Title

The Regenerative Medicine Laboratory: Facilitating Stem Cell Therapy for Equine Disease

Permalink

https://escholarship.org/uc/item/4vz106rt

Journal

Clinics in Laboratory Medicine, 31(1)

ISSN

0272-2712

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Publication Date

2011-03-01

DOI

10.1016/j.cll.2010.12.001

Peer reviewed

Introduction

The recent interest in equine stem cell biology and the rapid increase in experimental data, highlight the growing attention that this topic has been receiving over the past few years. Within the field of stem cell biology, the relevance of immunobiology is of particular interest. It appears that optimal and effective stem cell therapy for equine patients will require a thorough analysis of the immune properties of stem cells as well as their response to immune mediators. The main goal of this review is to discuss the biology of adult mesenchymal stem cells (MSCs) in the context of immunology.

MSCs are pluripotent, self-renewing cells with the potential for tissue regeneration. These cells have been implicated in the repair of bone, cartilage, tendon, ligament, skeletal muscle, and cardiac muscle. Data also suggest that MSCs may be able to trans-differentiate into cells of ectodermal origin, such as neurons. MSCs, also defined as non-embryonic stem cells, can be derived from a number of tissues including amniotic fluid, umbilical cord (blood and matrix), bone marrow, adipose tissue, synovium, synovial fluid, and periodontal ligament. MSCs have a bimodal effect on the immune system including an anti-inflammatory and an immune-enhancing response. MSCs regulate immune responses such as altering antibody production by B lymphocytes (B cells), promoting shifts in T lymphocyte (T cell) subtypes, and inducing immune tolerance to allogeneic transplants. MSCs also have the potential for gene delivery. This review explores the diverse clinical potential for MSCs and discusses MSC mechanisms for modulating the immune response and the limitations and advantages of their immunomodulatory properties. Within the context of this review we will highlight salient immunology concepts that may better guide the understanding of the interactions between MSCs and the various components of the immune system.

Immunomodulatory properties and Immunogenicity of MSCs in vitro and in vivo

Tissue repair and disease improvement mediated by MSCs have been shown to be

intimately related to MSC interaction with the host immune system in many disease models[1,2]. MSCs have clear immunomodulatory functions. It is increasingly recognized that the key to understanding MSC efficacy for tissue repair or inflammatory disease is to understand how MSCs modulate the inflammatory niche. MSCs have a distinct profile of bioactive mediators and adhesion molecules that work to inhibit scar formation, inhibit apoptosis, increase angiogenesis and stimulate intrinsic progenitor cells to regenerate function. To accomplish this, MSCs interact with nearly all the cells of the immune system including lymphocytes B cells, T cells, Natural Killer (NK) cells, dendritic cells (DC), macrophages/monocytes and neutrophils [3], suggesting far-reaching effects on early, innate and humoral immune responses. It is unclear if MSCs should be categorized as "immunosuppressive", implying a nonspecific down-regulation of the immune system, or rather if they induce an "immune tolerance", implying a more specific suppression of aberrant immune responses. What does appear clear is that the immunomodulatory ability of MSCs is dependent on a number of factors including: MSC activation, MSC tissue of origin, MSC dose, MSC time of administration and MSC contact with cells of the immune system.

MSC activation and preconditioning

MSCs do not secrete immunomodulatory proteins in the absence of activation [4,5]. In vitro, activated T cells, individual or combinations of cytokines such as interferon-γ (IFNγ) or TNFα are used to activate MSCs [5]. In vivo, injected MSCs will become activated by the local inflammatory milieu. This is one reason why it is so critical to understand the inflammatory niche into which MSCs are injected. Acute injury characterized by interleukin-6 (IL-6), IL-1 or TNFα would activate MSCs differently than chronic or immune-mediated lesions characterized by activated T cells or IFNγ.

Recent evidence suggests that preconditioning of MSCs may enhance their survival and,

as a result, enhance their regenerative and immunomodulating properties [6,7]. In fact, research efforts within the field of regenerative therapy have been dedicated to understanding the in vitro manipulation of cells prior to transplantation, in an attempt to maximize their biologic and functional properties [8,9]. Examples of these efforts include the use specific bioscaffolds which allow spatial and physical cell manipulation, increase cell survival and promote integration with the host. Furthermore, culture strategies have been evaluated to improve MSC viability by exposing cells to hypoxic conditions [10], specific growth factors [11,12], or a variety of biological agents [13].

Bone marrow (BM)-derived MSCs effectively migrate towards and enhance the healing processes that follow cardiac infarction and hind limb ischemia, both of which are characterized by a hypoxic gradient [14-16]. Although there is controversy regarding the effects of low oxygen tension on MSC survival, it appears that hypoxic conditions (1%–3% O_2) may be beneficial, as this oxygen tension is more similar to the physiologic niche of MSCs in the bone marrow (2%–7% O_2) [10]. MSCs appear to maintain their viability when cultured in low oxygen tension environments and increase their proliferation rate [10]. These studies may provide support for the use of MSCs in the treatment of equine tendonitis. Tendon injuries are characterized by hypoxic degeneration which leads to tenocyte apoptosis, particularly in chronic injury [17,18]. This hypoxic environment may be beneficial for MSC survival and may enhance their tissue regenerative potential.

Pretreatment of MSCs with bioactive compounds in vitro, such as growth factors, may decrease cell death and promote cell replication. It is likely that a similar phenomenon takes place in vivo. Based on such studies, the use of platelet rich plasma as a pre-treatment medium and a cell delivery vehicle may be of value. Depending on the disease condition and pending the results of similar research conducted in horses, MSCs could be pre-treated with bioactive compounds to allow them survive longer and enhance their performance in vivo.

MSC tissue of origin

In humans and rodents, the ability of MSCs to alter the immune system varies with the MSC tissue of origin. MSCs can be harvested from numerous tissues and locations and may have similar surface markers and growth characteristics, however these MSCs display distinct differences [19]. Some studies, including our own published studies [20], suggest that BM-MSCs and adipose-derived MSCs (ASCs) are more closely related than to MSCs derived from placental tissues [20,21]. In humans, cord blood derived-MSCs express genes involved in the cell cycle and in neurogenesis, consistent with their reported neuronal differentiation capacity; BM-MSCs appear to be primed towards developmental processes of tissues and organs derived from the mesoderm and endoderm; and ASCs are highly enriched in immune-related genes [21]. These inherent differences appear to be further enhanced by inflammation. These transcriptome studies are compatible with in vitro tissue source comparison studies [22-24] that find baseline and activated MSCs from BM, cord tissue, and ASC modulate the immune response and respond to inflammatory mediators distinctly [5,25]. Although research with MSCs from different tissue sources in horses is just beginning, equine MSCs appear to be remarkably similar to other species described to date. For example, equine MSCs derived from fat, BM, cord blood and cord tissue all respond to activating stimuli such as TNFα, with the secretion of prostaglandin E2 (PGE$_2$); however secretion of other mediators, such as nitric oxide (NO), may be dependent on tissue of origin (Borjesson, unpublished data). It is critical to define mediators associated with immunomodulation for all animal species individually, as it is clear that there are strong inter-species differences in MSCs responses to activation. For example, rodent MSCs and human MSCs differ slightly in their modulatory potential and mediators [26].

MSC dose

The inhibitory effects of MSCs are dose-dependent. MSCs act on un-stimulated T cells by preventing their activation. However, when T cells are already stimulated, MSCs reduce the

expression levels of their activation markers. Some studies have shown that high doses of MSCs possess immunosuppressive activity whereas low dose MSC therapy could be immunostimulatory [27,28] or fail to inhibit lymphocyte proliferation [29,30]. One study directly assessed MSC dose in a canine disc degeneration model and found that MSCs were least viable after injection at a low dose (10^5), that there were more apoptotic cells at the high dose of MSCs (10^7), whereas the structural microenvironment and extracellular matrix of the disc were maintained with an intermediate dose of MSCs (10^6) [31]. The ideal dose of MSCs for any given equine lesion is clearly an important area of study and the dual ability of MSCs to either sustain or suppress T-cell proliferation should be considered in the context of clinical applications [28].

MSC time of administration

There is very little information as to when MSCs should be administered, although some animal models support a time frame of one week post-injury after the acute inflammatory response recedes [32,33]. MSCs have shown dissimilar effects when applied at different stages of disease. As the inflammatory niche progresses from acute to chronic inflammation, the cells and mediators present could skew MSC activation in a number of ways. For example, in one study MSCs exhibited their typical suppressive phenotype when added early to cell cultures in the presence of CD4(+) T cell polarizing stimuli. However, once T cell activation had occurred, MSCs showed an opposite stimulating effect on Th17 cells, while leaving T regulatory (Treg) IL-10-producing cells unchanged [34]. These results suggest that the therapeutic use of MSCs in vivo might exert opposing effects on disease activity, according to the time of therapeutic application and the level of effector T cell activation, especially in autoimmune disease models [34].

MSC Contact with Cells of the Immune System

MSCs act as pleiotropic immune regulators to suppress immune responses through the production of multiple soluble factors and/or direct cell–cell contact in order to affect all the actors of immune responses: T cells, NK cells, B cells and DCs [4,28]. MSCs may act locally, however they may also accumulate in secondary lymphoid organs and attenuate delayed-type hypersensitivity response by inducing apoptotic cell death of surrounding immune cells in the draining lymph node. In one study, MSCs accumulated in lymph nodes, near the paracortical area and the germinal center, and markedly attenuated a delayed-type hypersensitivity response via increased apoptosis of activated T cells [35].

T lymphocytes:

MSCs interact with T cells in a number of ways. MSCs secrete soluble mediators and directly interact with T-cells to modulate their activity [29,36,37]. MSCs can induce apoptosis of activated T cells, induce cell cycle arrest, decrease T-cell proliferation [30,38] and alter T cell phenotype. MSCs target T-cell subsets (CD4+, CD8+, CD2+ and CD3+ subpopulations) equally [30]. MSCs in direct contact with lymphocytes may inhibit lymphocyte apoptosis via the secretion of IL-6 or nitric oxide (NO) [35-37]. MSCs activated by IFN-γ, up-regulate adhesion molecules, including intracellular adhesion molecule-1 (CD54; ICAM-1) and vascular cell adhesion molecule-1 (CD106; VCAM-1) [37]. In many models, direct contact between MSCs and T cells facilitates MSC immunosuppressive capacity. This direct association is presumed to facilitate the actions of locally produced, short-acting mediators such as NO [37].

Decreased lymphocyte proliferation

For murine and human MSCs, the ability of MSCs to inhibit T cell proliferation has been attributed to a variety of soluble mediators, adhesion molecules and matrix metalloproteinases. Soluble mediators are also critical for the ability of equine MSCs to inhibit lymphocyte proliferation (Borjesson, unpublished data). Soluble factors reported to suppress T-cell proliferation include: PGE2, hepatic growth factor (HGF), transforming growth factor-β (TGF-β), IFN-γ, IL-10, leukemia inhibitory factor (LIF), human leukocyte antigen-G (HLA-G) and

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indoleamine 2,3-dioxygenase (IDO) [4, 28, 30, 38-42]. MSC-derived matrix metalloproteinases can also cleave the IL-2 receptor (CD25) from the surface of activated T cells with a resultant reduction of IL-2 production [35,43]. This decreased production of IL-2 and IFNγ, mediated by the NF-κB signaling pathway, also results in decreased lymphocyte proliferation [35,43]. MSC activation or priming by IFN-γ, TNF-α and other pro-inflammatory cytokines increases their inhibitory effect [28].

Altered lymphocyte phenotype

MSCs are thought to induce lymphocytes to switch to a Treg phenotype [CD4+CD25+forkhead box P3 (FoxP3+) cells; 28,39,44-47]. Tregs are a specialized subpopulation of T cells that suppress activation of the immune system and promote tolerance to self-antigens. In humans and mice, MSC-derived IDO, IL-10, PGE$_2$ and TGF-β have been implicated in the induction of Tregs [39,45,47]. Tregs are at least partially responsible for the anti-inflammatory Th1- to Th2-dominant cytokine switch [44]. For human MSC-mediated allosuppression, some data support a sequential process of Treg induction involving direct MSC contact with CD4+ cells followed by both PGE$_2$ and TGF-β expression [45]. Interest in Tregs is increasing as data from mouse models demonstrate that these cells may be responsible for MSC efficacy in the treatment of autoimmune diseases and may facilitate allograft (transplantation) tolerance [39]. Equine Tregs are being defined [48,49], however the study of Tregs in the context of equine MSCs and a lymphocyte phenotype switch to Tregs has not yet been published.

Dendritic Cells

In humans and rodents, MSCs also modulate DC maturation, differentiation and function [42,50,51]. To date, there are no reports of MSC interaction with equine DCs. The ability of DCs to initiate an immune response depends on their transition from an antigen-processing to an antigen-presenting cell. During this transition, MHC class II and co-stimulatory molecules (CD80

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and CD86) are up-regulated on the cell surface, a process termed DC maturation. This transition is critical for mounting an immune response because immature DCs fail to prime T cells effectively but induce tolerance rather than immune rejection. MSCs appear to keep DCs in an immature state [51] and inhibit the maturation of myeloid-DCs and plasmocytoid-DCs [42]. MSCs also promote proliferation of mature DCs into a more immature "regulatory" phenotype [51,52]. Dendritic cells also interact with B cells and NK cells [52]. Similar to the themes described for T cells, MSC interaction with DCs depends on cell concentration, mechanism of activation, and the cohort of immune cells present. MSC modulation of DC function and maturation involve soluble factors, such as PGE_2, IL-6 or TGF-β, or cell-cell contact, or both [42].

B-Lymphocytes

In humans and rodents, MSCs promote the survival and inhibit the proliferation and maturation of B cells by arresting them in the G0/G1 phase of the cell cycle [53]. MSCs also induce both stimulation and impairment of immunoglobulin production by B cells without affecting co-stimulatory molecule expression and cytokine production [54,55]. As with their interaction with all types of immune cells (T cells and DCs), MSC immunomodulatory effects were dependent on the level of MSC activation (LPS or viral antigens) and whether MSCs were acting directly on (in contact with) un-fractionated lymphocytes or enriched B cells [55]. The interaction of equine MSCs with B cells has not yet been reported.

Natural Killer Cells

NK cells are the major effectors of innate immunity and their function is also inhibited by MSCs. MSCs alter NK cell phenotype, suppress cytokine-induced proliferation of NK cells and prevent the induction of effector functions [56,57]. MSCs inhibit both NK cell-mediated cytolysis and interferon-gamma secretion [41]. The inhibition of NK cell function is thought to be critical to the suppressive functions of MSCs, especially in therapeutic arenas such as graft-versus-host disease [41].

MSC Secretion of Soluble Factors

As is clear from the discussion above, MSCs mediate their effects via direct cell-cell contact with cells of the immune system and via the secretion of soluble factors which also act upon immune cell populations. There is a plethora of mediators that have been studied [1,2]. These mediators appear to act in concert (for example with chemokines and adhesion molecules) or they may act sequentially. Many of the mediators have redundant roles (and their roles may be determined by the inflammatory lesion) but others may act as sole effectors of a particular anti-inflammatory response. In some cases, cell-cell contact may dictate the types of mediators secreted. For example, in the absence of cell-cell contact, MSC-induced expression of the tolerogenic genes IDO, LIF, and HLA-G does not occur [40]. When MSCs contacted T cells, the expression of IL-10 and TGFβ are modulated [40]. Activated equine MSCs produce abundant PGE_2 and IL-6 and variable amounts of NO and TGFβ, depending on tissue source and activation stimuli (Carrade et al., in review). The significance of mediator secretion by equine MSCs has not been determined.

Inodoleamine 2,3 dioxygenase (IDO)

Human MSCs express the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), known to suppress T-cell responses [4,5,26]. IDO has been implicated in the induction of tolerogenic DCs, the switch to a Th2-dominant cytokine inflammatory response and the induction of Tregs. In short, IDO is thought to be a central mediator in almost all aspects of MSC interaction with cells of the human immune system and induction of immune tolerance. Inhibition of IDO in allograft receipts resulted in an inability to achieve allograft tolerance [39].

Human Leukocyte Antigens (HLA)

MSCs from all species described to date, including horses [58], express MHC class I but do not express MHC II. The regulation of MHC I and MHC II (or Human leukocyte antigens, HLA, the nomenclature of human MHC) on MSC can be altered by activation. Increased MHC

can increase the immunogenicity of MSCs (for example by up-regulation of HLA class I (MHC I) or HLA-DR (MHC II) expression [5]. Conversely, MSC activation by IFN-γ can enhance the immunosuppressive phenotype of MSCs by down-regulating MHC II (HLA-DR) expression and increasing IDO production. Similarly, increased intracellular HLA-G and surface HLA-E expression can induce immune tolerance by increasing TGF-β and IL-10 release, and inducing IDO expression. MSCs have also been shown to secrete a soluble isoform of HLA class I molecule (HLA-G). This secretion is IL-10 dependent and requires cell-cell contact between MSCs and allostimulated T cells. HLA-G5 contributes to the suppression of allogeneic T-cell proliferation and then to the expansion of Tregs [41]. Currently, very little is known about the family of MHC molecules in horses and how MHC regulation may contribute to MSC immunomodulation in horses.

Clinical impressions and future implications

Evidence suggests that MSCs play a role in cell survival and function in vivo. In lab animals with experimentally-induced autoimmune encephalomyelitis, MSCs administered during haematopoietic stem cell transplantation improve clinical outcomes by reducing the symptoms associated with grade 4 graft-versus-host response [59]. Similarly, there are many examples of a beneficial effect of MSCs in increasing the acceptability of co-infused haematopoietic stem cells and lessening the risk of graft-versus-host disease in people [60-62]. MSCs also enhance the longevity of co-transplanted MHC-incompatible skin grafts in baboons [63,64]. These studies indicate the need to expand the scientific knowledge and the use of MSCs in horses beyond regenerative applications. MSCs could be applied in chronic wound management where a dysregulation of the healing process creates an aberrant inflammatory and resultant hyperplastic response such as that seen with exuberant granulation tissue in horses. We have infused autologous MSCs via intravenous regional limb perfusion in three horses with "proud

flesh" and have observed encouraging healing responses. In these horses, intravenous

infusions of 20x10^6 MSCs suspended in physiological solution were carried out for three

consecutive days. Although traditional therapeutic strategies had previously failed, these

wounds continued to be treated with standard medical approaches such as bandaging and

surgical debridement.

The use of banked MSCs obtained from donor horses offers the advantage of an

expeditious treatment and the use of an established and homogenous cell population with

proven regenerative and differentiation capacity. Allogeneic BM-MSCs (obtained from donor

animals) may have inhibitory and anti-proliferative effects on T- and B cell function similar to that

seen in murine model of systemic lupus erythematous, with spontaneous and lethal auto-

immune responses [65]. Other diseases in which conventional immunosuppressive therapies

fail may provide a rationale for the use of MSC-based therapeutic approaches [66-68]. MSC

infusions may also play an important role in regulating inflammatory diseases of the central

nervous system, although their interactions with the blood brain barrier have not been fully

elucidated [67,69,70].

Studies aimed at assessing the efficacy and safety of allogeneic treatments in horses

are lacking, although pilot studies of surgically-induced lesions of the equine superficial digital

flexor tendon, have shown that inflammatory cell infiltration was no different regardless of

whether MSCs were allogeneic or autologous [71]. Furthermore, injection of allogeneic

placentally-derived MSCs into equine joints resulted in self-limiting inflammatory responses with

no difference in the type or severity of the inflammatory response elicited by autologous versus

allogeneic MSCs [72].

Our clinical impressions resulting from treating horses with intravenous or intralesional

injections of allogeneic BM-MSCs have been generally positive. Several horses within the

authors' respective institutions have been treated with banked BM- derived MSCs for a variety

of hard and soft tissue disorders, such as osteochondrosis, osteoarthritis and tendonitis. Local

reactions have been self-limiting and easily treated with the administration of non-steroidal anti-inflammatory medications. Interestingly, some of the best responses to the cell therapy were seen in those horses in which a reaction was observed following the intralesional injection. Large scale prospective studies are needed to optimize cell-based therapy in horses. These studies would ideally provide answers to key questions such as the route of administration, the appropriate cell dose and the necessary control to determine the value of the selected cell therapy.

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