetts General Hospital; Harvard Medical School; Boston, MA USA; 4Harvard Stem Cell Institute; Harvard University; Cambridge, MA USA

Stem cells have inherent tumor trophic migratory properties and can serve as vehicles for delivering effective, targeted therapy to isolated tumors and metastatic disease, making them promising anti cancer agents. Encapsulation of therapeutically engineered stem cells in hydrogels has been utilized to provide a physical barrier to protect the cells from hostile extrinsic factors and significantly improve the therapeutic efficacy of transplanted stem cells in different models of cancer. This review aims to discuss the potential of different stem cell types for cancer therapy, various engineered stem cell based therapies for cancer, stem cell encapsulation process and provide an in depth overview of current applications of therapeutic stem cell encapsulation in the highly malignant brain tumor, glioblastoma multiforme (GBM), as well as the prospects for their clinical translation.

Stem Cell Sources and Their Homing to Tumors

Stem cells are characterized by their capacity for self renewal and their ability to differentiate into specific cell types under the influence of their microenvironment. They are the natural sources of embriogenetic tissue generation and continuous regeneration throughout adult life. The embryonic stem cells originate from the inner cell mass (ICM) of the gastrula1 and form the three germ layers: endoderm, mesoderm and ectoderm, each committed to generating specified tissues of the forming body.2 Tissue specific stem cells, such as mesenchymal stem cells (mesoderm), hematopoietic stem cells (mesoderm) and neural stem cells (ectoderm), have been identified as present and situated between their germ layer progenitors and differentiated end organ tissues.2 Embryonic stem cells display indefinite self renewal capacity due to high telomerase expression. In contrast, telomerase activity in adult stem cells seems to be lower, limiting their perpetuation capacity in the long run.3 Recently, pluripotent stem cells have been shown to be generated from murine fibroblasts4 as well as from several human organs, such as heart, skin5 and bone marrow.6 More recently, stem cells derived from dental pulp7 and menstrual blood8 have also been isolated and studied to understand their potential applications in therapy. A number of different stem cell types have been used for the delivery of therapeutics to treat various cancers. These include mesenchymal stem cells (MSC), neural stem cells (NSC), umbilical cord derived stem cells (UCB SC) and adipose derived stem cells (ASC). However, bone marrow derived MSC have been widely studied for cancer therapy.

A number of studies have shown that various stem cell types migrate to sites of injury, ischemia and tumor microenvironments; and extensive studies have shown that migration of stem cells is dependent upon the different cytokine/receptor pairs SDF-1/CXCR4, SCF-c-Kit, HGF/c-Met, VEGF/VEGFR, PDGF/PDGFr, MCP-1/CCR2, and HMGB1/RAGE (reviewed in ref. 9). SDF-1/CXCR4 has been shown as the most prominent cytokine/receptor pair. The importance of the interaction between secreted SDF 1 and cell surface CXCR4 for stem cell migration has been displayed by experiments in which the activity of either the receptor or the cytokine has been inhibited.10-12 Recent studies on gene expression profiles of stem cells exposed to conditioned medium (CM) of various tumor cells, revealed the downregulation of matrix metalloproteinase 2 (MMP-2) and upregulation of CXCR4 in stem cells.13 This exposure to tumor CM-enhanced migration of MSC toward tumor cells, which was further confirmed by SDF-1 and MMP-2 inhibition studies. Another recent study has reported the involvement of a potent pro inflammatory cytokine, macrophage migration inhibitory factor (MIF) in stem cell migration. An activating antibody (CD74Ab) was employed in this study to examine the effect of one MIF receptor, CD74 (major histocompatibility complex class II associated invariant chain), on SC motility. Targeting CD74 to regulate migration and homing potentially may be a useful strategy to improve the efficacy of a variety of SC therapies including cancer.14 A recent study suggested that bioactive lipids, sphingosine-1-phosphate and ceramide-1 phosphate contribute directly toward the migratory properties of stem cells and also the presence of these priming factors leads to robust response of stem cells to very low SDF-1 gradients.15 Besides targeting the tumor main burden, different stem cell types have been shown to track tumor metastases and small intracranial microsatellite deposits of different tumor types. The stem cells have been shown to effectively treat these sites with either the factors they release, or in loco expression of tumorcidal transgenes that they have been engineered with.16-18 These findings provide a strong rationale for the development of therapies that capitalize on the tumoritropic properties of stem cells by engineering them into carriers for anti tumor therapy.
The unmodified stem cells, particularly MSC, have been shown to have anti tumor effects both in vitro and in vivo in different mouse models of cancer. This is attributed to the factors released by MSCs that have antitumor properties; reducing the proliferation of glioma, melanoma, lung cancer, hepatoma and breast cancer cells.19-22 Human bone marrow derived MSC injected intravenously (i.v.) in a mouse model of Kaposi’s sarcoma were shown to home to sites of tumorigenesis and potently inhibit tumor growth.23 MSCs have also been shown to have anti angiogenic effect both in vitro and in vivo models of melanoma.24 Direct injection of MSC into subcutaneous melanoma bearing mice induced apoptosis and abrogated tumor growth.24

**Stem cells based delivery of therapeutics.** Different stem cell types have been genetically modified mainly to introduce and overexpress target exogenous genes for expression/secretion of a desired therapeutic factor for targeted treatment of different cancer types (Fig. 1).25 A number of studies have recently been published describing the successful use of stem cell based delivery of cytokines for the management of various human cancers. A few of the recent developments in this area are discussed below.

**Interleukins.** Stem cells have been efficiently employed for the delivery of interleukins in order to improve the anti cancer immune surveillance by activating cytotoxic lymphocytes and natural killer cells. A number of previously published studies demonstrated the efficacy of stem cell delivered interleukin(IL)s such as IL-2,20-IL-7[2], and IL-1826 in various tumors. However, MSC engineered to express interleukin (IL)-12 were recently shown to prevent metastasis into the lymph nodes and other internal organs as well as increased tumor cell apoptosis in mice bearing pre established metastases of melanoma, breast and hepatoma tumors.27 Furthermore, in a separate study, human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) were successfully employed as delivery vehicles to deliver interleukin-12, a therapeutic gene in the management of malignant glioma.28 In another study, human umbilical cord blood stem cells (UCBSCs) were engineered to express interleukin-21 (IL-21) and were shown to have therapeutic efficacy in mice bearing ovarian cancer xenografts. This study suggested that the UCBSC-IL-21 therapy was safe and feasible in ovarian cancer therapy, and that the method would be a promising new strategy for clinical treatment of ovarian cancer.29

**Interferons.** Human bone marrow MSC secreting interferon IFNβ has been proven to have success in diminishing a number of tumors such as melanoma,30 breast cancer31 and lung metastases.32 In a recently published study, amniotic fluid derived mesenchymal stem cells (AF-MSCs) were isolated, investigated for their tumor tropism and capability to transport interferon-β (IFNβ) to the region of neoplasia in a bladder tumor model. A significant inhibition of tumor growth as well as prolonged survival of mice was observed in the presence of AF- MSC-IFNβ, thereby demonstrating the potential of engineered AF-MSCs as anti cancer vehicles.33 In another study, adipose tissue derived stem cells were engineered to secrete IFNβ and were used in combination with the chemotherapeutic agent, cisplatin to demonstrate a marked reduction in tumor growth in a mouse model of melanoma.34 Yi et al., recently demonstrated that genetically engineered stem cells are capable of migrating to and effectively eliminating lung cancer cells. Using a gene directed enzyme pro drug therapy, wherein human neural stem cells (NSC) were engineered to express the suicide gene, cytosine deaminase (CD) and also secrete IFNβ, Yi and his colleagues found a marked reduction in the growth of lung cancer cells in culture upon treatment with the prodrug for CD, 5 fluorocytosine.35

**Suicide gene therapy.** The suicide gene therapeutic approach is based on the conversion of non toxic prodrugs into active anticancer agents via introduction of non mammalian or mammalian enzymes. One of the earliest suicide gene therapies demonstrated is the herpes simplex virus thymidine kinase (HSV-TK) Ganciclovir (GCV) system.36,37 HSV-TK is a viral enzyme that catalyzes the phosphorylation of nucleotide analogs such as antiviral drug GCV.38 The phosphorylated GCV then enters the cell and is converted to a cytotoxic drug via the enzyme thymidine kinase (TK). The TK then catalyzes the conversion of the prodrug to an active form, which then interacts with DNA and inhibits DNA polymerase activity, leading to cell death. In addition to this system, there have been several other suicide enzyme-prodrug systems including cytosine deaminase (CD)/5 fluorocytosine (5-FC),40,41 cytosine deaminase (CD)/6-thioguanine (6-TG),42 carboxypeptidase/chloroethyl mesyloxethylaminobenzoyl-glutamic acid (CMA),43 and carboxylesterase (CE)/CPT-11.44 When human MSC-line HB1.F3 carrying CD enzyme gene (F3.CD) was transplanted intracranially at distant sites from the tumor, MSC were shown...
to migrate through normal tissue and selectively home to the glioblastoma tumor mass, which resulted in a significant reduction in tumor volume upon administration of prodrug 5-FC.45 In a separate study, HB1.F3 human NSCs carrying peroxin (PEX) gene, were found to surround the invading brain tumor cell population, chase down infiltrating tumor cells, and also significantly kill tumor cells as demonstrated by a reduction in tumor volume.46 Bone marrow derived MSCs expressing the suicide gene HSV TK, were demonstrated to potently eliminate prostate cancer cell mass both in culture and in vivo, in a study performed by Song et al.47 The different strategies that have been demonstrated so far reveal the promising potential of the combination of stem cell based therapies and suicide gene strategies to combat tumors.

Pro-apoptotic proteins. The delivery of pro apoptotic proteins such as TRAIL (tumor necrosis factor related apoptosis induced ligand) via stem cells is a relatively new approach toward tumor cell killing. TRAIL is an endogenous member of the TNF ligand family that binds to its death domain containing receptors DR4 and DR5 and induces apoptosis via activation of caspases, preferentially in cancer cells while sparing most other cell types.48 A number of studies have shown that the therapeutic efficacy of different adult stem cell types, including MSC, engineered to express TRAIL in either cell lines or mouse models of colorectal carcinoma,49 gliomas,50-52 lung, breast, squamous and cervical cancer53 result in induction of apoptosis and a subsequent reduction of tumor cell viability. Previous work from our laboratory has focused on designing a secretable version of TRAIL that consists of fusion between the extracellular domain of TRAIL and the extracellular domain of the hFlt3 ligand which binds to the Flt3-tyrosine kinase receptor. The re engineered recombinant protein named "secretable TRAIL" (S-TRAIL) is efficiently secreted into the producer cell's immediate microenvironment and exhibits higher cytotoxicity on glioma cells than the native TRAIL protein.17,54,55

Encapsulated Stem Cells for Therapy

Cell encapsulation technology refers to immobilization of cells within biocompatible, semipermeable membranes. The encapsulation of cells instead of therapeutic products, allows the delivery of molecules of interest for a longer period of time as cells release these molecules continuously. In addition, genetically engineered cells can be immobilized to express any desired protein in vivo without the modification of the host's genome.56 Encapsulation of cells presents an important advantage as compared with encapsulation of proteins, as the latter allows a sustained and controlled delivery of therapeutic molecules at a constant rate giving rise to more physiological concentrations.56 Due to their ability to provide a physiologic environment that promotes cell survival and prevent immune response while permitting easy in vivo transplantation and cell retention, biodegradable hydrogels and synthetic extracellular matrix (sECM) to encapsulate stem cells have been utilized.57,58 A number of different biomaterials such as alginate, hyaluronic acid, agarose and other polymers have been used for encapsulation.

Hyaluronic Acid Based Clinical Biomaterials

Hyaluronic acid (HA) is a non sulfated, linear polysaccharide with the repeating disaccharide,β-1,4-D-glucuronic acid-β-1,3- N-acetyl-D-glucosamine. HA is ubiquitous and highly hydrated polyanion and an essential component of the extracellular matrix (ECM); its structural and biological properties mediate cellular signaling, wound repair, morphogenesis and matrix organization.59 Although HA and its derivatives have been clinically used in the past, it has become recognized as an important building block for the creation of new biomaterials for use in cell therapy.60-62 Chemical modification of HA alters its material and biological properties,63 and targets three functional groups: the glucuronic acid carboxyl acid, the primary and secondary hydroxyl groups, and the N acetyl group (following deamidation). The chemical, mechanical, and biological criteria for clinical and preclinical biomaterials are design constraints that must be incorporated into the biomaterial design.60,64 HA based synthetic extracellular matrices (sECMs) have been developed for use in drug evaluation and regenerative medicine.65 These sECMs were based on modification of the carbohydrate groups of glycosaminoglycans (GAGs) and proteins such as gelatin using hydrazides containing disulfides.66,67 More importantly, in vivo injectable cell suspensions in the sECM macromonomers can be crosslinked with cyto compatible bifunctional polyethylene glycol (PEG) derived crosslinkers.58 The mechanical properties and rates of biodegradation could be altered by several varying parameters:69: (1) molecular weight of starting HA employed; (2) percentage of thiol modification; (3) concentrations of thiolated HA and thiolated gelatin; (4) molecular weight of the crosslinker polyethylene glycol diacrylate (PEGDA); and (5) ratio of thiols to acrylates. Living hydrogels allow control of gel composition and mechanics, and permit incorporation of cells and a wide variety of small molecules, large molecules, nanoparticles, and microparticles.63

Role of HA Based Gels in Different Disease Models

Due to their ability to provide a physiologic environment that promotes stem cell survival while permitting easy in vivo transplantation and cell retention, biodegradable HA based synthetic ECMs have been utilized in a variety of rodent models. A number of previous studies have shown that biodegradable sECM increase the viability of NSC and their differentiation into neurons in vitro.70 In models of intracerebral hypoxia ischemia and traumatic spinal cord injury, sECM acted as the necessary biomechanical substrate for endogenous neuro regeneration by increasing their stem cell viability and promoting differentiation into neurons.70-72 Subsequent studies have again highlighted the utility of biodegradable scaffolds in facilitating stem cell based therapy in the CNS.73,74 While sECM are ideally suited for retaining therapeutic stem cells at the site of repair as has been demonstrated by the previous studies.

A number of studies have been published recently, which have employed the hyaluronic acid based gels for developing novel therapies for various diseases. Ganesh et al. have developed a
novel hyaluronic acid based self assembling nanosystems wherein they have encapsulated siRNA targeting CD44 in these novel hyaluronic acid based systems to treat solid tumors.75 In another recent study, it was demonstrated that the addition of recombinant gelatin to hyaluronic acid based gels makes them unique scaffolds that can be injected for soft tissue growth.76 Chang et al. in their recent study, have demonstrated the use of a novel hyaluronic acid blood biodegradable hydrogel, which offers a unique potential of transplanting stem cells into the myocardium, thereby contributing to a better cardiac function following a myocardial infarction.77 Recent in vivo studies suggest considerable potential for transplanted biodegradable scaffolds containing stem (and other neuronal) cells in models of degeneration and hypoxia ischemia.78 Encapsulating human MSC has been described as a novel hypo immunogenic platform for cellular therapy and also demonstrated this strategy to release hemopexin like protein (PEX) an anti angiogenic agent for the treatment of a glioma tumor.79

Role of HA-Based Gels in Developing Novel Therapies for Malignant Brain Tumors

Glioblastoma (GBM) is the most common primary brain tumor in adults with a very poor prognosis.80-82 Currently, treatment for GBM is maximal surgical tumor resection (debulking)83 followed by radiation therapy, with concomitant and adjuvant chemotherapy.84,85 However, recurrence rates of GBM and the associated patient mortality are nearly 100%. Despite the numerous pre clinical studies, most in vivo GBM models do not mimic the clinical scenario of surgical debulking and focus on treating solid intact intracranial tumors. Therefore, in light of the central role tumor resection plays in clinical GBM therapy, development and implementation of mouse models of GBM resection are a necessity.86 In a recent study, we have developed a mouse resection model of GBM in cranial windows using malignant GBM cells engineered with fluorescent and bioluminescent proteins, which allow real time visualization of both growth and resection of tumors in vivo thereby simulating the clinical scenario of GBM resection. While resection of the primary tumor mass has shown clinical benefit, adjuvant chemotherapy has provided limited additional benefit (Fig. 2A).86,87 One of the major impediments to the efficient delivery of many therapeutic molecules is the blood brain barrier (BBB)87 and vascular dysfunction in the tumor,88 which prevents many drugs from reaching brain tumor cells. One of the approaches to overcome the drug delivery problems to intracranial tumors is to develop on site means to deliver novel tumor specific agents. There are a number of limitations to effectively test stem cell based therapeutic interventions in a mouse model of GBM resection, including developing methods to introduce stem cells into the resection cavity to prevent rapid “wash-out” of a significant number of cells by cerebrospinal fluid (CSF). Additionally, it is critical to allow efficient secretion of anti GBM therapies and retain the ability of stem cells to migrate from the resection cavity into the parenchyma toward invasive tumor deposits. Due to their ability to provide a physiologic environment that promotes stem cell survival while permitting easy in vivo transplantation and cell retention, we utilized sECMs that are based on a thiol modified hyaluronic acid (HA) and a thiol reactive cross linker (polyethylene-glycol-diacylate), which provides biocompatibility, physiological relevance, and customizability (Fig. 1).89

In our recent study, we first assessed the influence of sECMs on stem cell survival in vivo and showed that there was a significant increase in cell viability in mice bearing sECM encapsulated NSC as compared with the non encapsulated NSC (Fig. 2). In order to follow migration of sECM encapsulated NSC, we used intravital imaging on mice bearing GBMs in a cranial window and implanted with mNSC GFP Rluc encapsulated in sECMs 1 mm away from an established tumor. It was revealed that sECM encapsulated NSC migrate out of the sECMs and specifically home to tumors in the brain over a period of 4 d (Fig. 2). In order to assess the therapeutic potential of NSC expressing therapeutic proteins that specifically kill tumor cells, we engineered NSC to express secretable (S)-tumor necrosis factor related apoptosis inducing ligand (TRAIL). TRAIL is a cytotoxic agent that is known to induce apoptosis in about 50% of GBM.89,90 We observed a significant reduction in GBM cell viability when NSC-S-TRAIL encapsulated in sECMs were placed in the culture dish containing TRAIL sensitive human GBM cells. Our in vitro studies thus revealed that sECM encapsulated engineered NSC survive longer in mice brains, migrate to tumors in the brain and induce apoptosis in cultured GBM cells (Fig. 2).

To assess survival of NSC encapsulated in sECMs in mouse model of GBM resection, we implanted NSC expressing a fusion of a fluorescent (GFP) and bioluminescent (Fluc) protein (NSC-GFP Fluc) either in suspension or encapsulated in the resection cavity of U87 GBMs. sECM encapsulated NSC were retained in the tumor resection cavity at high local concentrations adjacent to the residual tumor cells and their survival in the tumor resection cavity over a period of 1 mo was significantly higher as compared with the non encapsulated NSC in the resection cavity (Fig. 2). When sECM encapsulated NSC S-TRAIL were implanted intracranially in the tumor resection cavity to assess the therapeutic potential of sECM encapsulated NSC-S-TRAIL in mouse resection models of GBM, the sECM encapsulated NSC S-TRAIL suppressed regrowth of residual tumor cells through 49 d post resection. Highlighting the survival benefit of this approach, 100% of mice treated with NSC-S-TRAIL encapsulated in sECM after GBM resection were alive 42 d post treatment as compared with the controls which showed a median survival of 14.5 d after GBM resection. Our studies thus revealed that sECM encapsulated therapeutic NSC are retained in the tumor resection cavity, kill residual GBM cells and result in significantly increased survival of mice. Our studies on freshly isolated primary GBM lines from GBM patients and human MSC expressing S-TRAIL more accurately recapitulate the clinical scenario of GBMs and show that sECM encapsulated engineered human MSC have therapeutic benefits against primary patient derived GBMs. These studies clearly reveal that sECM encapsulated engineered stem cells are effective by way of increasing the concentration of therapeutic stem cells at the site of tumor resection to extend the drug exposure time to tumor cells.
Prospects and Caveats on the Way to the Clinics

Encapsulating therapeutic stem cells provides numerous advantages and helps overcome a number of caveats in the current cell based therapies. Stem cells have an incredible potential in developing novel targeted therapies for various diseases including cancer. The evidence that stem cells have the potential to migrate efficiently to lesions, tumors or other sites of damage has led to the development of unique stem cell based therapeutics which are gaining significant importance in today’s scientific world. This has triggered the urge in various researchers to study and obtain a detailed understanding of these mechanisms which thereby have led to the emergence of targeted therapeutics that are robust and effective. However, in spite of significant advances in this field, the translation of such therapies from bench to bedside still remains a daunting task. The appropriate disease models that exactly mimic the clinical scenario are very crucial to demonstrate the usefulness and applicability of novel therapeutic approaches. Emerging technologies like the encapsulation strategy are much needed to supplement the cell transplantation therapeutics, and the incorporation of such strategies aid in better delivery and longer duration of the desired therapy and thereby boost the probability of the success of these proposed therapies. The use of clinically employed biodegradable hydrogels like the HA based gels for encapsulation of “armed” stem cells will not only have a great impact on therapeutic outcomes but will also make the translation of therapies into clinics a reality.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We would like to thank Deepak Bhere and Tracy Twombly for help with the preparation of this manuscript. This work was supported by NIH grants CA138922, NS071197, CA173077 (KS) and James McDonald Foundation (KS).

Figure 2. Stem Cells engineered to express S-TRAIL have therapeutic efficacy in mouse tumor model of GBM resection. (A) Photomicrographs of mice bearing established U87-mCherry-Fluc GBM tumors in the cranial window that were injected with a blood pool agent, AngioSense-750 before (top) and after (bottom) tumor resection. (B) Kaplan-Meier survival curves of mice with and without resected U87-mCherry-Fluc tumors. (C-G) Stem cell (SC) (green) expressing a secretable in vivo marker, Ss-Rluc(o) or therapeutic S-TRAIL were encapsulated in sECM and placed in a culture dish containing U87-mCherry-Fluc tumor cells (red). Photomicrographs of SC at 8 h (C, E) and 24 h (D, F) and plot showing tumor cell viability (G). (H-J) SC-GFP-Fluc in suspension or encapsulated in sECM were implanted intracranially in the resection cavity of the mouse model of resection, injected with Angiosense-750 i.v. and mice were imaged by intravital microscopy and by serial imaging. Photomicrograph showing fluorescent images of sECM encapsulated SC-GFP-Fluc implanted in the resection cavity (H) and SC (green) targeting residual GBM cells (red) indicated by arrows in a tumor resection cavity with leaky vasculature (blue) (I). (J) Plot and representative figures of the relative mean Fluc signal intensity of SC-GFP-Fluc in suspension or encapsulated in sECMs placed in the GBM resection cavity. (K) SC-S-TRAIL or SC-GFP-Rluc encapsulated in sECM or SC-S-TRAIL in suspension were implanted intracranially in the tumor resection cavity and mice were followed for survival. Kaplan Meier survival curves are shown (adapted from ref. 86 with permission).


Hyaluronic acid hydrogels for vocal fold wound healing

Joel Gaston and Susan L. Thibeault

Department of Biomedical Engineering; University of Wisconsin Madison; Madison, WI USA; Division of Otolaryngology Head and Neck Surgery; Department of Surgery; University of Wisconsin Madison; Madison, WI USA

Keywords: vocal fold, tissue engineering, hyaluronic acid hydrogel, progenitor cell

The unique vibrational properties inherent to the human vocal fold have a significant detrimental impact on wound healing and scar formation. Hydrogels have taken prominence as a tissue engineered strategy to restore normal vocal structure and function as cellularity is low. The frequent vibrational and shear forces applied to, and present in this connective tissue make mechanical properties of such hydrogels a priority in this active area of research. Hyaluronic acid has been chemically modified in a variety of ways to address cell function while maintaining desirable tissue mechanical properties. These various modifications have had mixed results when injected in vivo typically resulting in better biomechanical function but not necessarily with a concomitant decrease in tissue fibrosis. Recent work has focused on seeding mesenchymal progenitor cells within 3D architecture of crosslinked hydrogels. The data from these studies demonstrate that this approach has a positive effect on cells in both early and late wound healing, but little work has been done regarding the biomechanical effects of these treatments. This paper provides an overview of the various hyaluronic acid derivatives, their crosslinking agents, and their effect when implanted into the vocal folds of various animal models.

Introduction

Vocal folds are two strips of tissue housed in the larynx, whose vibration results in voice. Voice disorders secondary to injury to these strips are the most common communication disorder seen across the lifespan. Further, conservative estimates suggest that 3 to 9% of the general population has some type of voice abnormality at any given moment in time, and that 29% of that 3 to 9% of the general population has some type of voice disorder at least once in their life. Vocal fold scarring, a specific vocal fold injury is accompanied by a marked decrease in voice quality and control secondary to pathophysiologic changes of the vocal fold lamina propria extracellular matrix (ECM). These changes directly alter vocal quality and create debilitating dysphonias due to loss of normal vibratory function.

Fibrosis induced vis-à-vis vocal fold scarring significantly increases stiffness and viscosity of the lamina propria, contributing to glottic incompetence. Treatment outcomes for patients with vocal fold ECM injury, loss, or scarring remain largely ineffective despite substantial remediative efforts that have been taken to date. For more information on these efforts, see refs 8 and 9.

The foremost reason for the inability to adequately treat vocal fold scarring is that current surgical options disrupt ECM biomechanical tissue properties and injectable gels or implants do not mimic the complex composition of the ECM. ECM composition and organization is a central issue due to its crucial contributions to vocal fold biomechanical properties and resultant voice quality. Collagen injections, fat injections, and microflaps have all been tried in an effort to remediate scarring with diminishing success. None of these interventions have been reported to yield appropriate biomechanical properties or long-term success. Human vocal fold lamina propria has an elastic shear modulus ranging from 10 Pa to 1 kPa over a frequency range of 0.01 to 10 Hz. Dynamic viscosity of the same tissue ranges from 1 to 0.1 kPa-s over the same frequency range. Ideally, hydrogels for injection should attempt to match these ranges, a goal which inhibits the usefulness of some current materials. For example, collagen has a dynamic viscosity that is an order of magnitude or greater than normal vocal folds. In addition, long-term collagen injection results have been compromised due to foreign body reaction and resorption. Most importantly, these materials have been unable to regenerate lost ECM when scarred.

In recent years, tissue engineering strategies for repair of vocal fold injury such as scarring have been introduced and centered on the use of injectable hydrogels and their use as delivery vehicles for stem cells. Injectable biomaterials overcome a major limitation of most scaffold materials used for tissue engineering, the need for surgical implantation. For the vocal folds, injectable hydrogels are strongly preferred for three main reasons. First, an injectable material could be formed into any desired shape at the site of injury upon injection. Second, crosslinkable polymer mixtures would adhere to the tissue during gel formation and the resulting mechanical interlocking would strengthen the tissue-hydrogel interface. Third, introduction of a crosslinkable hydrogel could be accomplished by injection, thereby minimizing the invasiveness and potential trauma of the procedure. The lamina propria of the vocal folds is only 3 mm thick, so the possibility of creating vocal scar and therefore impairing the mucosal wave is present with every microlaryngeal procedure. An injectable...
treatment would not increase the incidence of additional scarring and have greater applicability. The use of hydrogels to promote cell growth, differentiation, and organization is a common strategy in tissue engineering. Hydrogels, defined as hydrated polymer materials, are pliable, hydrophilic networks composed of synthetic or natural materials. Due to their pliable nature, hydrogels are commonly employed as a synthetic ECM for soft tissues, such as skin or cartilage. These characteristics also make them ideal for the vocal fold. Several important physical and chemical properties must be considered when designing or selecting appropriate materials for the vocal folds. These properties include the biomechanical properties of the hydrogel, its interactions with cells and tissues, and its ability to be easily injected through a small gauge needle.

Physical hydrogel properties are governed by repeating units of the main polymer backbone, crosslinking conditions, and processing environment. The most important physical properties are typically the mechanical properties of the material, including elastic and viscous moduli. Hydrogels can also be used as space filling scaffolds to fill defects and promote wound healing, with an emphasis on matching native tissue mechanical properties, including elastic and viscous moduli. Hydrogel mesh size, typically controlled by crosslinker concentration, can also play a critical role in cell fate processes. Different cell types may react to scaffold mesh size in different ways, which can affect proliferation rates, protein synthesis, ECM deposition and myofibroblast differentiation. Processing conditions, such as temperature and pH, also play an important role in the degree of crosslinking and gelation time. Chemical properties, governed by the interaction of cell surface receptors of resident cells with the chemical groups present on the hydrogel polymers, must also be considered for any implantable biomaterials. Downstream effects of the interaction between cell surface receptors and scaffold can influence inflammation, cell attachment, and proliferation. Scaffolds designed to avoid or even mitigate inflammation upon injection have met with some success in other parts of the body. Attachment motifs, such as the RGD peptide, are critical for adhesive cell survival and function. As such, scaffolds designed to interact with cells must have the necessary groups present.

A hydrogel scaffold designed for the human vocal fold lamina propria must take into account several unique considerations. The viscoelastic properties are especially crucial for the human vocal fold, due to the high frequency vibration required for voicing. When the viscoelastic properties of implantable biomaterials are used to treat vocal fold mucosa are greater than those of the vocal fold tissues being replaced then vocal fold oscillation and phonation becomes more difficult. This is particularly true when the vocal fold mucosa is directly involved in repair because the mucosa is the major vibratory portion of the vocal fold, especially in small-amplitude oscillations like phonation onset and offset. Viscoelastic shear property is one of the most important factors in the choice for optimal biomaterial for mucosal repair. Further, inflammation associated with injection and foreign substances in this tissue can have severe negative effects on wound healing and even life threatening airway edema. Finally, the resident cells within the lamina propria must be taken into account. The lamina propria consists primarily of vocal fold fibroblasts (VFF), which share many properties with mesenchymal stem cells (MSC), including differentiation potential. As such, any injectable hydrogel scaffold should be able to interact with VFF, and maintain them in their native, undifferentiated state.

Hyaluronic Acid

Hyaluronic acid (HA), a linear nonsulfated glycosaminoglycan, is a major component of the vocal fold ECM and is found in most tissues in the human body. Structurally, HA is a long chain composed of a disaccharide repeating unit containing D-glucuronic acid and N-acetyl-D-glucuronic acid (Fig. 1). The number of repeating units determines the molecular weight of the HA molecule, which can range from 1x10^5 Da to 2x10^6, as well as cellular interaction. Short chain HA fragments, typically in the 200-kDa range, elicit a response in inflammatory macrophages, causing expression in a number of inflammatory mediators. Long HA chains play an integral role in ECM organization and mechanical properties. The considerable chain length formed by the repeating structure allows HA to form an extensive hydrogen bond network with water across its entire length, resulting in a high viscosity solution. This not only restricts the diffusion of small molecules within the matrix, but also has a significant impact on the mechanical properties of tissue with high HA content. In the human vocal fold lamina propria, HA is the most prevalent glycosaminoglycan present, with...
roughly 6.4 μg of HA for each mg of total protein. This high concentration contributes significantly to the observed biological and mechanical properties and its subsequent effect on vibration. In particular, the large, loosely coiled molecular structure of HA allows it to function as a shock absorber, resisting tissue compression and cellular trauma. In this capacity, HA acts as a tissue damper that may protect the vocal fold edges from the oscillatory trauma experienced during phonation. Moreover, the osmotic, viscoelastic and space-filling properties of HA are important in voice because they directly affect the thickness and viscosity of the vocal fold. Maintenance of HA distribution, and ECM organization by local VFF is therefore important in voice production and wound healing. HA interacts with cells through various cell surface receptors, including CD44, which is present in both VFF and MSC. The major pathway for HA degradation in the vocal fold is through local metabolism by the hyaluronidase family of enzymes. As a result, HA injected into the vocal fold is typically rapidly degraded in as little as 3–5 d. The rapid degradation of HA injections makes its natural form unsuitable for tissue engineering, and necessitates chemical modifications. The most common modification is covalent bonding to the carboxylate or hydroxyl residues. The inherent properties of HA make it a promising candidate as a hydrogel platform for the delivery of progenitor cells, as well as providing a matrix for cell growth.

**HA Hydrogels for Vocal Fold Augmentation**

Several engineered HA hydrogels, ranging in complexity and purpose, have been investigated as potential scaffolds for progenitor cell delivery specifically for the vocal fold. One of the simplest hydrogels, the divinyl sulfone crosslinked HA derivative Hylan-B, has been shown to be non-antigenic, non-toxic, and non-inflammatory in animal models. Further modifications can be performed to add photo-polymerizable groups to Hylan-B, thereby creating a simple method to adjust the swelling ratio and degradation rate. To date, Hylan-B and its derivatives have only been used as a space filling hydrogel injection, not as delivery vehicles for progenitor cells to the vocal fold. Human use with Hylan B, or Hylaform (Allergan, Inc.), in the vocal fold lamina propria has not been reported in the literature and is no longer being marketed in the United State for clinical use. Recently, a new process utilizing a latent crosslinking agent has produced thiol-modified HA with considerable advantages, including tunability. Under appropriate conditions, HA can be reacted with thiol cross-linker 3,3'-dithiobiis (DTP), to produce HA-DTPH (Fig. 2). The DTP crosslinker, a non-cytotoxic agent, allows for hydrogel formation through the formation of disulfide bonds at room temperature and reduced pH. Due to the disulfide bond nature of the cross-linked network, a simple reducing agent such as dithiothreitol can be used to dissolve the gels. The addition use of this crosslinking agent, as well as the mild conditions necessary for reaction to occur makes HA-DTPH capable of cell encapsulation. Indeed, murine fibroblasts seeded within the cross-linked gel not only showed 95% viability after 96 h of culture, but proliferated as well. The clinical use of HA-DTPH for cell encapsulation is limited however, with gels taking up to 120 min to form. This period of time is not ideal for clinicians or during a surgical operation, curbing its clinical usefulness. The use of disulfide bonds as crosslinkers readily lends itself to adding in functional groups capable of significantly altering hydrogel properties, such as gelation time. The ability to rapidly and easily alter hydrogel properties allows researchers, and eventually clinicians, to tailor HA hydrogels to specific applications as they are needed.

In order to promote faster gelation times, polyethylene glycol diacrylate (PEGDA) was incorporated into HA-DTPH hydrogels. By varying the ratio of PEGDA to HA-DTPH, gelation time can be decreased to as low as five minutes. As the ratio of HA-DTPH to PEGDA was increased, crosslinking density decreased and swelling ratio increased, with the 1:1 ratio having the highest crosslink density and the lowest swelling ratio. Thus, the mesh size, gelling time, and swelling ratio can all easily be altered simply by varying the ratio, offering a significant degree of control over the resultant hydrogel. Crosslinked gels can be naturally degraded by hyaluronidases in a manner consistent with non-crosslinked HA. This is a significant finding, as it allows cells to interact with and remodel the crosslinked HA using normal pathways and molecules. Like HA-DTPH, it is possible to encapsulate cells in the hydrogel by adding them to the solution before adding the crosslinker, in this case PEGDA. Using this method, human tracheal scar fibroblasts were shown to be viable for 28 d in vitro, increasing their number by nearly 10-fold. Similarly, cell-seeded hydrogels implanted

![Figure 2. Hyaluronic acid backbone with attached DTP crosslinker. The 2-carboxylic acid group on D-glucuronic acid (GlcA) serves as the site for covalent attachment of the DTP crosslinker.](image-url)
subcutaneously in nude mice were shown to not induce necrosis or damage surrounding tissue, as well as maintain cellular phenotype. Recently, HA-DTPH has been slightly modified to include additional carboxylate groups on the HA backbone, effectively altering the viscosity and reducing the rate of degradation. This new HA derivative has been named CMHA-S or Carbylan-S, and will be referred to as CMHA-S from this point forward. The extra carboxylate groups on the HA backbone also allow further ease of chemical modification and crosslinking possibilities. For example, the crosslinking of CMHA-S to thiolated gelatin (Gtn-DTPH), resulting in a material designated Extracel® (a.k.a., HyStem-C®) allows for cell interaction through natural attachment motifs present on the denatured collagen present in the gelatin.

Mechanical Effects of HA Gel Injection

Injection of HA gels, as well as their crosslinked derivatives, has shown to be a successful method for restoring normal vocal fold mechanical properties in vivo. In vivo vocal fold studies with gel injection have been performed primarily in rabbits, due to their similarity of vocal fold tissue to human vocal fold and ease of access to the larynx intraorally. Early studies with Hylan-B show that injections into rabbit vocal fold lamina propria have mechanical results similar to normal vocal fold. Normal vocal folds from animals sacrificed 6 mo after injection have a dynamic viscosity lower than other injectable biomaterials such as collagen or Teflon and similar to non-injected vocal fold tissue, demonstrating efficacy.

In vitro rheology results on CMHA-S and HA-DTPH-PEGDA detailing the elastic shear viscous moduli of both materials indicated that the CMHA-S is the stiffer of the two materials, yet still within normal range to that of human vocal fold lamina propria. Excised tissue from injured rabbit vocal fold injected with CMHA-S had a lower elastic shear modulus than both the injured tissue treated with saline controls and injured tissue injected with HA-DTPH-PEGDA. A significant difference was also observed in the viscous modulus; both CMHA-S and HA-DTPH-PEGDA were significantly less viscous than the saline-treated samples. Overall, both gels were biomechanically compatible to human vocal fold mucosa, with the possibility of CMHA-S providing a better environment for subsequent biomechanical outcomes as determined by histological outcomes, as discussed below.

Fibrotic Effects of HA Gel on VFF and MSC

Injection of HA hydrogels without cells has been shown to have a positive effect on resident VFF response to wound healing. One of the earliest such methods employed CMHA-S and a HA-DTPH-PEGDA hydrogel, effectively comparing the effects

<table>
<thead>
<tr>
<th>HA derivative</th>
<th>Type of crosslinking</th>
<th>Animal model and vocal fold condition</th>
<th>Seeded cell type</th>
<th>Biomechanical properties, compared with saline injection</th>
<th>Fibrotic effects compared with saline controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-DTPH-PEGDA</td>
<td>PEGDA</td>
<td>Injured rabbit&lt;sup&gt;29&lt;/sup&gt;</td>
<td>None</td>
<td>-No change in G'</td>
<td>-Moderate fibrosis, no difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Lower G''</td>
<td>-No difference for Col</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-No difference for HA</td>
</tr>
<tr>
<td>Carbylan-S&lt;sup&gt;*&lt;/sup&gt;</td>
<td>PEGDA</td>
<td>Injured rabbit&lt;sup&gt;29&lt;/sup&gt;</td>
<td>None</td>
<td>-Lower G'</td>
<td>-Mild fibrosis, significant difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Lower G''</td>
<td>-No difference for Col</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-No difference for HA</td>
</tr>
<tr>
<td>Extracel&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Thiolated gelatin (Gtn-DTPH)</td>
<td>Scarred rabbit&lt;sup&gt;24&lt;/sup&gt;</td>
<td>None</td>
<td>-Lower G'</td>
<td>-Increased Col</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Lower G''</td>
<td>-Increased FN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Increased Procollagen</td>
</tr>
<tr>
<td>Extracel&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Thiolated gelatin (Gtn-DTPH)</td>
<td>Scarred rabbit&lt;sup&gt;24&lt;/sup&gt;</td>
<td>Autologous VFF</td>
<td>-Lower G'</td>
<td>-Increased Col</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Lower G''</td>
<td>-Increased FN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Increased Procollagen</td>
</tr>
<tr>
<td>Collagen-HA cogel</td>
<td>Physical entanglement</td>
<td>Injured rabbit&lt;sup&gt;38&lt;/sup&gt;</td>
<td>Rabbit adipose MSC</td>
<td>-No data available</td>
<td>-Increased Col for 3 mo, then normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Increased HA for 3 mo, then normal</td>
</tr>
</tbody>
</table>

G', elastic modulus; G'', viscous modulus; Col, collagen; FN, fibronectin; MSC, mesenchymal stem cell; VFF, vocal fold fibroblasts.
of each on cell survival in an in vivo environment. Trichrome staining performed on sections of the excised larynges showed that the average fibrosis levels for animals injected with the HA-DTPH-PEGDA were moderate and not significantly different from saline-treated controls (Fig. 3). Average fibrosis levels for the CMHA-S treated group displayed only mild fibrosis, with a significant difference compared with controls, indicating less tissue fibrosis. It should also be noted that an ELISA assay determined that HA levels in the hydrogel treated vocal fold samples were the same as those in the saline controls, indicating that the injected gels had degraded. In an in vitro investigation to study the effects of various engineered HA hydrogels on progenitor cell fate, MSCs isolated from abdominal fat were encapsulated in several hydrogels, including Restylane® (crosslinked HA), and a cogel of Restylane® and fibrin. Cell morphology and proliferation in these gels showed increased elongation and DNA content in the fibrin-HA cogel, compared with the HA gel. An increase in elastin expression was also observed in the cogel samples, but decorin levels remained similar in both gel types. Finally, CD44 significantly decreased in the cogel sample, indicating a potential deregulation of support for the stem cell maintenance of the undifferentiated state. This is supported by lower expression levels of CD105, a cell surface marker highly expressed in undifferentiated MSCs. These results indicate that fibrin-HA cogels may be useful as an MSC delivery system while also increasing cell proliferation and elastogenesis. The lower expression levels for CD44 and CD105 may indicate that it is possible to affect MSC differentiation within the cogel as well.

Fibrotic Effects of Cell-Seeded HA Gel Injection

The first method to employ both a hyaluronic acid hydrogel and progenitor cells in vivo showed the potential of a cell-seeded scaffold to alter tissue viscoelastic properties to a comparable degree as hydrogel alone. Scarred rabbit vocal fold tissue was injected with one of four groups: saline, autologous VFF, Extracele®, or Extracele® with autologous VFF. Extracele® injected vocal fold tissue demonstrated increased collagen and fibronectin deposition compared with saline controls, but no increase in procollagen staining was observed. The tissue injected with seeded Extracele® had decreased elastic shear modulus and viscous modulus compared with untreated vocal folds, but lacked any significant difference when compared with any of the other treatment groups. Both viscous and elastic shear moduli for the autologous VFF group was significantly decreased compared with the moduli measured in saline controls and the other treatment groups. Decreased moduli are typically related to an improved mucosal wave and decrease in tissue fibrosis. Overall, the results indicate that injection of autologous VFF alone represents the best option for improved viscoelastic properties of vocal fold scar. The results demonstrating that VFF seeded Extracele® injections improve biomechanical properties, as wound healing strategies employing cell-matrix injections are viable without a loss of viscoelasticity.

The benefit of cell seeded Extracele® matrices, with respect to wound healing, has also been demonstrated in a rat model.

In a fashion similar to the 2008 study, Johnson et al. seeded bone marrow derived mouse MSCs in an Extracele® matrix and injected it in a scarred rat animal model. This effect of the seeded Extracele® was compared with injections containing saline, stem cells alone, or Extracele® alone, with respect to gene expression and apoptosis. The cell-seeded matrix showed a significantly higher level of collagen III, fibronectin, and TGF-β1 over any of the other treatment groups. The increased expression of collagen and fibronectin, both fibrous proteins with extensive cell adhesion motifs, indicate the early establishment of a lattice for wound healing events. The concomitant upregulation of TGF-β1 further supports the establishment of an early lattice,
as TGF-β1 plays a significant role as a promoter of ECM protein expression. Importantly, injection of the cell seeded scaffolds did not cause a rise in myofibroblasts within the lamina propria, as was evidenced by a lack of increase in smooth muscle actin expression and staining over saline controls. Increased myofibroblast levels and persistence of these levels can lead to the onset of hypertrophic scar formation. These investigations suggest that cell-seeded Extracel® promotes early wound healing by increasing the production ECM proteins necessary for early wound healing while simultaneously decreasing the likelihood of scar formation.

Most recently, adipose-derived MSCs (AdMSC) were injected into injured rabbit vocal folds with a collagen-hyaluronic acid composite hydrogel, with histological effects being investigated after 15 d, 40 d, 3 mo, 6 mo and 1 y after injection. This study differs from the other cell-seeded gel studies in several important ways, including gel crosslinking and time points. The collagen-hyaluronic acid gel utilized in this investigation was not chemically crosslinked in any way, but simply consists of collagen and hyaluronic acid gels mixed together. Further, gels seeded with AdMSC were cultured in vitro for one week with an air-liquid interface, indicative of promoting epithelial cell differentiation and stratification. Results demonstrated that collagen content in the vocal folds injected with cell seeded scaffolds increased until the 3 mo time point, where it peaked. At 12 mo, collagen content and distribution were close to that of normal controls. Hyaluronic acid content following injection followed a similar trend, with levels peaking at day 40, then declining and finally stabilizing at 12 mo. After injection of the cell-seeded HA-collagen composite, the fibronectin content was highest at 40 d, and then decreased until stabilizing around 12 mo. Hematoxylin and eosin staining also revealed physiologic differences between AdMSC-seeded HA-collagen, adipose-derived MSC implantation, and untreated (but still injured) vocal fold tissue. At 15 d, inflammatory cell migration and infiltration were present in all treatment groups. At 6 mo, untreated controls had large amounts of fibrosis and disorganized lamina propria ECM was observed. Tissue implanted with adipose-derived MSC showed a gradual decrease in fibrous tissue starting at 6 mo to nearly normal levels at 12 mo, with some irregular distribution. Finally, the cell-seeded HA-collagen composite gel showed the most improvement, with normal levels of fibrous tissue at 6 mo and normal organization at 12 mo. This study lends further proof of concept to the theory that progenitor-seeded HA supports in early wound healing, while not inducing fibrosis and scar formation. Despite the results demonstrated in this study, little was done to characterize the gel utilized itself. No data was reported on the hydrogel residence time in vivo nor the rheological properties of the gel in vitro or in vivo. Further an uncrosslinked gel is likely to be degraded quickly. Given the positive histological results reported, further investigation into this material is warranted.

Taking a step back to better understand why cell seeded Extracel is beneficial in vivo, a recent in vitro study has also shown this combination may have an impact on key players in inflammation. VFF cultured on the 2D surface of Extracel® hydrogels had a higher expression of IL-8 and TNF-α, two pro-inflammatory cytokines, compared with polystyrene controls. VFF seeded within 3D Extracel constructs have also been shown to increase IL-8 and TNF-α mRNA levels compared with polystyrene. It should be noted that increased expression of COX-2 and IL-6, two pro-inflammatory cytokines, was not observed in either condition. A similar study investigating the effect of macrophages grown on Extracel® or VFF-seeded Extracel® found differences between macrophage inflammatory phenotype. Macrophages grown on the cell-seeded Extracel® showed decreased CD116 and increased HLA-DR, indicative of an anti-inflammatory phenotype. Translation of these findings to in vivo work may have a significant effect on clinical vocal fold injections via modulation of the macrophage phenotype. Recent in vitro work found that CMHA-S could enhance the role TNF-α in remodeling the lamina propria layer via significantly downregulating TIMP3 and extracellular matrix-related mRNA transcript levels for collagen III and fibronectin and upregulation MMP1 and MMP2 expression, resulting in increased MMP/TIMP3 ratios. Taken together, the anti-inflammatory properties of cell-seeded Extracel® hydrogels show translational potential for clinical use.

Conclusions

Hyaluronic hydrogels has been investigated for vocal fold regeneration and wound healing since the early 2000s. They can be easily chemically modified to provide the necessary viscoelastic properties that match the vocal fold lamina propria which a paramount consideration for this tissue type. Early in vitro and in vivo animals studies have demonstrated that most HA hydrogels alone improve wound healing in injured and scarred models. More recently the delivery of mesenchymal stem cells to the vocal fold using a hyaluronic acid hydrogel augments and amplifies improved wound healing and minimizing scarring. Unique in vitro investigations have demonstrated benefits of these hydrogels in terms of inflammatory effects on both resident VFF and recruited macrophages. The in vitro and in vivo studies reported herein provide the necessary data to move forward with FDA approval for human clinical trials with hyaluronic hydrogels injections in isolation and with cell therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors would like to acknowledge funding from NIH NIDCD R01 4336 and T32 DC009401.


Enhancing retention and efficacy of cardiosphere-derived cells administered after myocardial infarction using a hyaluronan-gelatin hydrogel

Rachel Ruckdeschel Smith,1,* Eduardo Marbán2 and Linda Marbán1

1Capricor Inc.; 8700 Beverly Boulevard; Los Angeles, CA USA; 2Cedars-Sinai Heart Institute; Los Angeles, CA USA

Keywords: cardiosphere-derived cells, myocardial infarction, hyaluronan-gelatin hydrogel, CADUCEUS trial, ALLSTAR trial

Cardiosphere-derived cells (CDCs) are under clinical development and are currently being tested in a clinical trial enrolling patients who have undergone a myocardial infarction. CDCs are presently administered via infusion into the infarct-related artery and have been shown in early clinical trials to be effective agents of myocardial regeneration. This review describes the administration of CDCs in a hyaluronan-gelatin hydrogel via myocardial injection and the subsequent improvements in therapeutic benefit seen in animal models. Development of a next generation therapy involving the combination of CDCs and hydrogel is discussed.

Introduction

Cardiosphere-derived cells (CDCs) have been under clinical development since 2009. The ongoing ALLSTAR trial (NCT01458405) is examining the safety and efficacy of allogeneic CDCs administered by intracoronary infusion in patients who have suffered a myocardial infarction (MI). Findings from the CADUCEUS trial, in which autologous CDCs were administered to post-MI patients, have already foreshadowed the potential clinical utility of CDCs in this patient population. Both cell therapies are believed to act via the same mechanisms, to stimulate endogenous regeneration and attenuate fibrosis, and do so without eliciting an immune response, in the case of allogeneic CDCs. The effects manifest preclinically as a decrease in cardiomyocyte apoptosis, recruitment of cardiac stem cells, stimulation of cardiomyocyte proliferation, increase in blood vessel density and decrease in collagen deposition; clinically as a reduction in infarct size, accumulation of viable myocardium and attenuation of left ventricular remodeling.

CDCs will experience a nearly 50% reduction in infarct size over the course of a year, commensurate with the addition of new myocardial mass. Despite the sizeable observed and expected benefits of CDC therapy in clinical studies, preclinical studies have shown that no more than 5% of cells survive longer than 24 h after intracoronary delivery in either saline or a cryopreservation solution containing DMSO (dimethyl sulfoxide). Presumably, poor cell retention and engraftment can be attributed to multiple factors, such as: the use of a minimally-invasive delivery approach, intracoronary infusion, which is not as effective as intramyocardial injection, the harsh ischemic microenvironment making transplanted cells susceptible to apoptosis, and the lack of space and anchorage sites available for transplanted cells making them susceptible to interstitial clearance by the lymphatic system. Furthermore, these retention and engraftment issues are common to most cell therapies, not specific to CDCs, although solutions may need to be tailored to cell type. While many possible solutions do exist, in the case of CDCs, intramyocardial injection in a hyaluronan-gelatin hydrogel has been shown to meaningfully improve retention, engraftment and efficacy in preclinical studies. A next generation therapy for MI patients may involve the combination of CDCs and hydrogel.

The current status of cell therapy for MI is summarized herein along with the preclinical data supporting the use of a CDC-hydrogel combination therapy. Plans to move that combination product toward the clinic are described as well.

Cell Therapy for Myocardial Infarction

This year 1.3 million Americans will have a new or recurrent MI. Only 15% of MI sufferers will die as an immediate result, a mortality rate that has declined in recent years thanks to advances in the acute management of MI. However, 36% of MI survivors will develop heart failure (HF), and will consequently be at increased risk for death. Following an MI, ejection fraction (EF), end-systolic and end-diastolic volumes (ESV and EDV), and to a greater extent infarct size have been shown to predict subsequent HF development, adverse left ventricular (LV) remodeling, MACE (major adverse cardiac events), and
all-cause mortality for patients.\(^{12-16}\) Infarct size alone is a rigorous, independent predictor of MACE-free survival that can be used to classify patients as at-risk (e.g., infarct size \(\geq 18.5\%\)) or not-at-risk.\(^{14}\) Even with maximum medical care, however, once an infarct is established its size does not change.\(^{17}\) While long-term adverse neurohormonal responses can be countered with \(\beta\) blockers and ACE-inhibitors and the likelihood of recurrent ischemic events can be decreased with aggressive secondary prevention,\(^{18}\) no therapy currently available can reduce the size of an established infarct.

Cell therapy aims to alter this fixed trajectory for MI survivors: to intervene in the process of adverse LV remodeling, to reduce infarct size and to actually regenerate viable myocardial tissue in its place. The field to date has focused primarily on when to administer cells and what cells to administer, while relying on minimally-invasive delivery approaches (i.e., intracoronary infusion) that could also be readily and widely adopted by clinicians. More novel delivery approaches (i.e., transcendocardial injection) have begun to establish a decent clinical safety profile,\(^{19}\) but seem to offer marginal added efficacy benefits. The result of all attempts to date has been partial restoration of cardiac structure and function. On the whole (in a meta-analysis considering 50 studies enrolling 2625 patients) autologous bone marrow cells, by far the cell type most extensively studied clinically, have led to a 4.0% increase in EF, an 8.9 mL reduction in ESV, a 5.2 mL reduction in EDV, and a 4.0% reduction in infarct size compared with control.\(^{20}\) These primary efficacy data can be termed marginally positive at best. Although one of the first and most positive studies\(^{21}\) is now reporting unanticipated benefits on long-term clinical endpoints (e.g., death, recurrent MI, HF development, revascularization),\(^{22}\) room for improvement undeniably still exists.

**Clinical Use of Cardiosphere-Derived Cells**

Cardiosphere-derived cells have yet undergone limited clinical use, but may have come the closest to achieving the goals of cell therapy, including viable tissue regeneration. The CADUCEUS (CArdiosphere-Derived AUtologous Stem CELls to Reverse VentricUlar DySfunction) trial demonstrated the safety and efficacy of autologous CDC administration via intracoronary infusion in patients with LV dysfunction post-MI.\(^{2}\) In the randomized, controlled, dose-escalating Phase I trial, autologous CDCs manufactured from endomyocardial biopsy specimens were infused into the infarct-related artery in 17 patients. Eight patients were followed as standard-of-care controls. In >12 months of follow-up, safety endpoints were equivalent. Contrast-enhanced magnetic resonance imaging (MRI) revealed reductions of infarct size (scar mass normalized to total LV mass) in CDC-treated patients (-7.7 ± 4.8%), but not in controls (+0.3 ± 5.4%) over a period of 6 mo. The treatment effect in CDC patients nearly doubled at 12 mo (-12.3 ± 5.0%), amounting to a 46% relative reduction of infarct size (from a baseline of 24%), but remained unchanged in controls (-2.2 ± 7.1%). In comparison to the overall effect reported for bone marrow cells on infarct size,\(^{20}\) CDCs elicited much larger reductions. Theoretically, tissue regeneration should be manifested not only by scar shrinkage but also by an increase in viable tissue (measured independently by MRI). Accordingly, while changes in scar mass mirrored changes in infarct size, viable tissue mass increased in CDC-treated patients (+13.0 ± 11.4 g at 6 mo), but not in controls (+0.9 ± 6.2 g at 6 mo), and the correlation between scar shrinkage and increased viability was highly significant \((r = -0.59, p = 0.0007)\). This novel finding indicates that CDCs may in fact be truly regenerative.

Following the discovery that autologous and allogeneic CDCs act via the same mechanisms of action, and furthermore, that allogeneic CDCs could be safely administered in the setting of MI without eliciting an immune response,\(^{3,4}\) the ALLSTAR trial was initiated. ALLSTAR (ALLogeneic Heart STem Cells to Achieve Myocardial Regeneration) is a Phase I/II randomized, double-blinded, placebo-controlled safety and efficacy study. The ongoing study is evaluating intracoronary infusion of allogeneic CDCs or placebo in 248 patients with LV dysfunction post-MI. Allogeneic CDCs are manufactured from a single donor for use in many recipients, and several donors will be utilized during the course of ALLSTAR, so as to demonstrate product comparability. The study will carefully monitor patients

### Figure 1.

CDC surface markers compatible with hydrogel. Representative flow cytometry histograms showing expression of CD49a (A), CD49b (B), CD49c (C) and CD44 (D) in CDCs (in blue). Isotype controls are shown in red.
for the development of inflammation or an immune reaction in response to allogeneic CDC administration, while simultaneously assessing changes in infarct size, cardiac function, quality-of-life and cardiac biomarkers. ALLSTAR will establish much about the effectiveness of CDC therapy for post-MI patients. In the meantime, efforts to improve upon the therapy will continue.

**Approaches to Enhance Efficacy of Cell Therapy**

CDCs, much like other cell types, are retained in the heart more effectively when intramyocardial injection as opposed to intracoronary infusion is employed for cell administration. In a clinically-relevant preclinical model, use of a minimally-invasive catheter-based transendocardial injection system resulted in ~15% engraftment 24 h after delivery, as opposed to the ~5% achievable with intracoronary infusion. Prior preclinical studies have shown also that preventing mechanical loss and washout of CDCs from the contracting, perfused myocardium (by completely arresting the heart), can lead to a further 4-fold increase in 24 h engraftment. Ultimately, efficacy scales with engraftment, and novel approaches aimed at reducing or preventing mechanical loss while enhancing cell survival and subsequent engraftment could contribute greatly to the efficacy of CDC therapy.

One such approach combines CDCs with an in situ polymerizable hydrogel (Hystem™, BioTime Inc.) that can be delivered intramyocardially, either by direct surgical injection or by a transendocardial catheter. Multiple hydrogels alone have demonstrated a capacity for improving cardiac function in preclinical models and at least one is undergoing clinical testing in a post-MI patient population (NCT01226563). Cell-hydrogel combinations of various sorts have also been characterized preclinically as therapies for myocardial repair. A hyaluronan-gelatin hydrogel not dissimilar to that selected for use with CDCs has been shown to be particularly well-suited for withstanding the contractile forces of the heart. Hystem-C™ is a hyaluronan-based hydrogel crosslinked using thiol-reactive poly(ethylene glycol) diacrylate and covalently linked to thiolated collagen to aid cell attachment. The base product is chemically-defined and nonimmunogenic and the collagen is porcine derived. Hyaluronan is a glycosaminoglycan component of the extracellular matrix of all connective tissues, making it an attractive vehicle for cell delivery. Hyaluronan-based hydrogels can be formulated with varying gelation times depending on the concentrations of the individual monomers, making them suitable for catheter delivery and in situ polymerization. Collagen is a major component of the heart’s natural extracellular matrix. Furthermore, hyaluronan-gelatin hydrogels biodegrade in vivo over the course of four to eight weeks due to the action of hyaluronidases and collagenases produced naturally by cells. It has been demonstrated that Hystem-C™ promotes tissue repair in various organ systems, but our study represents its first use in the heart.

The CDC-hydrogel combination therapy was intended to: (1) reduce cell loss due to leakage by virtue of hydrogel viscosity and by acting as a substrate to which CDCs can anchor; (2) bolster cell survival by reducing the level of apoptosis following transplantation by offering an environment in which CDCs are temporarily protected from the in vivo elements; (3) allow for the gradual migration of CDCs out of the hydrogel, concurrent with its degradation, and into the myocardium where they can form new cardiomyocytes and endothelial cells; and (4) improve cardiac function beyond the level seen with cells delivered in a saline vehicle due to improved cell engraftment and prolonged paracrine effects.
A mouse model of MI was next employed to investigate the combination product in vivo. CDCs were incorporated within Hystem-C™, Hystem™ or PBS (phosphate-buffered saline) and delivered as an aqueous solution (such that gelation occurred in situ), using a needle and syringe, intramyocardially in mice. Cell retention 24 h after delivery was dramatically increased in the Hystem-C™ condition (Fig. 3b), by more than 7-fold compared with both the Hystem™ and PBS conditions (Fig. 3a), resulting in an average retention of ~35% of the total cells delivered. Long-term cell engraftment (3 weeks after delivery) was significantly increased for the Hystem-C™ group compared with the PBS group (Fig. 3C), though expectedly reduced compared with 24 h. The results in terms of cardiac function and structure revealed improvements in left ventricular ejection fraction (LVEF; Fig. 4a) and additions of viable myocardial mass (Fig. 4b) for the Hystem-C™ group that exceeded the effects seen in all other groups (p < 0.05 vs. all other groups). The two other treatment groups included for comparison, Hystem-C™ alone (no cells) and CDCs in PBS, showed a preservation of LVEF over the study period, as opposed to the clear improvement seen in the CDCs in Hystem-C™ group, while the PBS only control group deteriorated. Severe

Hyaluronan-Gelatin Hydrogel Delivery of Cardiosphere-Derived Cells

CDCs, which express multiple collagen-binding integrins (α1, α2, α3; Fig. 1A–C) as well as the receptor for hyaluronic acid (CD44; Fig. 1D), were found to be highly compatible with Hystem-C™ when incorporated within the hydrogel and cultured for up to one week. Cells loaded with viable and dead cell fluorescent indicators, allowed for the visualization of CDC morphology and the quantitative assessment of viability over time. CDCs adopted a spread morphology, typical of that seen in culture, in Hystem-C™ (Fig. 2B), while CDCs in Hystem™ (the base product without collagen; Fig. 2A) remained rounded. Furthermore, more than 80% of CDCs embedded in Hystem-C™ remained viable for one week, while more than 50% of CDCs embedded in Hystem™ were dead within the week (Fig. 2C). Additionally, in vitro migratory capacity of the CDCs was greatest when Hystem-C™ was the material from which they migrated, with Hystem™ acting no differently than culture media alone in terms of a migration platform. These data indicated that CDCs could survive embedded in Hystem-C™ short-term, for the amount of time it may take for the hydrogels to begin to biodegrade in vivo, and that Hystem-C™ as a delivery vehicle may in fact stimulate CDC migration into the surrounding myocardium in vivo at such a time when environmental cues are favorable.

Figure 4. Cardiac function and heart morphometry. (A) Changes of left ventricular ejection fraction (LVEF) measured by echocardiography from baseline to 3 weeks in each group. (B) Quantitative analysis and LV morphometric parameters of Masson’s trichrome images (n = 3–5 mice per group). * indicates p < 0.05 when compared with Control. **indicates p < 0.05 when compared with any other group.

Figure 5. Promotion of angiogenesis by CDC/hydrogel transplantation. (A) Representative confocal images showing α smooth muscle actin-positive vasculature in the hearts receiving various treatment products. (B) Quantitation of α smooth muscle actin-positive vasculature in various groups (n = 5 mice per group). *indicates p < 0.05 when compared with Control. **indicates p < 0.05 when compared with any other group. Bar = 200 μm.
adverse remodeling (chamber dilatation and infarct wall thinning) and a limited amount of viable mass were observed in the control group. The treatment groups showed significantly reduced degrees of adverse remodeling and significantly greater amounts of viable mass, with benefits increasing across the Hystem-C™ alone, CDCs in PBS and CDCs in Hystem-C™ groups. An analysis of CDC differentiation capacity revealed that delivery in Hystem-C™ did not impair their ability to form new cardiomyocytes or endothelial cells, that more differentiation occurred as a consequence of greater engraftment. An analysis of the angiogenic effect, one of several manifestations of the paracrine effects of CDC treatment, demonstrated that neovascularization was improved when Hystem-C™ was used for CDC delivery (Fig. 5). These data illustrated that Hystem-C™ as a delivery vehicle could in fact improve both short-term retention and long-term engraftment of CDCs in the setting of MI, and could also lead to improvements in treatment efficacy as assessed by cardiac function and cell activity in vivo. These data in total serve as compelling proof-of-concept for the CDC-hydrogel combination therapy.

Advancing a Cardiosphere-Derived Cell and Hydrogel Combination Therapy to the Clinic

Next steps for the CDC-hydrogel combination therapy will include compatibility testing with one of several catheter-based transcatheterial injection systems and large animal studies to evaluate safety and efficacy in a clinically-relevant model. An appropriate patient population, perhaps one in which intracoronary infusion in a previously infarcted artery poses a safety risk, can then be targeted for a first clinical study. In the arena of cell therapy for MI, a new product that can overcome the widespread issues affecting cell engraftment should ultimately result in greater clinical benefits for patients. Cardiosphere-derived cells paired with Hystem-C™ have shown great promise thus far in preclinical testing. Such a product may also reduce the manufacturing time and cost needed to generate an adequate therapeutic dosage, making the therapy more accessible to patients. The general techniques developed and knowledge gained from this study may be applicable to other cell types as well, and delivery with Hystem-C™ may in fact benefit the field of cell therapy for MI as a whole.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Delivery of EPC embedded in HA-hydrogels for treatment of acute kidney injury

Brian B. Ratliff and Michael S. Goligorsky*
Departments of Medicine, Pharmacology and Physiology; New York Medical College; Valhalla, NY USA

Keywords: endothelial progenitor cells, hyaluronic acid based hydrogels, stem cell therapy, acute kidney injury, endotoxemia, sepsis, Adriamycin-induced kidney injury

Adoptive transfer of stem cells has shown potential as an effective treatment for acute kidney injury (AKI). The current strategy for adoptive transfer of stem cells is by intravenous injection. However, this conventional method of stem cell delivery is riddled with problems causing reduced efficacy of the therapeutic potential of delivered stem cells. This review summarizes the recent advancements in an alternative method of stem cell delivery for treatment of AKI, embedding stem cells in hyaluronic acid (HA-) based hydrogels followed by their implantation. Furthermore, one stem cell type in particular, endothelial progenitor cells (EPC), have shown remarkable therapeutic benefits for treatment of AKI when delivered by HA-hydrogels. The review also summarizes the delivery of EPC by HA-hydrogels in the setting of AKI.

Stem Cells for Therapeutic Use

While the potential of stem cells for tissue repair and regeneration in treatment of disease and injury has been subject to intense investigation over the past 20 years, the full therapeutic potential of these cells has yet to be fully realized. During this time, various stem cell lines have been isolated and characterized including embryonic and adult stem cells such as hematopoietic, mesenchymal, cardiac, neuronal and retinal. All these various stem cell lines have been examined for their therapeutic benefits including their adoptive transfer for treatment of injuries and diseases as diverse as post-chemotherapy blood disorders, myocardial infarction, burns, spinal cord and brain injuries, eye injury, diabetes, Crohn disease and muscular dystrophy, to name a few.1 The kidney has been no stranger to this ever expanding and evolving field. Stem cells have been demonstrated to possess a notable repair and regenerative potential when delivered to the injured kidney.2-8 However, currently much more advancement is needed before stem cell therapy is successfully applied clinically for broad scale use in the treatment of kidney disease.

One type of stem cells that has shown remarkable renoprotective potential without significant side effects are endothelial progenitor cells (EPC). EPC have been shown to improve renal function, attenuate the pro-inflammatory response associated with renal injury, and improve damage to tubules and renal vascular segments during kidney injury while providing enhanced neoangiogenesis.2,4,7,8 The beneficial attributes associated with EPC delivery for treatment of kidney damage counter the vascular impairment that occurs in the course of various episodes of acute kidney injury (AKI) that leads to the progressive nature of renal dysfunction and disease.2,7-9 An intact and healthy EPC niche, residing in the bone marrow but also found locally in renal vascular beds such as in the area of the adventitia layer of vessels, is relied on to maintain normal vascular function including maintenance and possible replacement of the endothelium.10-12

The loss of EPC integrity during kidney disease was illustrated by our group in an Adriamycin model of nephropathy,4 in which the progressive nature of renal injury was heavily influenced by the destruction of competent endogenous EPC. The deterioration of the bone marrow EPC niche prevented both the mobilization of these cells to the sites of renal injury and ensuing repair of damage. When exogenous EPC were adoptively transferred, renal function considerably improved. These results are not exclusive to Adriamycin-induced nephropathy, but have also been seen in other models of AKI such as sepsis-induced AKI.2

Problems with Current Methods of Cell Therapy: Treatment of AKI

One of the major problems confronting current stem cell (including EPC) therapy is the method for cell delivery. When stem cells are delivered by IV injection, less than 3% of the delivered cells find their way to the injured kidney and engraft, while majority of delivered cells undergo programmed cell death (anoikis) before they are capable of providing any therapeutic benefits to

---

*Correspondence to: Brian B. Ratliff; Email: ratlifbb@gmail.com
Submitted: 10/25/12; Revised: 12/13/12; Accepted: 12/14/12
Citation: Ratliff BB, Goligorsky MS. Delivery of EPC embedded in HA-hydrogels for treatment of acute kidney injury. Biomatter 2013; 3:e23284; http://dx.doi.org/10.4161/biom.23284

www.landesbioscience.com Biomatter e23284-35
damaged tissues. Many current trials examining the delivery of stem cells for treatment of kidney disease use IV injection of large quantities of cells (usually around 1 million cells per injection), administered all at once into the circulation by a bolus IV injection. Often times these delivered cells become trapped in the pulmonary vasculature causing embolism or suffer from anoikis before ever making it to the injured kidneys. Furthermore, if the impairment of kidney function is due to circulating factors such as cytokotoxins, then stem cells introduced into the circulation by IV injection become susceptible to the harmful effects of such circulating toxins.

Another major problem with IV delivery of stem cells is related to integrin dependent activation and homing of delivered cells. While β2 integrins are the major regulators of EPC transendothelial migration, integrins α5β1, α6β1, αvβ3 and αvβ5 are major determinants of EPC homing, invasion, differentiation and paracrine factor production with integrin α4β1 being a key regulator of EPC retention and/or mobilization from the bone marrow. The expression and activation of these integrins on the surface of stem cells is critical for their homing to proper sites of damage, adherence and exertion of their renoprotective paracrine influence.

In essence, integrins give EPC a “guidance mechanism” to find their way around the circulation and locate the targeted damaged tissue. Furthermore, activation of integrins on the surface of EPC and subsequent effects on intracellular cytoskeleton properties within the EPC, has also been shown to enhance the release of both anti-inflammatory and pro-regenerative substances from EPC enhancing their reparative capabilities. Integrins are activated once these cells bind to substrates that contain a RGD sequence (an arginine-glycine-aspartic acid conserved motif found in cell adhesion substrates such as fibronectin). When EPC are delivered by IV injection, because of the aqueous nature of both the delivery vehicle solution and the circulating plasma, delivered cells lack substrate RGD adherence cues that would trigger the expression and activation of integrins on their surface. The lack of integrin activation on delivered EPC prevents these cells from fully eliciting their protective effects. This presents yet another problem with conventional methods of IV delivery of cells.

Bioengineered Scaffolds for Stem Cell Delivery: Treatment of AKI

In attempt to protect delivered stem cells and to provide them with a microenvironment conducive to their viability while maximizing their therapeutic potential, our laboratory has experimented with the delivery of EPC embedded in bioengineered scaffolds. Scaffolds provide substantial flexibility because they can be constructed from many different substances and their properties chemically changed to allow for creation of alternative niche-like microenvironments. For instance, variable scaffold compositions can be manipulated to affect embedded stem cell retention, differentiation and proliferation while also influencing the release of anti-inflammatory molecules from embedded cells. The selection of specific biomaterial compositions for scaffold engineering can provide embedded cells with surroundings resembling the endogenous extracellular matrix and offer many advantages in regard to cell therapy applications. The use of such biomaterials allows for direct delivery and retention of EPC at the precise location of tissue damage leading to the improved delivery efficiency, a more intense therapeutic response, and avoidance of side effects related to systemic IV delivery.

Various bioengineered scaffolds have been constructed and tested for cell therapy purposes. For embedding of stem cells, it is highly desirable to create scaffolds that closely mimic the endogenous stem cell niche. Collagen, gelatin, fibrin, silk, agarose, alginate, dextran, cellulose, chitosan, heparin, chondroitin sulfate and hyaluronic acid engineered scaffolds are the more commonly and successfully used natural biomaterials for stem cell delivery in animal models. Recently, hyaluronic acid (HA) based hydrogels have emerged as a bioengineered scaffold that holds potential for its application in cell therapy. An advantage of stem cell delivery by embedding in HA-hydrogels is the ease of delivery. HA-hydrogels are implanted before their solidification, which allows for hydrogels with embedded cells to be delivered by a simple syringe injection. Once injected, the hydrogel concludes the solidification process within a few minutes. The delivery of HA-hydrogel scaffolds (without embedded cells) composed of thiol-modified gelatin (derived from collagen I and IV) results in the least inflammatory response, as compared with various other bioengineered scaffolds. In experiments by our lab, we observed greatest response from embedded EPC when pronectin (50mg/ml) was added to these denatured collagen HA-hydrogels and crosslinked at 4% with polyethylene (gloyl) diacrylate (PEGDA). Addition of pronectin introduced the critical RGD binding element required by EPC for adhesion and activation of cell surface integrins including α5β1, α6β1, αvβ3, αvβ5 and α4β1. The use of 4% PEGDA crosslinker provides optimal hydrogel rigidity and permits significant cell retention and viability while preventing infiltration of toxins present in the external milieu.

An advantage of HA-hydrogels is their ability to be readily dissolved on demand. Since the main constituents of hydrogels are denatured collagen and HA, these scaffolds can easily and rapidly be dissolved with hyaluronidase and collagenase, subsequently allowing the release and mobilization of embedded stem cells. Used in the correct concentrations, these digesting enzymes do not cause damage or phenotypical change to embedded cells. The application of digesting enzymes is simple and consists of a mere injection directly into the implanted HA-hydrogel, followed by dissolution of the gel and complete mobilization of embedded cells within 24–48 h. The ability to release embedded EPC on demand provides greater flexibility of cell therapy. For instance, HA-hydrogels with embedded EPC can be implanted into desired tissues where they remain until the release of EPC is required for local tissue repair. Meanwhile, the considerable retention of EPC in HA-hydrogels means embedded cells will stay in their implant locality (usually up to two weeks), while retaining their capability to secrete anti-inflammatory and pro-angiogenic molecules that can escape the scaffolding into the surrounding tissue and circulation. The ability to regulate the release of EPC from implanted scaffolds also allows flexibility in the timing of hydrogel implantation for treatment of kidney injury. The kidney is especially useful for HA-hydrogel delivery of stem cells.
because during kidney injury, the injured tissue endogenously releases hyaluronidase. The intrinsic release of hyaluronidase means the kidney, when injured, will automatically digest implanted HA-hydrogels even without injection of enzymes, allowing release of embedded stem cells. Since hyaluronidase is released when kidneys are initially injured, the digestion of HA-hydrogels and subsequent EPC release occurs at an optimal time during kidney injury allowing for EPC therapeutic effects to engage before damage becomes too extensive and irreparable. A schematic summary of the implantation of HA-hydrogels and subsequent release and mobilization of embedded EPC for treatment of AKI is provided in Figure 1.

In studies by our lab, we have conducted multiple experiments using HA-hydrogels as a bioengineered scaffold for delivery of stem cells (EPC) for the treatment of various forms of AKI (the results of these experiments are summarized in Tables 1 and 2). The first AKI model we examined was a model of Adriamycin (doxorubicin) induced nephropathy, which is characterized by tubulointerstitial damage, glomerulosclerosis with accompanying glomerular visceral epithelial cell damage and proteinuria. In preliminary in vitro experiments, we first assessed if HA-hydrogel scaffolds offered protection to embedded EPC against toxins such as Adriamycin. Indeed, at concentrations of Adriamycin (1–50 μM) that normally caused cell damage and death, HA-hydrogel embedding protected cells against cytotoxicity and resulted in 6-fold enhanced cell viability of EPC. This effect was primarily the result of the impedance for toxins to readily infiltrate the HA-hydrogel and damage embedded EPC. In a murine in vivo model, HA-hydrogels with embedded EPC were delivered to Adriamycin-induced AKI mice. In these in vivo experiments, HA-hydrogels were implanted at two locations, subcutaneously in the ear (for ease of microscopic monitoring of fluorescently labeled embedded cells) and subcapsularly in the kidney. While HA-hydrogels were implanted prior to Adriamycin-induced AKI, EPC were not released from these scaffolds until after administration of Adriamycin. Embedded EPC were released into the systemic circulation from ear implanted HA-hydrogels by direct injection of digestive enzymes within 1–2 h after Adriamycin administration. HA-hydrogels implanted in the kidney were digested by endogenous renal secretion of hyaluronidase allowing for release of embedded EPC locally into the injured kidney. Adoptive transfer of EPC into mice with Adriamycin-induced AKI reduced both short- and long-term elevation in serum creatinine levels by 50–60% and long-term proteinuria by 60–75%, with greater improvement observed with EPC delivery by HA-hydrogel as compared with IV injection. Engraftment of EPC into the damaged kidney was enhanced by as much as 6-fold when EPC were delivered by HA-hydrogel as opposed to IV delivery. Only slight differences were observed when HA-hydrogels were implanted in the ear as compared with the kidney. These differences included increased renal engraftment of EPC when HA-hydrogels were implanted in the kidney while ear implants and subsequent EPC release resulted in a slightly better improvement of systemic functions, such as blood pressure.

We further examined the effects of EPC delivery by HA-hydrogel to determine if the enhanced therapeutic effect of these bioengineered scaffolds was applicable to other kidney injury models in addition to Adriamycin nephropathy. In a mouse renal

Figure 1. Schematic of the implantation of HA-hydrogels and subsequent release and mobilization of embedded EPC for treatment of AKI in a mouse model. 1) HA-hydrogels with embedded EPC are implanted either superficially into ears or subcapsularly into kidneys. 2) Induction of AKI (cyto-/endotoxins). 3a) Kidney implants are digested by endogenous release of hyaluronidase from the kidneys during AKI and embedded EPC are mobilized into the kidney, or 3b) ear implants are digested by direct injection of hydrogel-digesting enzymes and embedded EPC are mobilized into the circulation. 4) Released EPC generate therapeutic effects (see Tables 1 and 2).
ischemia-reperfusion injury (IRI) model of AKI, in which renal blood flow was occluded for 30 min causing microcirculatory and tubular damage, the delivery of EPC by HA-hydrogel resulted in 50% attenuation of the associated rise in serum creatinine 36 h post-IRI, demonstrating the beneficial effects of HA-hydrogel delivery. EPC have been shown to mediate re-vascularization and angiogenesis in models of vascular injury. Improvement in microvascular competence and function plays a role in the renoprotective effects offered by EPC during AKI. When EPC were delivered into a model of vascular injury (a murine model of hind limb femoral ligation), conventional IV delivery of EPC improved neovascularization by 25% while HA-hydrogel delivery of EPC improved neovascularization and angiogenesis by 45%, further illustrating enhancement of vascular reparative effects mediated by HA-hydrogel delivery.

The improvement in the therapeutic efficacy of adoptive transfer of EPC by HA-hydrogel delivery prompted us to examine the effects of this phenomenon more elaborately in another model of AKI. Endotoxemia and related sepsis is a growing health problem in our society today and is a significant contributor to AKI. During sepsis, circulating endotoxins cause damage to the vascular endothelium and promote a catalytic immune system response that involves substantial release of chemokines and cytokines causing multiple organ damage including AKI. Since sepsis-induced AKI is a result of microvascular damage and the immune response, the ability of EPC to mediate both vascular repair/regeneration while also modulating the immune response makes these stem/progenitor cells attractive candidates for cell therapy of this form of AKI. A critical effect that occurs during sepsis is that intrinsic EPC niches and reparative stem cell pools are adversely affected by circulating endotoxins including loss of endogenous EPC competence, integrity and viability leading to the inability of these stem cells to mediate vascular and renal repair and regeneration. While adoptive transfer of EPC is an attractive approach to combat sepsis-induced AKI, conventional IV delivery of these cells is problematic because once EPC are introduced into the circulation, they are predisposed to damage due to their exposure to the circulating endotoxins initially responsible for both systemic and local tissue damage.

Similar to our studies on Adriamycin, HA-hydrogels protected embedded EPC from the endotoxin LPS in in vitro experiments. In in vivo studies, adoptive transfer of EPC to septic mice improved systemic functions within 24 h after injection of LPS. Sepsis is typically characterized by hypotension and elevation of circulating hepatic enzymes. EPC delivery by IV injection significantly improved arterial blood pressure and reduced hepatic release of ALT and AST, however, EPC delivery by HA-hydrogel completely normalized both blood pressure and hepatic enzyme release to healthy baseline levels. EPC adoptive transfer also afforded renoprotection, both short- and long-term during sepsis. Impaired renal hemodynamics has been found to be crucially involved in kidney injury during sepsis. IV administration of EPC improved renal blood flow, both cortical and medullary, by 40% and 22%, respectively; however, this improvement was markedly potentiated to 75% and 56% when EPC were delivered by HA-hydrogel. In addition to improving renal microcirculation, renal function (as measured by serum creatinine) was improved by 55% upon HA-hydrogel EPC delivery. In long-term studies, EPC delivery by HA-hydrogel cut in half the renal interstitial fibrosis that was associated with sepsis-induced AKI and endogenous bone marrow EPC competence was restored. While adoptive transfer of EPC in general led to improvement of all sepsis-induced systemic and renal abnormalities, the therapeutic ability of EPC was greatly enhanced (both short- and long-term) when these cells were delivered by HA-hydrogel scaffolding. The duration of a latent period before cells are released from the implanted HA-hydrogel scaffold and become exposed to the circulating endotoxins represents the major difference between the adoptive transfer of cells via HA-hydrogel vs. IV delivery.

In the latest series of hydrogel experiments by our lab, we have combined delivery of EPC with renal mesenchymal stem cells (MSC). Our lab has previously isolated MSC from the murine kidney. These MSC are positive for typical stem cell markers such as Sca-1, CD29, CD44, CD73, negative for CD117 and CD45, and are able to differentiate into multiple cell lineages including chondrocytes, osteocytes and adipocytes. Intrinsic stem cell niches, such as in bone marrow and in cardiac tissue, contain multiple progenitor cell types and lineage committed cells which serve as supporting cells for the niche. Supporting cells help maintain the quality and stability of the niche and play a role in asymmetric stem cell division and renewal of daughter cells. Introducing renal MSC into HA-hydrogels to support EPC may improve the quality of the bioartificial niche and enhance the regenerative response of the embedded cells. MSC are especially attractive candidates for the supporting cell role because they have been reported to improve stem cell niche quality and stem cell mobilization and homing through release of SDF-1, SCF and LIF. MSC also possess immuno-modulatory properties due to their release of anti-inflammatory molecules, such as IL-10, and are capable of inducing neighbor cells to secrete cytokines which

| Table 1. The improvement in various systemic and local parameters during sepsis- and Adriamycin-induced AKI after treatment by adoptive transfer of EPC by either conventional IV delivery or implantation of HA-hydrogels with embedded EPC. |
|-----------------|-----------------|
| Parameter       | IV delivery     | HA-hydrogel delivery |
| Mean blood pressure | ++              | +++                  |
| Hepatic enzymes (ALT and AST) | +               | +++                  |
| Bone marrow EPC colony forming ability | ++              | +++                  |
| Serum creatinine | ++              | +++                  |
| Intrarenal microcirculation | ++              | +++                  |
| EPC engraftment | +               | +++                  |
| Cytokines/chemokines | ++              | +++                  |
| Proteinuria     | ++              | +++                  |
| Long-term fibrosis | +++             | +++                  |

All parameters were measured 18–36 h after induction of AKI, except long-term proteinuria and fibrosis, which were measured 3 weeks and 2 mo, respectively, after AKI induction. + denotes improvement.
anti-inflammatory and pro-angiogenic paracrine molecules such as IL-10, IL-8, IP-10, MMP’s and various growth factors including VEGF, bFGF and HGH.42

Conclusions

EPC delivery in bioengineered scaffolds, such HA-hydrogels, offers significant advantages over conventional cell delivery by IV injection. Pronectin-coated HA-hydrogels closely resemble

Table 2. The summary of systemic and renal effects when EPC are delivered by HA-hydrogel embedding and transplantation as compared with conventional IV injection during AKI

<table>
<thead>
<tr>
<th>Systemic therapeutic effects</th>
<th>Renal therapeutic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized mean blood pressure</td>
<td>Increased renal EPC engraftment</td>
</tr>
<tr>
<td>Reduced release of ALT/AST</td>
<td>Enhanced cortical and medullary microcirculation</td>
</tr>
<tr>
<td>Improved competence of endogenous stem cells</td>
<td>Improved proteinuria</td>
</tr>
<tr>
<td>Reduced release of pro-inflammatory cyto-/chemokines</td>
<td>Improved serum creatinine</td>
</tr>
<tr>
<td>Enhanced release of anti-inflammatory cyto-/chemokines</td>
<td>Decreased fibrosis</td>
</tr>
<tr>
<td>Enhanced release of pro-angiogenic molecules</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Schematic representation (left) and corresponding images (right) (40x magnification) of EPC treated with 10 μg/ml LPS for 24 h. During LPS treatment, EPC were plated on culture plates (A) (without HA-hydrogel embedding), embedded in HA-hydrogels (B), or embedded in HA-hydrogels along with MSC (C). Embedding EPC in HA-hydrogels improved EPC viability and resistance to endotoxins, an effect that was considerably enhanced when EPC were co-embedded with MSC. To determine cell viability, cells were subject to a LIVE/DEAD assay in which live cells were stained green with calcein and dead cells were stained red with ethidium homodimer.
endogenous stem cell niches and thus provide a microenvironment conducive for viability and expansion of embedded cells while also providing protection from circulating cytokine and endotoxins. HA-hydrogels are readily implantable within tissues and organs, including the kidney. The composition of HA-hydrogels allows them to be easily dissolved on demand permitting mobilization of embedded cells. In experiments using multiple models of AKI, the use of HA-hydrogels for delivery of EPC enhances the therapeutic efficacy of these stem cells by providing substantial systemic and renal protective effects. The enhanced therapeutic performance of HA-hydrogel embedded EPC is the result of multiple factors. Increased cellular αβ1 integrin binding to RGD sequences present in HA-hydrogels activate EPC causing potentiated release of anti-inflammatory and pro-angiogenic molecules, engraftment and transdifferentiation. Despite current advancements, continued development of the adoptive transfer of EPC by HA-hydrogel delivery is needed before its application is clinically realized for treatment of AKI.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Studies from the authors’ laboratories were supported in part by the grants from the AHA 12SDG0800606 (BBR) and NIH DK54602, DK50278, DK45462 and Westchester Artificial Kidney Foundation (MSG).

References
4.  Park HC, Yasuda K, Ratliff B, Stoessel A,ershkovski Y, Yamamoto I, et al. Postobstructive regeneration of kidney is derailed when surge in renal stem cells dur-

Volume 3 Issue 1

Biomatter


Immunotherapeutic organoids
A new approach to cancer treatment

Marta Compte, Natalia Nuñez-Prado, Laura Sanz and Luís Álvarez-Vallina*
Molecular Immunology Unit; Hospital Universitario Puerta de Hierro Majadahonda; Madrid, Spain

Keywords: antibody, immunotherapy, gene-therapy, mesenchymal stem cells, cell factories, organoids

Abbreviations: AAV, adeno-associated virus; bsAb, bispecific antibody; CEA, carcinoembryonic antigen; ECM, extracellular matrix; HSC, hematopoietic stem cells; mAbs, monoclonal antibodies; MSC, mesenchymal stem cells; scFv, single-chain antibody fragment

Therapeutic monoclonal antibodies have revolutionized the treatment of cancer and other diseases. However, several limitations of antibody-based treatments, such as the cost of therapy and the achievement of sustained plasma levels, should be still addressed for their widespread use as therapeutics. The use of cell and gene transfer methods offers additional benefits by producing a continuous release of the antibody with syngenic glycosylation patterns, which makes the antibody potentially less immunogenic. In vivo secretion of therapeutic antibodies by viral vector delivery or ex vivo gene modified long-lived autologous or allogeneic human mesenchymal stem cells may advantageously replace repeated injection of clinical-grade antibodies. Gene-modified autologous mesenchymal stem cells can be delivered subcutaneously embedded in a non-immunogenic synthetic extracellular matrix-based scaffold that guarantees the survival of the cell inoculum. The scaffold would keep cells at the implantation site, with the therapeutic protein acting at distance (immunotherapeutic organoid), and could be retrieved once the therapeutic effect is fulfilled. In the present review we highlight the practical importance of living cell factories for in vivo secretion of recombinant antibodies.

Introduction

Monoclonal antibodies (mAbs) have revolutionized the field of biology and medicine since their first description in 1975.1 However, the development of therapeutic monoclonal antibodies has been complicated by a number of technical challenges including the appearance of immunogenic responses against murine antibody domains, and their inability to trigger human effector functions.2 These drawbacks were overcome initially by the generation of chimeric and humanized antibodies and now can be completely avoided by using fully human antibodies.2

However, several limitations hamper native mAb-based treatments, such as low tumor-to-blood ratio, due to long serum half-life and limited tissue penetration, and the need for high doses over a long period of time. There is a wide range of different recombinant antibodies fragments with differences in molecular weight, valence, specificity and format. Thus, half-life and tumor penetration can be fine-tuned by adjusting these parameters.3 There remain, however, at least two major concerns: the extremely high cost of therapy and the achievement of sustained plasma levels, since the recommended dosage and administration involve repeated bolus injections, with fluctuating plasma concentrations ranging from subtoxic to subtherapeutic.

In Vivo Secretion of Therapeutic Antibodies

Gene therapy has the potential to overcome some of the shortcomings associated with conventional bolus protein therapy by producing a sustained release of the antibody with syngenic glycosylation patterns, that makes the antibody potentially less immunogenic and better tolerated.4 Two main approaches to gene therapy include in vivo and ex vivo gene transfer methods (Fig. 1). In vivo gene therapy implies direct injection of genetic material into the human body, usually by using viral vectors. Ex vivo gene therapy involves modifying target cells, prior to implanting these into the tissues of the living body.

In Vivo Secretion of Full-Length mAbs

Pioneering work by Noel et al.5 demonstrated that several types of non-lymphoid cells have the ability to secrete full-length IgG antibodies in vitro after retroviral gene transfer. Furthermore, implantation of ex vivo retroviral-modified myoblasts resulted in detectable mAb serum levels (~1–3 μg/ml) for long periods of time. Four years later, the same group demonstrated that in vivo administration of high doses of a recombinant adenovirus encoding the same antibody gene resulted in a 100- to 200-fold increase in mAb serum levels (~200 μg/ml). However, adenoviral vectors are highly immunogenic and trigger an innate immune response that reduces therapeutic effect and causes inflammation-related side effects.6,7 On the other hand, adeno-associated virus (rAAV) is a weak innate immunogen and it does not elicit...
the immune response observed for adenoviral vectors, although both type of viral vectors share the drawback of prevalence of neutralizing antibodies in the human population.8 Using this expression system, Fang et al.9 generated a rAAV serotype 8 encoding a full-length VEGFR-2 neutralizing mAb (DC101). The mAb is expressed from a single open reading frame by linking the heavy and light chains with a self-processing peptide 2A derived from the foot-and-mouth disease virus. A furin cleavage site was introduced to remove 2A-derived residues. A single dose of rAAV8-DC101 resulted in long-term expression of high-levels (> 1,000 μg/ml) of mAb, demonstrating significant anti-tumor efficacy. Watanabe et al.10 reported that adenoviral vectors and rAAV encoding a full-length anti-VEGF mAb equivalent to bevacizumab (Avastin®) effectively suppresses the growth of human tumors.

Sustained high serum levels of a full-length anti-HER2 (also referred to as HER2/neu or ErbB-2) mAb have also been reported after intramuscular administration of a rAAV vector incorporating the furin/2A technology for monocistronic expression of both heavy and light chains. This strategy achieved significant tumor growth inhibition when rAAV was administered prior to tumor challenge, and demonstrated antitumor efficacy against pre-established tumors when AAV was administered up to 20 d after tumor challenge.11 Also, long-term therapeutic levels of an anti-HER2 mAb have been documented after a single intravenous injection of an AAV vector based on the non-human primate AAV serotype rh.10 containing the furin/2A expression system, which reduced the growth of HER2 positive tumors and increased the survival of tumor-bearing mice.12

A different strategy for cancer therapy used a systemically administered bidirectional lentiviral vector for the in vivo secretion of a full-length anti-Met mAb. This approach resulted in substantial inhibition of tumor growth.13 Recently, Balazs et al.14 showed that a single intramuscular injection in mice of a specialized AAV vector containing a self-processing 2A sequence induces lifelong expression of high concentrations of a HIV neutralizing full-length mAb (b12), and it is possible to reach sustained protection against HIV infection.

In Vivo Secretion of Novel Recombinant Antibody Formats

In an attempt to improve tumor penetration, recombinant antibodies with modified properties have been generated. Novel antibody formats, such as the single-chain antibody (scFv), exhibit better pharmacokinetics than intact IgG.3 However, scFv antibodies exhibit rapid blood clearance and poor retention times on the target owing to small sizes and monovalent binding properties, which results in the necessity of frequent delivery of such fragments.3 To circumvent these limitations, several gene therapy approaches have been developed to express antibody fragments in vivo.

In 2002, Arafat et al.15 demonstrated for the first time the therapeutic effect of a scFv secreted by eukaryotic cells. Effective concentrations of scFv were achieved following in vivo administration of an adenoviral vector expressing an anti-erbB2 scFv. Furthermore, in vivo gene transfer via the anti-erbB2 scFv encoding adenovirus resulted in substantial inhibition of tumor growth. A few months later Sanz et al. demonstrated that in vivo secretion of the L36 scFv,16 that recognizes an angiogenesis-associated laminin epitope,17 inhibited tumor growth in vivo.18 In 2006, the same group reported that genetically modified human cells efficiently secreted trivalent and hexavalent antibodies, based on fusion of L36 scFv to different portions of the C-terminal noncollagenous domain of collagen XVIII.19 In vivo secretion of the multivalent L36 antibodies was more effective in preventing tumor growth than the monomeric scFv counterpart.19

Afanasieva et al.20 demonstrated that a single systemic administration of recombinant adenovirus encoding an anti-VEGF scFv (V65 scFv) or bivalent derivatives (minibody and scFv-Fc) resulted in tumor inhibition and had a therapeutic effect equivalent to that of multiple injections of high amounts of purified V65 scFv. Systemic administration of an adenoviral vector has also been used to deliver in vivo an immunotoxin comprising an anti-HER2 scFv as targeting moiety.21

Bispecific antibodies (bsAbs) represent promising approaches to more efficacious antitumor therapy.22 BsAbs targeting tumor-associated antigens (TAA) and effector cell trigger molecules have been generated and shown to redirect cellular cytotoxicity toward target cells.2,22,23,24 The potential of T-cell activating anti-TAA x anti-CD3 bsAbs in cancer therapy has been demonstrated in a variety of in vitro and in vivo models and several bsAbs

Figure 1. Strategies for in vivo secretion of therapeutic antibodies: direct injection of genetic material using non-viral or viral vectors (in vivo gene therapy), and implantation of genetically modified cells (ex vivo gene therapy).
have been tested in clinical trials.\textsuperscript{22} In fact, an anti-EpCAM x anti-CD3 full-length IgG (catumaxomab) has been approved for intraperitoneal treatment of malignant ascites.\textsuperscript{25,26} However, maximum tolerated dose is low due to the toxicity caused by induction of ‘cytokine storm’, a consequence of cross-linking of T cells with accessory cells bearing Fc receptors, followed by cytokine release-related symptoms.

For these reasons, it is highly recommended using the use of recombinant Fc-lacking bsAbs such as tandem scFvs [(scFv).\textsubscript{2}] and diabodies. Numerous studies have demonstrated the potency of these formats in preclinical and clinical studies.\textsuperscript{27,28} However, these recombinant bsAbs present a very short serum half-life and must be administered by continuous infusion using portable minipumps.

In 2003, Blanco et al.\textsuperscript{29} demonstrated for the first time therapeutic effect of recombinant Fc-lacking anti-carcinoembryonic antigen (CEA) x anti-CD3 two-chain diabody secreted by mammalian cells. They generated a bicistronic vector that enable the secretion of functionally active diabody by gene-modified human cells, and promoted unstimulated human primary T cells to proliferate and kill CEA-expressing cancer cells. Importantly, locally produced diabody showed significant cytotoxic activity in vivo against established tumors.\textsuperscript{29} Four years later, the same group generated a bicistronic lentiviral vector and demonstrated that primary human lymphocytes can be efficiently transduced to secrete high levels of functional anti-CEA x anti-CD3 diabody. Importantly gene-modified lymphocytes significantly reduced in vivo tumor growth rates in xenograft studies.\textsuperscript{30}

**Ex Vivo Gene-Modified Cells as Factories for Long-Term In Vivo Secretion of Therapeutic Proteins**

The application of ex vivo gene-modified human cells for in vivo secretion of therapeutic proteins offers several advantages over viral vector-mediated in vivo gene therapy. Viral vectors are highly efficient as gene delivery vehicles, but raise concerns about safety and limitation of the effect due to immune response. The use of ex vivo gene-modified cells as “factories or biological pumps” eliminates the risk of non-specific diffusion, allows in vitro quantification of protein secretion, and offers the possibility of selection for high-level expression clones prior to administration. Furthermore, cells can be retrieved if administered in certain formats, and/or armed with a “suicide” gene (e.g., HSV thymidine kinase), which can be activated to ensure the destruction of the cell inoculum.

Terminally differentiated mature cells possess a short lifespan, and this implies an obvious limitation to their application in cell-based gene therapy strategies for cancer immunotherapy. In contrast, stem/progenitor cells have greater expansion capacity and constitute a more appropriate cellular source. Since the advent of gene therapy, hematopoietic stem cells (HSC) have been a delivery cell of choice. However, the ex vivo expansion of adult HSC is expensive and time-consuming, and they are difficult to transduce. On the contrary, mesenchymal stem cells (MSC) can be found in virtually all postnatal tissues, are easily transduced and exhibit a unique in vitro expansion capacity using a simple medium formulation.

**Local or Systemic Injection of Ex Vivo Gene-Modified MSC for Cancer Immunotherapy**

MSC suspended in PBS or solution saline, have been administered in preclinical models of cancer through a wide variety of routes (Table 1), in the proximity or not of the tumor. Although MSC are supposed to be endowed with tumor-targeting properties, most studies showing therapeutic effect of gene-modified MSC use one of these two strategies: either coinjection or
### Table 1. Ex vivo gene-modified mesenchymal stem cells for cancer immunotherapy

<table>
<thead>
<tr>
<th>Gene</th>
<th>MSC source</th>
<th>Route of administration</th>
<th>Disease model</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX3CL1</td>
<td>(M) BM</td>
<td>i.v.</td>
<td>(M) melanoma/colon cancer lung metastasis</td>
<td>(M) C57BL6/BLBc</td>
<td>45</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>(M) BM</td>
<td>intratracheal</td>
<td>(M) colon cancer lung metastasis</td>
<td>(M) C57BL6/BLBc</td>
<td>46</td>
</tr>
<tr>
<td>IFN-α</td>
<td>(M) BM</td>
<td>i.v.</td>
<td>(M) melanoma lung metastasis</td>
<td>(M) C57BL6</td>
<td>47</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) BM</td>
<td>i.v.</td>
<td>(H) melanoma (s.c.), lung metastasis</td>
<td>(M) athymic nude</td>
<td>35</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) BM</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) athymic nude</td>
<td>48</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) BM</td>
<td>i.v.</td>
<td>(H) breast cancer lung metastasis</td>
<td>(M) SCID</td>
<td>49</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(M) BM</td>
<td>i.v.</td>
<td>(M) prostate cancer lung metastasis</td>
<td>(M) C57BL6</td>
<td>36</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) UCM</td>
<td>i.v.</td>
<td>(H) breast cancer lung metastasis</td>
<td>(M) SCID</td>
<td>50</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) UCM</td>
<td>i.v.</td>
<td>(H) bronchioloalveolar cancer (orthotopic)</td>
<td>(M) SCID</td>
<td>51</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(H) BM</td>
<td>i.p.</td>
<td>(H) pancreatic carcinoma (orthotopic)</td>
<td>(M) SCID</td>
<td>52</td>
</tr>
<tr>
<td>IFN-β</td>
<td>(C) AT</td>
<td>s.c.</td>
<td>(M) melanoma (s.c.)</td>
<td>(M) C57BL6</td>
<td>53</td>
</tr>
<tr>
<td>IL-2</td>
<td>(R) BM</td>
<td>i.t.</td>
<td>(R) glioma (intracranial)</td>
<td>(R) Fisher 344</td>
<td>54</td>
</tr>
<tr>
<td>IL-7</td>
<td>(R) BM</td>
<td>i.t.</td>
<td>(R) glioma (intracranial)</td>
<td>(R) Fisher 344</td>
<td>55</td>
</tr>
<tr>
<td>IL-12</td>
<td>(H) BM</td>
<td>i.t./i.p.</td>
<td>(M) melanoma (s.c.)/lung metastasis</td>
<td>(M) C57BL6</td>
<td>56</td>
</tr>
<tr>
<td>IL-12</td>
<td>(M) BM</td>
<td>p.t.</td>
<td>(M) glioma (intracranial)</td>
<td>(M) C57BL6</td>
<td>57</td>
</tr>
<tr>
<td>IL-12</td>
<td>(M) BM</td>
<td>i.v.</td>
<td>(M) tumors (s.c.), spontaneous metastasis</td>
<td>(M) C57BL6/BLBc</td>
<td>58</td>
</tr>
<tr>
<td>IL-12</td>
<td>(M) BM</td>
<td>i.v.</td>
<td>(H) Ewing’s sarcoma (s.c.)</td>
<td>(M) athymic nude</td>
<td>59</td>
</tr>
<tr>
<td>IL-12</td>
<td>(H) BM</td>
<td>i.v.</td>
<td>(H) renal carcinoma (s.c.)</td>
<td>(M) athymic nude</td>
<td>60</td>
</tr>
<tr>
<td>IL-12</td>
<td>(H) UCB</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) C57BL6</td>
<td>61</td>
</tr>
<tr>
<td>IL-18</td>
<td>(R) BM</td>
<td>i.t.</td>
<td>(R) glioma (intracranial)</td>
<td>(R) Sprague-Dawley</td>
<td>62</td>
</tr>
<tr>
<td>IL-21</td>
<td>(H) UCB</td>
<td>i.v.</td>
<td>(H) ovarian cancer</td>
<td>(M) athymic nude</td>
<td>63</td>
</tr>
<tr>
<td>TNFSF2 (TNF α)</td>
<td>(H) UCB</td>
<td>p.t.</td>
<td>(H) gastric cancer (s.c.)</td>
<td>(M) athymic nude</td>
<td>64</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) UCB</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) athymic nude</td>
<td>31</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) SCID</td>
<td>32</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>p.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) athymic nude</td>
<td>65</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.t./i.v.</td>
<td>(H) breast cancer (s.c.)/lung metastasis</td>
<td>(M) NOD–SCID</td>
<td>66</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) AT</td>
<td>i.t./i.v.</td>
<td>(H) cervix carcinoma (s.c.)</td>
<td>(M) NOD–SCID</td>
<td>67</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.t./i.v.</td>
<td>(H) colorectal carcinoma (s.c.)</td>
<td>(M) athymic nude</td>
<td>34</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.v.</td>
<td>(H) pancreatic cancer (s.c.)</td>
<td>(M) athymic nude</td>
<td>68</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) UCB</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) athymic nude</td>
<td>69</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.t.</td>
<td>(H) colorectal carcinoma (s.c.)</td>
<td>(M) athymic nude</td>
<td>70</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) AT</td>
<td>i.t.</td>
<td>(R) glioma (intracranial)</td>
<td>(R) Fisher 344</td>
<td>71</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>i.t.</td>
<td>(H) glioma (intracranial)</td>
<td>(M) athymic nude</td>
<td>72</td>
</tr>
<tr>
<td>TNFSF14 (LIGHT)</td>
<td>(H) UCB</td>
<td>p.t.</td>
<td>(H) gastric cancer (s.c.)</td>
<td>(M) athymic nude</td>
<td>73</td>
</tr>
<tr>
<td>TNFSF14 (LIGHT)</td>
<td>(M) BM</td>
<td>s.c. (contralateral)</td>
<td>(M) breast cancer (s.c.)</td>
<td>(M) BALB/c</td>
<td>39</td>
</tr>
</tbody>
</table>

**Confined cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>MSC source</th>
<th>Route of administration</th>
<th>Disease model</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CEA x anti-CD3 dAb</td>
<td>(H) BM</td>
<td>Hydrogel-embedded</td>
<td>(H) colorectal carcinoma (s.c.)</td>
<td>(M) athymic nude</td>
<td>40</td>
</tr>
<tr>
<td>IL-2</td>
<td>(M) BM</td>
<td>Matrigel-embedded</td>
<td>(M) melanoma (s.c.)</td>
<td>(M) C57BL6</td>
<td>74</td>
</tr>
<tr>
<td>IL-12</td>
<td>(M) BM</td>
<td>Matrigel-embedded</td>
<td>(M) breast cancer (s.c.)</td>
<td>(M) BALB/c</td>
<td>75</td>
</tr>
</tbody>
</table>

MSC, mesenchymal stem cells; M, mouse; H, human; R, rat; BM, bone-marrow; UCM, umbilical cord matrix; UCB, umbilical cord blood; AT, adipose tissue; dAb, diabody; s.c., subcutaneous; i.v., intravenous; i.t., intratumoral; p.t., peritumoral; i.p., intraperitoneal.
intratumoral/peritumoral injection for localized tumors, or intravenous administration for lung metastasis, taking advantage of the fact that most of the intravenous-inoculated MSC (especially human MSC) are physically retained in the mouse pulmonary filter as a consequence of their size.\textsuperscript{31,32}

In fact, Bexell et al.\textsuperscript{33} found no evidence of MSC homing to intracranial gliomas following intravenous injections and suggested that MSC should be administered by intratumoral implantation to achieve a therapeutic effect. In a work by a different group, MSC genetically modified for the expression of TNFSF10/TRAIL (MSC\textsuperscript{TRAIL}) inhibited colon carcinoma tumor growth after subcutaneous coinjection, but systemic application of MSC\textsuperscript{TRAIL} had no effect, which appeared to be due to lung entrapment and low rate of tumor grafting.\textsuperscript{34}

Intratumoral/peritumoral MSC have been validated for the delivery of CX3CL1, IFN-β, IL-2, IL-7, IL-12, IL-18, TNF α and TRAIL to subcutaneous or intracranial tumors. Using a different experimental approach, Studeny et al.\textsuperscript{35} reported that intravenous-injected, IFN-β expressing MSC (MSC\textsuperscript{IFNβ}) significantly extended animal survival in mice with human melanoma lung metastases. These results were supported by Ren et al.\textsuperscript{36} who demonstrated the therapeutic effect of systemically administered murine MSC\textsuperscript{IFNβ} in a mouse model for prostate cancer metastasis.

A therapeutic effect was also reported by Chen et al.,\textsuperscript{37} who injected different tumor cells into the footpad of syngenic mice and after intravenous administration of MSC\textsuperscript{IL-12} observed inhibition of tumor growth and spontaneous metastases. MSC could be detected into the tumors five weeks after administration but interestingly they were absent from normal tissues such as lung and liver. As an alternative route of systemic delivery, MSC\textsuperscript{IL-12} were administered intraperitoneally before intravenous inoculation of melanoma cell.\textsuperscript{38} Treatment led to a considerable decrease in the number of lung metastases, but unfortunately no data about potential MSC homing or increased IL-12 serum levels were reported. Recently, it was shown that adipose-derived MSC expressing TRAIL, intratumoral- or intravenous-administered in mice bearing subcutaneous tumors, caused a reduction in tumor burden.\textsuperscript{38} Presence of MSC\textsuperscript{TRAIL} in tumors after intravenous administration was demonstrated, but no data on potential localization of MSC in normal tissues were provided. In a different setting, MSC\textsuperscript{LIGHT} exhibited a notable prophylactic and therapeutic effect when administered subcutaneous in the flank contralateral to tumors. However, migration of MSC from the left flank to the right one where tumor cells were inoculated is not addressed in this work.\textsuperscript{39}

Considering that it is difficult to estimate the percentage of MSC effectively homing to tumors in these models (probably very low), and given the antitumoral effect observed in most of them, these results could be attributed, at least in part, to therapeutic protein production in locations other than tumors. Therefore, perhaps MSC as cell factories for soluble proteins can be “outsourced” from the tumor while preserving their therapeutic effect.

If MSC tumor homing is not a requirement, perhaps we could implant them in a determined location, within a controlled environment providing clues that could enhance their engraftment and survival. Moreover, some MSC properties (e.g., immunosuppresion, metastasis and angiogenesis promotion) would strongly recommend avoiding the direct contact between MSC and tumor cells.\textsuperscript{40,41}

### Confined Administration of Ex Vivo Gene-Modified MSC for Cancer Immunotherapy

In fact, for strategies where MSC are used as cell factories for therapeutic antibodies, ex vivo gene-modified producer cells can be confined within a controlled environment providing clues that could enhance their engraftment and survival. The scaffold would keep cells at the implantation site, with the therapeutic protein acting at distance (immunotherapeutic organoid), and could be retrieved once the therapeutic effect is fulfilled (Fig. 2). A seminal work by Eliopoulos et al. reported that transduced MSC secreting EPO (MSC\textsuperscript{EPO}), when delivered freely in the subcutaneous or intraperitoneal spaces led to a temporary hematocrit increase.\textsuperscript{42} In contrast, subcutaneous implantation of MSC\textsuperscript{EPO} embedded in Matrigel led to a sustained pharmacological effect. This systemic effect of locally produced proteins (IL-2, IL-12, PEX, sIGF-IR and TRAIL) has also been reported in the context

### Table 1. Ex vivo gene-modified mesenchymal stem cells for cancer immunotherapy (continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>MSC source</th>
<th>Route of administration</th>
<th>Disease model</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>(R) BM</td>
<td>Matrigel-embedded, s.c., i.t., i.v.</td>
<td>(M) melanoma (s.c and i.v.)</td>
<td>(M) C57BL6, beige, SCID</td>
<td>76</td>
</tr>
<tr>
<td>PEX</td>
<td>(H) BM</td>
<td>Alginate-PLL microcapsules</td>
<td>(H) glioma (s.c.)</td>
<td>(M) athymic nude</td>
<td>77</td>
</tr>
<tr>
<td>sGF-1 R</td>
<td>(M) BM</td>
<td>Matrigel-embedded</td>
<td>(M/H) colon/lung cancer liver metastases</td>
<td>(M) C57BL6/ athymic nude</td>
<td>43</td>
</tr>
<tr>
<td>TNFSF10 (TRAIL)</td>
<td>(H) BM</td>
<td>Silk scaffold, i.t., i.v.</td>
<td>(H) breast cancer (orthotopic)</td>
<td>(M) NOD–SCID</td>
<td>78</td>
</tr>
</tbody>
</table>

MSC, mesenchymal stem cells; M, mouse; H, human; R, rat; BM, bone-marrow; UCM, umbilical cord matrix; UCB, umbilical cord blood; AT, adipose tissue; dAb, diabody; s.c., subcutaneous; i.v., intravenous; i.t., intratumoral; p.t., peritumoral; i.p., intraperitoneal.
of cancer therapy (Table 1). For example, Wang et al.43 reported that Matrigel-embedded sIGF-IR-secreting MSC subcutaneous implanted, provided sustained delivery of this decoy receptor in vivo. The protein achieved therapeutically effective concentrations, resulting in marked reductions in the ability of three different highly metastatic tumor cell types to colonize the liver.

Matrigel is a reconstituted extracellular matrix (ECM) preparation extracted from a murine sarcoma cell line, not suitable for MSC immobilization in a clinical setting. For this reason we used a chemically defined and non-immunogenic synthetic ECM (sECM) for in vivo MSC engraftment in a bsAb-based cancer immunotherapy approach. MSC were transduced ex vivo with a lentiviral vector expressing a recombinant anti-CEA x anti-CD3 diabody, embedded in a sECM crosslinkable in situ (Extracel-X) and inoculated in the ventral subcutaneous space of nude mice. The antibody was released into the bloodstream at detectable levels for at least 7 weeks. MSC-secreted diabody activated tumor-specific T cells and reduced the growth of CEA-expressing human colon carcinoma cells.40

In previous works, two immunotherapeutic MSC-based organoids had been reported for the expression of IL-2 and IL-12.74,75 Matrix-embedded IL-2-producing MSC inoculated in the vicinity of B16 melanoma led to inhibition of tumor growth.74 Similarly, gene-modified MSC to express IL-2, embedded in a matrix, and implanted peritumorally in a model of breast cancer caused a significant decrease of tumor growth.75 Although MSCIL-12 scaffolds determined increased IL-12 plasma levels, the observed therapeutic benefit was not due to a systemic effect, since MSCIL-12 implanted contralaterally did not inhibit significantly tumor growth. In contrast, MSC-secreted diabody demonstrated the systemic effect of a locally produced protein.40

In summary, the necessary components for generating a long-lasting immunotherapeutic organoid (Fig. 2) are: (1) gene/s encoding a soluble protein/s with immune-modulating activity, (2) an efficient and safe transfer vector, (3) a suitable an easily transduced long-lived cell and (4) a non-immunogenic synthetic matrix scaffold that guarantees the survival of the cell vehicle at the point of implantation.

Future Prospect

AAV have demonstrated excellent safety and tolerability in human trials and two phase 1 trials currently ongoing involve the use of AAV for the expression of anti-HIV antibodies from muscle tissues.79 However, the possibility of rare adverse events is still a concern. On the other hand, cell-based approaches face challenges such as loss of transgene expression over time due to limited life-span of producer cells or host immune responses against them. MSC are emerging as the best cell choice for the generation of long-lasting cell factories (Fig. 2). Perhaps the safest and most practical approach might be the use of scaffolds that keep genetically modified autologous MSC at the implantation site (immunotherapeutic organoids). However, in terms of cost-effectiveness, the only potential approach to apply these cell factories to the clinical setting would imply the use of “off-the-shelf” stocks of gene-modified MSC ready to be used in a series of patients. In principle, MSC would be perfect candidates for this strategy due to low immunogenicity and immunomodulatory properties, but some reports point out that allogenic MSC are not so “invisible” to immunocompetent hosts.43,44 Therefore, the use of encapsulation systems (Fig. 2) to shield producer MSC from the host immune system (immunotherapeutic capsules) would be highly desirable in order to obtain long-term systemic protein delivery. In a recent work, Goren et al.77 demonstrated the feasibility of this approach for the in vivo production of PEX, an inhibitor of angiogenesis, by encapsulated MSC.77 The technical and biological advances may lead to the realization of the full potential of cell encapsulation.60

In summary, the transfer of genes encoding antibodies, both in vivo and ex vivo, is a promising strategy that can be applied to treat clinical conditions in which continuous production of antibodies is required. Administration of ex vivo gene-modified cells embedded in appropriate scaffolds, such as hydrogels, can help to improve their therapeutic potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported by grants from the Ministerio de Ciencia e Innovación (BIO2011-22738 and PSE-01000-2009-11), the Comunidad de Madrid (S2010/BMD-2312), and the European Union (SUDOE-FEDER, IMUNONET-SOE1/P1/E014) to L.A-V; and from the Fondo de Investigación Sanitaria/Instituto de Salud Carlos III (PI08/09856 and PS09/00227) and Fundación Investigación Biomédica Hospital Puerta de Hierro to L.S.

References


Defining cell-matrix combination products in the era of pluripotency

Hal Sternberg, Jeffrey Janus and Michael D. West*

BioTime, Inc.; Alameda, CA USA

Keywords: embryonic stem cells, matrices, tissue engineering, embryonic progenitor cells, hyaluronic acid, differentiation, cartilage

Human pluripotent stem (hPS) cells provide an attractive opportunity for the manufacture of a wide array of therapeutic cell types. The challenges to commercialization include the thousand-fold diversity of cell types emerging from hPS cells and the associated difficulties in validating processes to reliably generate cells with precise identity and purity. Improved methods of controlling the dosage and migration of hPS-derived cells in solid tissues are also needed. To directly address these issues, we clonally expanded proliferating lineages of cells that were intermediate in regard to their state of differentiation between hPS and terminally differentiated cells. These cells called monoclonal embryonic progenitors (hEP), are expandable mortal lineages with diverse site-specific homeobox gene expression and multipotentiality. In this review, we discuss methods of generating combination products wherein the fate space of precisely identified monoclonal hEP cells is mapped by differentiating the cells in vitro in HyStem-4D bead arrays in the presence of diverse growth factors. This combination of discovery processes has the potential to translate directly into cell-matrix formulations that can be used to generate pre-clinical data leading to human clinical trials and potentially new medical therapies.

Introduction

The rising prevalence of age-related degenerative disease in many countries is triggering significant interest in the development of novel cell-based therapies to regenerate tissue function—a prospect that has long escaped the capabilities of drug and antibody-based therapeutic strategies. Of particular interest is the use of human pluripotent stem (hPS) cells such as human embryonic stem (hES) and induced pluripotent stem (iPS) cells to generate the diverse cellular components of the human body on an industrial scale for use in therapy. Pluripotent stem cell technologies offer at least two distinct advantages over previous approaches to the manufacture of living-cell therapies. First, pluripotent stem cells in the undifferentiated state are capable of indefinite proliferation due to the abundant expression of the telomerase catalytic component TERT. This allows a manufacturing scheme wherein master cell banks of hPS cells are precisely characterized and used for the indefinite manufacture of differentiated cell types consistent with good manufacturing practices (GMP). The second advantage of the pluripotent stem cell platform is the ability of these cells to differentiate into all somatic cell lineages.

In the adult human, certain cells types are proposed to have multipotency; such cells include hematopoietic stem cells (HSCs), bone marrow-derived mesenchymal stem cells (MSCs), and brain subventricular zone neuronal stem cells (NSCs). However, no adult stem cell sources have been identified to date that are effective at regenerating most vital tissues of the body and adult stem cell cultures typically have limited scale up potential. Taken together, the unique capacity of hPS cells to be expanded in the immortal state while maintaining long telomere length, as well as the ability of hPS cells to cascade into all body cell types are leading to the increasing interest in pluripotent stem cells.

Challenges of Pluripotency

While hPS cells offer these exciting prospects, there remain significant challenges to making these products compatible for human clinical use. As opposed to multipotent cells, such as HSCs or MSCs, hPS cells and their immediate derivatives are: (1) unique in displaying the potential to differentiate into derivatives of all three embryonic germ layers (a complexity of > 1,000-fold), (2) are often highly proliferative, (3) appear to occasionally express regenerative patterns of gene expression lost in subsequent fetal and adult tissues, and (4) and can generate both parenchymal and associated stromal cell types capable of the cross-talk required to generate tissue structures.

The above complexity is at once as much a challenge to researchers as it is an opportunity. The opportunity is obvious, the challenge is often overlooked. Formulations of targeted differentiated cells derived from hPS cells may, for instance, contain contaminating cell types, many of which are currently uncharacterized, resulting in unpredictable ectopic tissues developing at the site of engraftment. While most of the undesired ectopic tissues reported appear to be benign (cystic structures and cartilage), they may not manifest during the relatively short period of animal model preclinical testing, and therefore, their full risk to a human patient over years or decades is currently unknown. Similarly, the manifestation of such ectopic cells may require higher dosages than is routine or even possible in animal models, but required for efficacy in humans. Lastly, human hPS-derived formulations may respond differently in a human host.

*Correspondence to: Michael D. West; Email: mwest@biotimemail.com
Submitted: 03/22/13; Revised: 03/26/13; Accepted: 03/28/13
Citation: Sternberg H, Janus J, West M. Defining cell-matrix combination products in the era of pluripotency. Biomatter 3: e24496; http://dx.doi.org/10.4161/biom.24496
this deficiency in assaying for problematic contaminants compromises the ability to effectively purify out and assay for the cells. The impurity of these products, and the associated costs of attempting to further purify them and/or to demonstrate the safety of the impure formulations in very large numbers of models has historically been a significant challenge to the industry. In addition, heterogeneous differentiation protocols typically result in poor lot-to-lot reproducibility, since they typically involve numerous temporally oriented differentiation steps. With each fate decision, there is a cumulative potential that the cells will differentiate into an undesired cell lineage rather than into the intended cell type. When the multiple differentiation steps are linked, the probability of obtaining a product lot of cells of the intended differentiated state is often relatively low. Thus, efforts are ongoing to map the gene expression markers of all cells derived from pluripotent stem cells, which would potentially provide more reliable differentiation protocols as well as more definition of cell-surface antigens to improve the affinity purification process (http://discovery.lifemapsc.com). In the meantime, there is an urgent need to identify alternative protocols to enable a rapid, reliable, and economic scale-up process of hPS-derived cells that meet the regulatory criteria of purity and identity.

One such alternative strategy is the clonal expansion of cells intermediate in their differentiated state between hPS cells and terminally differentiated cells. These intermediate cells are designated human embryonic progenitor (hEP) cells (trade name PureStem™ progenitors http://discovery.lifemapsc.com). In vivo examples of hEP cells would be the neuroepithelial cells of the neural tube, propagating and migrating neural crest in the...
cranial mesenchyme, paraxial mesoderm and lateral plate limb bud mesenchyme. Like hPS cells, monoclonal embryonic progenitor cell lines do not necessarily correspond to stem cells in vivo, capable of propagating in a niche while maintaining their relatively undifferentiated state, or making fate decisions to differentiate. In that sense, technically, hES cells are not “stem cells,” but rather reflect an in vitro artifact, or stasis, wherein the culture of the cells in particular conditions results in the expansion of the cell in number without further downstream differentiation. Thus, an account of the propagation of monoclonal embryonic progenitor cell lines may rest instead with the possibility that both hPS and hEP cell lines represent cells that are propagating within developmental stasis; in other words, neither type of cell progresses to differentiation due to the lack of a requisite signal.

According to the hEP cell protocol, hPS cells are not initially scaled up, but instead are partially differentiated under a variety of differentiation conditions to obtain heterogeneous cultures of hEP cells. From these heterogeneous cultures, hPS cells are clonally isolated and expanded under a combinatorial array of propagation conditions (Fig. 1B). An initial “ shotgun cloning” of hEP cells demonstrated that the resulting cells, if continually propagated in their relatively undifferentiated state, will exhibit a broad diversity of gene expression profiles, including diverse site-specific homeobox gene expression and an estimated diversity of 140 distinct cell types within the 242 cell lines tested.1 These hEP cells typically display a uniform morphology, high levels of mitotic activity, and appear to telomerase negative (mortal). But since most hES cell lines, when properly cultivated, maintain telomeres at a long and stable length, the clonal progenitor cells in our hands are often capable of clonal expansion from a single initial cell into stable cultures (approximately 20 doublings to generate one million cells), as well as capable of further expansion to create master and working cell banks, and direct scale-up to produce > 10^11 cells before the cultures become impaired by telomere shortening (replicative senescence). The hEP cell line 4D20.8, for example, remains multipotent up to passage 33,6 far in excess of the limited capacity to propagate bone marrow-derived MSCs before losing chondrogenic potential. At late passage (passage 38) copy number variations are detected including a trisomy in chromosome 16 and a monosomy in chromosome 17, similar to that reported in long-term cultures of hES cells. However, at earlier passages, the cells displayed only minor variations common to all cultured cells. In the case of a more limited scale up of this line, for instance to a maximum of passage 30, it would be possible to generate an estimated 10 billion doses of 100 million cells for potential therapeutic use from only one existing clonal cell line. Therefore, clonally-purified hEP cells can be directly expanded, cryopreserved, thawed and expanded again as a point of scale up as opposed to the scale up of hPS cells typically planned in the case of heterogeneous differentiation protocols.

**Defining an Optimum Cell Transplantation Matrix**

The use of clonal and expandable hEP cells may provide a means of manufacturing diverse types of human progenitors from the hPS cell platform in vitro, however, the ultimate goal is to define the dosage and potency of cells engrafted in vivo. Ideally, the cells would be co-developed with an injectable matrix that would improve the reliability of survival of the engrafted cells by providing key cell attachment sites as well as a hyaluronate-rich environment similar to that prevalent in early embryonic development.7,4 This co-development would increase the understanding of the effectiveness (potency) of the cells in the defined matrix. HyStem-C hydrogels are an example of such a matrix.8,10 Composed of thiol-modified gelatin and thiolated hyaluronic acid crosslinked with polyethylene glycol diacrylate (PEGDA), HyStem-C hydrogels appear to increase the reliability of cell viability in diverse target tissues such as myocardium,11 brain,12 vocal cords13 and adipose tissue.14 HyStem-C also appears to be capable of safely crosslinking in vivo to potentially anchor the introduced cells at the injection site.15 We therefore undertook studies to determine whether this matrix could be utilized in screening the differentiation potential of hEP cell lines to streamline the translation from bench top experiments to relevant animal in vivo transplantation studies.

**In Vitro Testing of hEP Cell-Matrix Combination Products: Fate Space Screening**

To map the fate space of the diverse clonal hEP cell lines, we utilize two methods to generate high-density cultures, thereby predisposing hEP cells to differentiation in the presence of exogenous factors. The first approach, commonly referred to as micromass differentiation,16 is a system wherein 10 μL aliquots of 2.0 × 10^7 cells/mL (200,000, cells/micromass) are plated in growth medium to allow for attachment, then the micromasses are incubated in a differentiation cocktail. The second approach, referred to herein as HyStem-4D bead arrays, is a system by which high density is achieved with the plating of 25 μL aliquots of 2.0 × 10^7 cells/mL in 1% w/v HyStem-C (500,000 cells/construct).6 then following polymerization into hemispherical beads arrayed to the tissue culture dish, the constructs are similarly exposed to growth/differentiation factors.

As shown in **Figure 2A**, micromass conditions provide a relatively heterogeneous environment in which a subset of cells are typically observed spreading at the edges of the mass, while the majority are embedded in a dense mound. Immunocytochemical staining of the micromasses with antibodies directed to antigens expected in the micromasses, often show evidence of heterogeneity, such as different differentiation pathways taken in the peripheral cells than the majority of cells in the micromass (not shown). In contrast, the cells embedded in HyStem-4D bead arrays (Fig. 2B) in our experience, typically appear to be more uniformly dispersed in a three-dimensional matrix, and allow the additional temporal dimension of extended incubation or cryopreservation (the fourth dimension hence the designation “4D”). The more uniform constructs generated using HyStem-4D bead arrays combined with the potential to directly translate the combination product into preclinical animal models wherein the same hydrogel is designed to increase viable engraftment and prevent undesired migration, may therefore provide significant advantages of...
the other differentiated cell lines in order to detect evidence of genes that are differentially expressed.

When 100 diverse clonal hEP cell lines were thawed and then plated under high density micromass conditions in the presence of TGFβ, dexamethasone, and ITS for 14 d, seven of the lines were observed to undergo chondrogenesis as judged by the abundant upregulation of collagen type II, α1 (COL2A1) transcript and related chondrocyte markers. The seven lines displayed distinctive site-specific homeobox gene expression in the undifferentiated progenitor state, including differential expression of markers such as BARX1, HOXB2, LHX1, LHX8, MSX2, PITX1 and TBX15. Expanded fate-space mapping of these seven lines in HyStem-4D bead arrays in combinations of TGFβ family members showed induction of diverse differentiation markers corresponding to the diverse site-specific origins of the clones, including varied expression of the cartilage markers COL2A1 and CRTAC1, osteogenic markers IBSP and ALPL, and the tendon/ligament marker TNMD.

LHX8 is a homeobox gene associated with perioral mesenchyme. When the LHX8+ BARX1+ cell line 4D20.8 with the most distal HOX gene expression of HOXB2 was screened in HyStem-4D bead arrays using diverse combinations of TGFβ family members, the line showed higher COL2A1 and CRTAC1 expression than early passage mesenchymal stem cells (MSCs) and markedly lower expression of the hypertrophic marker COL10A1 when differentiation occurred in a cocktail containing both TGFβ3 and GDF5. MSCs typically differentiate in vitro in a manner mirroring their role in the growth plate and healing bone fractures in vivo. Differentiating MSCs often express markers of hypertrophic cartilage which provides a transient cartilaginous matrix that degrades and attracts vasculature and associated osteoblasts and bone formation. This hypertrophic phenotype of MSCs complicates the potential use of MSCs in regenerating definitive chondrocytes in at the joint surface or intervertebral disc. The discovery that clonal hEP cell lines cultured in high density conditions such as micromasses or HyStem-4D bead arrays in the presence of diverse differentiation conditions such as physiological concentrations of TGFβ family members, FGFs, retinoic acid, and modulators of Wnt signaling. After 14–21 d, RNA is analyzed by gene expression microarrays.

**Figure 2.** Phase-contrast photographs of a representative hEP cell line micromass and HyStem-4D bead used in fate-space screening (A). hEP cell micromass from the cell line T42 cultured in the presence of BMP4. (B) hEP cell HyStem-4D bead constructs from the cell line T42 cultured in the presence of BMP4 (Scale bar, 100 microns).

**Figure 3.** Strategy of fate-space screening. Diverse clonal hEP cell lines are cultured in high density conditions such as micromasses or HyStem-4D bead arrays in the presence of diverse differentiation conditions such as physiological concentrations of TGFβ family members, FGFs, retinoic acid, and modulators of Wnt signaling. After 14–21 d, RNA is analyzed by gene expression microarrays.
Illumina gene expression microarray data from more than 3,000 differentiation experiments, we searched for genes frequently up and downregulated in HyStem-4D beads and compared those profiles to those obtained under micromass conditions. For example, we observed that cells cultured in HyStem-4D beads with BMP4 frequently exhibited a marked decrease in myofibroblast markers such as *MYH11*, and increased expression of adipocyte markers such as *FABP4* and anti-inflammatory markers such as *TIMP4*. A representative experiment is shown below.

![Figure 4](image)

**Figure 4.** Representative histology of differentiated HyStem-C constructs in vitro and tracking of human hEP-derived differentiated cells in HyStem-C in tissue sections. The hEP cell line E15 (P19) was differentiated for 14 d in HyStem constructs in the presence of 100 ng/ml of BMP7 and 10 ng/ml of TGFβ3. The results show hematoxylin and eosin (H&E) and Safranin-O staining as expected for cartilage differentiation (**A** and **B** respectively, previously unpublished data).

While we observed that hEP cell lines can be successfully differentiated in alternative injectable matrices such as calcium alginate beads, we have observed that transplantation of the cells in vivo in alginate is problematic in that alginate stains brightly with multiple reagents including Safranin-O. While HyStem-C stains slightly with Safranin-O stain, we found that cells transplanted in vivo in HyStem-C were easily stained by H&E and Safranin-O, and could be localized in the tissue with human-specific antibody. For example, as shown in **Figure 4C**, the chondrogenic progenitor 4D20.8 transplanted in HyStem-C in vivo into femoral trochlear defects, could be easily tracked in tissue sections using anti-human mitochondrial antibody.

To determine whether this cell-matrix combination can also be used to generate tissue engineered cartilage constructs useful in measuring mechanical strength parameters, 5 x 10⁷ cells/ml were incorporated into 4 mm HyStem discs with and without collagen for three or six weeks. In the presence of TGFβ3 and BMP7, for example, the hEP cells designated 4D20.8 at six weeks showed an equilibrium modulus of 9.6 kPa while the cells in the presence of TGFβ3 and GDF5 at the same time point showed an equilibrium modulus of 29.2 kPa. The dynamic modulus observed in the presence of TGFβ3 and BMP7 was 824 kPa and that in TGFβ3 and GDF5 at the same time point was 1311 kPa. These observations replicated in other chondrogenic hEP cell lines suggest that various in vitro modeling can also be performed in the clonal hEP cell lines in the same matrix planned for in vivo application.

HyStem-4D bead arrays not only allowed for improved definition of the fate space of these novel clonal hEP cell lines, it documented their ability to differentiate in HyStem-C, and enabled the accumulation of a large amount of data on the biological influence of HyStem-C on diverse cell types. With
shown in Figure 5. The cell line E15, which in other conditions was shown to have chondrogenic potential, and the line W10 strongly induced MYH11 in micromass conditions supplemented with 10 ng/mL BMP4, but this induction was essentially ablated in HyStem-4D culture supplemented with BMP4. Instead, in HyStem-4D beads, the line markedly upregulated expression of DCN, a marker of meninges. This physiological effect on myofibroblastic differentiation seen in many lines cultured in HyStem-4D beads (i.e., the strong reduction in MYH11 expression) may have therapeutic implications in vivo, such as in inhibiting fibrosis or adhesions. This may also be of benefit in surgical settings where cells could be transplanted to regenerate tissue function while inhibiting adhesions and related fibrotic process at the surgical site.

In addition to decreasing myofibroblast gene expression, the culture of hEP cells in HyStem-4D beads and BMP4, as opposed to micromasses and BMP4, frequently showed an upregulation of adipocyte differentiation markers as shown for the cell line E69 in Figure 5. These observations support the view that co-development of hPS-derived cells and matrices in combination early in the discovery process may provide important information on gene expression, dosage, and viability that would be useful in designing optimal animal preclinical models, thereby streamlining product development.

Summary

The increasingly rigorous standards imposed by regulatory agencies for the approval of cellular therapeutics, and the requisite financial costs involved, demands a fresh perspective on the process development strategies of manufacturing products from hPS cell sources. The combination of in vivo crosslinkable hydrogels such as HyStem-C together with clonally-purified hPS-derived EP cell lines may simplify and improve the economics of the scalable manufacture of highly identified and purified cell types with improved standards of viability and immobilization post-implant. While hPS cells possess the advantage of indefinite scalability, hPS-derived clonal EP cells have suitable proliferative lifespan for widespread therapeutic applications. The use of a single culture of scaled clonal EP cells may allow, for example, the manufacture of very large product lot sizes capable of delivering, for instance, millions of doses of 100 million cells. Since it is also possible to generate additional clonal lines from the frozen stocks of the heterogeneous cultures from which the clones were initially isolated, very large numbers of cells from expanded hEP cell lines is easily achievable.

In contrast to the use of clonal hEP cells, the process of heterogeneous differentiation results in impure populations of cells with contaminating and uncharacterized embryonic progenitor cells. Because such lots of cells vary from lot-to-lot, it is only feasible to test the behavior and gene expression profiles of these cells in candidate matrices after the fact. This may lead to uncertainty regarding the behavior of the cells in the novel matrix, and the potential clonal expansion of the contaminating progenitor cells to form ectopic tissues.

In summary, there appear to be at least two advantages the use of clonal hEP cell lines in HyStem-4D bead arrays. First, stable and expandable clonal hEP cell lines may provide an improved method of reliably generating diverse, purified, and
scalable human embryonic cell types. Second, HyStem-C may lead to a more direct translation into formulations suited for human transplantation since HyStem-C is capable of safely crosslinking in the presence of cells in vivo. Together, clonal hEP cell lines formulated in HyStem-C may accelerate the pace of translation from discovery to the use of the combined products in preclinical and clinical testing.

References