Mesenchymal Stem Cells as Trophic Mediators

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Abstract   Adult marrow-derived Mesenchymal Stem Cells (MSCs) are capable of dividing and their progeny are further capable of differentiating into one of several mesenchymal phenotypes such as osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and adipocytes. In addition, these MSCs secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. These secreted bioactive factors suppress the local immune system, inhibit fibrosis (scar formation) and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-intrinsic reparative or stem cells. These effects, which are referred to as trophic effects, are distinct from the direct differentiation of MSCs into repair tissue. Several studies which tested the use of MSCs in models of infarct (injured heart), stroke (brain), or meniscus regeneration models are reviewed within the context of MSC-mediated trophic effects in tissue repair. J. Cell. Biochem. 98: 1076–1084, 2006.   © 2006 Wiley-Liss, Inc.

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Several progenitor cells can be found in human adult bone marrow. One class of multi-potent adult progenitors is referred to as mesenchymal stem cells (MSCs) [Caplan, 1991]. It is well documented that these cells are capable of differentiating into bone [Haynesworth et al., 1992], cartilage [Yoo et al., 1998], muscle [Wakitani et al., 1995], marrow stroma [Majumdar et al., 1998], tendon and ligament [Young et al., 1998], fat [Dennis et al., 1999], and a variety of other connective tissues [Studeny et al., 2004]. Like the hematopoietic stem cells (HSCs) of marrow, the differentiation of MSCs involves multi-step cell lineages controlled by bioactive factors found in the local micro-environment or supplied in the culture environment of ex vivo cultivated cells (Fig. 1). This controlled differentiation scheme was evolutionarily selected because it comprises a sequential process that can be modulated both in time and end-stage outcome; a multi-step pathway allows a large number of regulatory elements to be used to safeguard the final outcome. By having various checks and balances along the formative route of differentiation, this multi-step lineage progression sequence insures that short lapses in whole animal physiology or traumatic events do not result in aberrant production of undesirable end-stage phenotypes.

It is important to understand that all adult stem cell systems exist to insure that when cells within tissues expire naturally, these cells can be expeditiously replaced to provide physiological balance in the organism. Every cell in the body has a lifespan ranging from 20 minutes to many years depending on the cell. Some circulating blood cells expire in 20 minutes after forming, while neurons exist for many years. The expiration allows the tissue to rejuvenate itself and provides the tissue with a mechanism to slowly change its properties as a function of age and/or use. Thus, the stem cells are the source of replacement parts for expired units, but also serve as reserve cells for damaged or compromised adult tissues requiring more extensive repair, regeneration, or expansion as depicted in Figure 1. Such stem cells are, therefore, remarkably different from their embryonic parents, whose purpose was to segregate and form unique morphologies and functional units that are integrated into the whole physiology of the organism [Caplan et al., 1983; Macdonald et al., 2002]. It follows that

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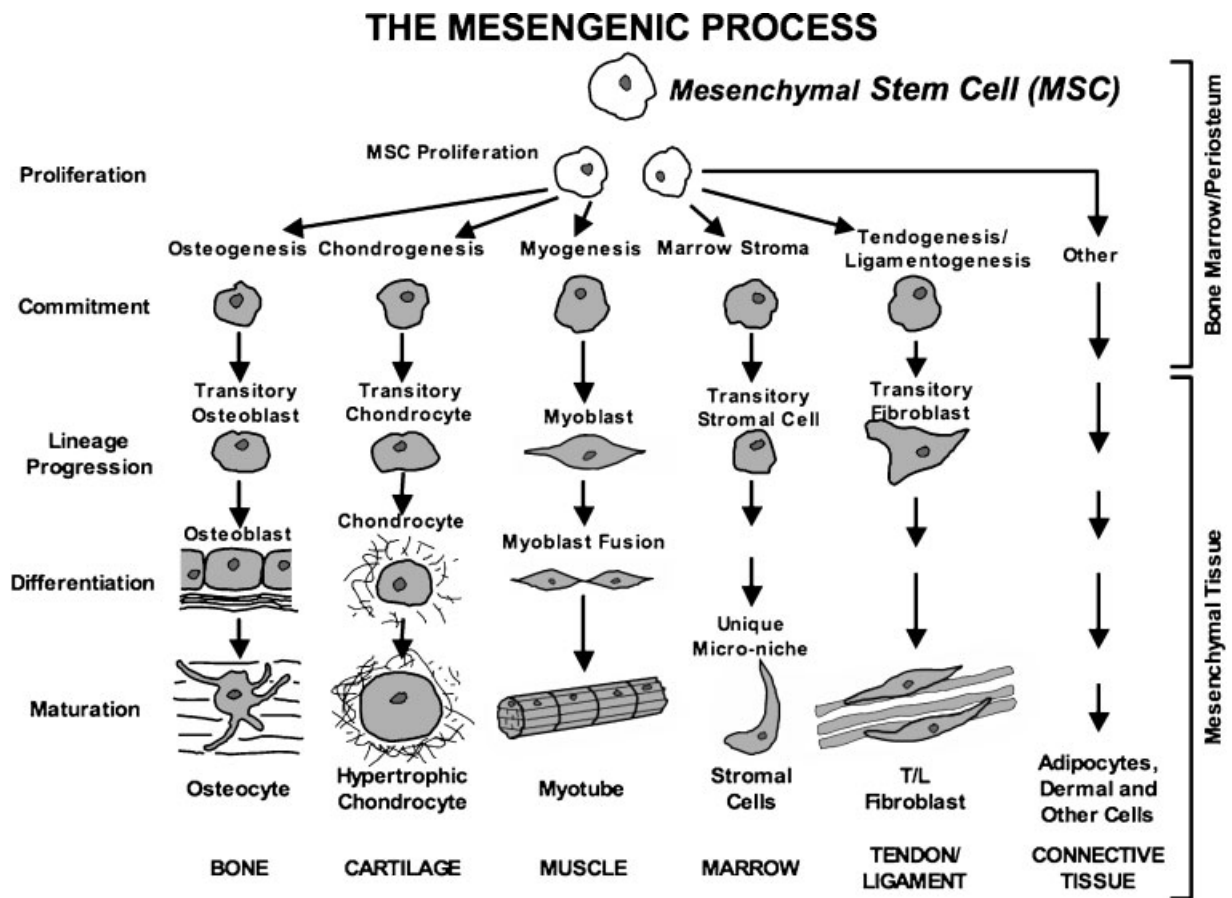
Fig. 1. Mesengenesis: A multi-potent stem cell found in adult bone marrow and other depots is capable of replicating and having its progeny differentiate along distinctive lineage pathways to produce highly specialized synthetic phenotypes that fabricate bone, cartilage, muscle, marrow stroma, tendon/ligament, and other connective tissues.

adult stem cells do not function in the embryonic microenvironment and do not respond to embryonic signaling molecules that specify tissue edges (i.e., morphologies) or those that induce an embryonic function. Lastly, the continuous turnover of cells and adult tissue components insures that the genomically controlled sequence of maturation and aging will take place within the confines of these stem cell/ tissue compartments.

MSCS AS SECRETORY SOURCES

MSCs do more than respond to stimuli and differentiate. Long ago we documented [Haynesworth et al., 1996] that newly committed progenitors synthesize a broad spectrum of growth factors and cytokines that have effects on cells in their vicinity (Fig. 2). When comparing the cytokines and growth factors which are released from MSCs that are placed into different developmental pathways, the percent change (increase or decrease) of individual bioactive factors is relatively constant between different donors, regardless of age or health status of the donor. Some donor-specific levels of secreted bioactive factors can be tenfold different in assays of their constitutive secretion. Indeed, all cells secrete various bioactive agents that reflect both their functional status and the influence of their local microenvironments. Clearly, as MSCs enter and progress toward an end-stage phenotype, the quantity and array of secreted bioactive factors changes as the descendants of MSCs enter new lineage stages. The pattern and quantity of such secreted factors is well known to feed back on the cell itself and govern both its functional status and physiology.

Such functional (paracrine and autocrine) secretions of bioactive factors can have profound effects on local cellular dynamics. For
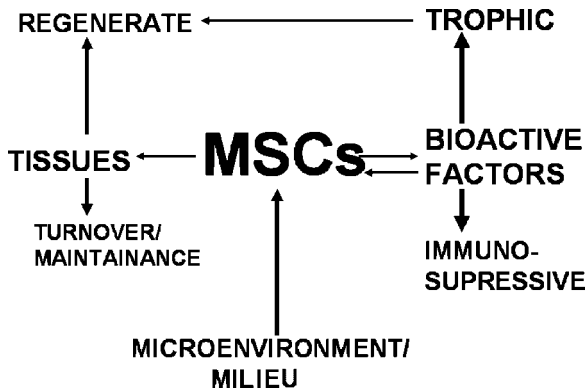
Fig. 2. The dual role of MSCs: MSCs play a central role in aspects of tissue regeneration and repair, maintenance and turnover and the control of hematopoiesis in the bone marrow. The MSCs accomplish this function by directly differentiating into specific CELLULAR phenotypes such as osteoblasts, chondrocytes, myocytes, etc. (Fig. 1) which fabricate bone, cartilage, muscle, etc. Of equal importance is the secretion of bioactive factors by MSCs that influence regeneration, turnover, and hematopoiesis. This influence is referred to as TROPHIC because the MSCs do not themselves differentiate but rather their bioactive factor secretion mediates the functional tissue outcomes.

example, the marrow stroma derived from MSCs not only provides the informational connective tissue floor space for cell anchorage, but also is hugely instructive for both vascular and hematopoietic cells [Dexter, 1989; Dexter et al., 1990]. In stroma-limited niches in marrow, different arms of the hematopoietic pathway will flourish. This growth factor/cytokine-mediated control of hematopoiesis is highly regulated, and lawns of MSCs in culture are consequently capable of supporting full hematopoietic lineage progression [Majumdar et al., 1998].

The effects of MSC-secreted bioactive molecules can be either direct or indirect or even both: direct by causing intracellular signaling or indirect by causing another cell in the vicinity to secrete the functionally active agent. We refer to this indirect activity as TROPHIC, consistent with the original use of this word by neurobiologists to indicate the bioactive molecules released from nerve terminals that are not neurotransmitters [Singer, 1964, 1974]. As seen in Figure 2, MSCs can have two distinct functions: MSCs can provide replacement units for expired cells in mesenchymal tissues, and MSCs can have TROPHIC effects on cells in their vicinity without generating newly differentiated mesenchymal phenotypes and, thus,

also influence the regeneration of cells or tissues by purely a bioactive factor effect.

In support of this new MSC functional capacity, we review three experimental tissue repair models where introduction of MSCs can cause dramatic trophic effects without extensive differentiation of MSCs into new phenotypes: Myocardial Infarct, Stroke and Meniscus Regeneration. First, the details of how MSCs influence hematopoiesis set the stage for these three tissue effects.

HEMATOPOIESIS

MSCs are now known for their ability to differentiate into a number of different phenotypes or, more precisely, to express markers for a range of different cell phenotypes [Jiang et al., 2002]. Prior to the more recent discoveries of the multiple potential of MSCs, bone marrow was known primarily as a source of osteogenic cells, and cultures of bone marrow stromal cells were known to support hematopoiesis in culture [Friedenstein et al., 1974; Dexter et al., 1977]. It came to be recognized that there is a dual role of stromal cells in hematopoiesis and osteogenesis [Owen, 1985], and the repertoire of phenotypic expression was soon expanded to include adipocytes [Bennett et al., 1991], chondrocytes [Yoo et al., 1998], and then a variety of other phenotypes, such as cardiomyocytes [Jiang et al., 2002; Hattan et al., 2003] and nerve tissue [Sanchez-Ramos et al., 2000; Woodbury et al., 2000]. Hematopoietic researchers primarily focused on MSCs as "stromal cells" whose function was to assist HSCs and their derivative progenitors to survive and thrive. This nourishment, or provision of a stromal microenviroment, is an example of MSCs (or stromal cells) exerting a trophic effect, "trophic" from the Greek *troph*, to nourish.

A specific example of the interaction between MSCs and hematopoietic cells is the production of RANK ligand by MSCs, which stimulates osteoclast formation, and the production of another molecule, osteoprotegrin, which is a decoy receptor for RANK that inhibits bone formation [Takahashi et al., 1999]. Trophic effects are, as we define them here, those chemotactic, mitotic, and differentiation-modulating effects which emanate from cells as bioactive factors that exert their effects primarily on neighboring cells and whose effects never result in differentiation of the producer cell. As already stated, a prime example of this trophic

action of marrow stromal cells is the support of hematopoiesis via the production of an assortment of hematopoietic cytokines, such as G-CSF, stem cell factor, M-CSF, and IL-6 [Haynesworth et al., 1996], while the MSCs do not themselves become hematopoietic.

Since these trophic effects are at play in multiple biological systems and MSCs exhibit the ability to produce a trophic effect on hematopoietic cells, we focus on how this trophic capacity might come into play for MSCs as they have been applied to the repair of different tissues. Certainly, the demonstrated ability of MSCs to differentiate into different phenotypes makes MSCs excellent candidates as therapeutic cells for the repair of damaged tissues; several laboratories have applied MSCs to tissue repair models. The results from these experiments have been mixed, such that where MSCs are sometimes shown to express differentiated markers at exceedingly low numbers, there is often a measurable therapeutic effect. In other instances MSCs may show no overt differentiation capabilities, yet still show some therapeutic value. Often, it is the trophic effect of MSCs that is likely the primary cause of the observed positive therapeutic effects. The following examples focus on MSC-mediated trophic effects in stroke, myocardial infarct, and meniscus repair.

STROKE

The brain is a complex, multi-cellular, multi-compartmented organ with its various layers and compartments nurtured by the vascular system. An interruption in vascular flow or blockade results in an injury response associated with the resulting ischemia. The cumulative effect of a vascular blockage, ischemia, and injury is summarily referred to as "stroke" which, if severe enough, is recognized after a loss of coordinated neurological function that can be cognitive and/or physical; some restoration of function is achieved by new neurological circuits or regeneration of previous pathways.

Because published reports assert that MSCs are capable of differentiating into neural elements [Sanchez-Ramos et al., 2000; Woodbury et al., 2000], it could be argued that if MSCs were introduced into an ischemic sector of brain, newly differentiated neural elements from the MSCs could play a role in restoring pre-existing pathways or provide neural support cells that

could help to re-establish or circumvent degenerated neural components. Indeed, Chopp and his collaborators and others have shown that MSCs can promote gain of coordinated function when introduced directly or systemically into the affected brain [Chen et al., 2003; Li et al., 2005]. The most powerful data in this regard come from the MSC-mediated restoration of coordinated function in old rats by mechanisms that seem to suggest that these introduced MSCs do not differentiate into neurons or neuronal support cells (such as astrocytes, etc.). It is proposed that the MSCs supply bioactive agents that inhibit scar formation, inhibit apoptosis, increase angiogenesis, and stimulate the action of intrinsic neural progenitor cells to regenerate functional neurological pathways (synaptogenesis, neurogenesis) with the resulting gain of coordinated function.

In one study from Chopp's group, MSCs were introduced after permanent middle cerebral artery occlusion was induced by a method of intraluminal vascular occlusion in 10- to 12-month-old Wistar rats [Li et al., 2005]. Either primary rat marrow-derived or human MSCs were introduced into the affected brains by direct injection or via tail vein injection of 3×10^6 MSCs in 1 ml of PBS 1 week after the occlusion. The systemic introduction of MSCs relies on the ability of MSCs to circulate and travel to injured tissues [Chen et al., 2003]. MSC-treated rats showed reduced thickness of the scar wall that usually forms, reduced numbers of microglia macrophages within the scar wall, and increased numbers of reactive astrocytes in the scar boundary zone and in the subventricular zone. Brain tissue repair appears to persist for at least 4 months, as noted in ongoing long-term neurological studies in these older animals. It would appear that reactive astrocytes respond to MSC treatment of ischemia and promote axonal regeneration during long-term recovery [Li et al., 2005].

The experimentally introduced MSCs appear to mediate a complex sequence of events. For example, the ischemia-damaged tissue consists of a central, liquid-filled zone surrounded by a scar wall next to a transition zone that contains morphologically intact neurons. Both this liquid zone (presumably the end-product of massive cell death) and scar wall thickness are significantly reduced in MSC-treated animals. The active replacement of these zones and healing of

the neurological tissue requires many weeks with new astrocytes (as shown by BrdU labeling) and with increased cellular dimensions compared to normal astrocytes [Li et al., 2005]. In seven of nine MSC-treated rats, long axons were oriented parallel to the processes of reactive astrocytes with axonal sprouting in the subventricular zone. As these morphological events progressed, afflicted animals recovered coordinated function as shown by the Adhesion-Removal Test (coordinated removal of small adhesive pad placed on the paw).

The MSCs had their major effects in the days following their introduction and they expired within the first weeks after introduction. These effects summarily involve inhibiting scar formation, decreasing apoptosis, promoting angiogenesis, and stimulating the intrinsic cells to support the re-establishment of the complex neurological pathways (reactive gliosis) resulting in coordinated motor and neural activity. These sequential events involve MSC-mediated parenchymal cell expression of growth and trophic factors in the post-ischemia environment that enhanced Connexin43 gap junction intercellular communication and resulted in functional recovery. Again for emphasis, the labeled MSCs did not differentiate into neural elements.

HEART

There are multiple studies on the use of HSCs as a cell therapeutic for the repair of heart tissue, many of them with conflicting results about whether HSCs have the capacity to differentiate into cardiomyocytes at all or, if they do have cardiomyocyte potential, whether they differentiate into cardiomyocytes at a high enough graft efficiency to have a significant effect on heart function. Whether HSCs differentiate into cardiac cells is a subject of debate [Orlic et al., 2001; Balsam et al., 2004; Kawada et al., 2004; Murry et al., 2004]; for an extensive review on this debate and on cardiac repair in general, see Laflamme and Murry [2005]. This debate aside, we focus here on the therapeutic effects of MSCs on heart function, which leads to similar questions of whether MSCs differentiate into cardiomyocytes or provide therapeutic effects via trophic interactions.

With respect to cardiomyocyte differentiation, there is evidence that MSCs have the potential to differentiate into cardiomyocytes when injected into mouse embryos [Jiang et al., 2002] and to express cardiomyocyte markers in vitro when cultured in the presence of the DNA demethylating agent 5-azacytidine [Hattan et al., 2003] or low levels of dexamethasone $(10^{-9}\,M)$ [Shim et al., 2004]. Several groups studying the systemic or local administration of MSCs in cardiac repair models show co-localization of cardiac markers with implanted MSCs [Min et al., 2002; Shake et al., 2002; Mangi et al., 2003; Kawada et al., 2004; Nagaya et al., 2004]. However, the therapeutic contribution of MSCs to increased heart function can be caused by multiple factors including: neo-vascularization, inhibition of scarring, decreased cardiomyocyte apoptosis, increased nerve sprouting [Pak et al., 2003], and direct differentiation into cardiomyocytes. Even in studies where the contribution of donor MSCs differentiating into cardiomyocytes has been quantified [Thiele et al., 2004], it is difficult to assess what portion of the increased heart function can be ascribed to MSC-derived cardiomyocytes themselves and what contribution is attributable to those other factors. What is clear from these studies is that the administration of MSCs to the defect site resulted in increased heart function compared to controls, as shown by hemodynamic measurements (systolic and end-diastolic pressure), echocardiography, blood flow [Min et al., 2002], and systolic wall thickness and function [Shake et al., 2002].

The trophic effects of MSC administration in infarct models has not been lost on the authors of these various studies. At least a portion of the therapeutic improvement of MSC administration has been ascribed to attenuation of ventricular wall thinning [Shake et al., 2002] and increased angiogenesis [Min et al., 2002]. Recent studies [Mangi et al., 2003] document the effects of transplanted MSCs expressing the pro-survival gene Akt1, and the authors recognized that the molecules released by transplanted MSCs are important. The trophic effects of MSCs have been documented by Tang et al. [2004], who showed that MSCs implanted into ischemic myocardium simulated an increased production of vascular endothelial growth factor (VEGF), increased vascular density and blood flow, and decreased apoptosis, all of which were likely influenced by the secretion of bioactive molecules; the authors also present evidence that some MSCs differentiated directly into endothelial cells.
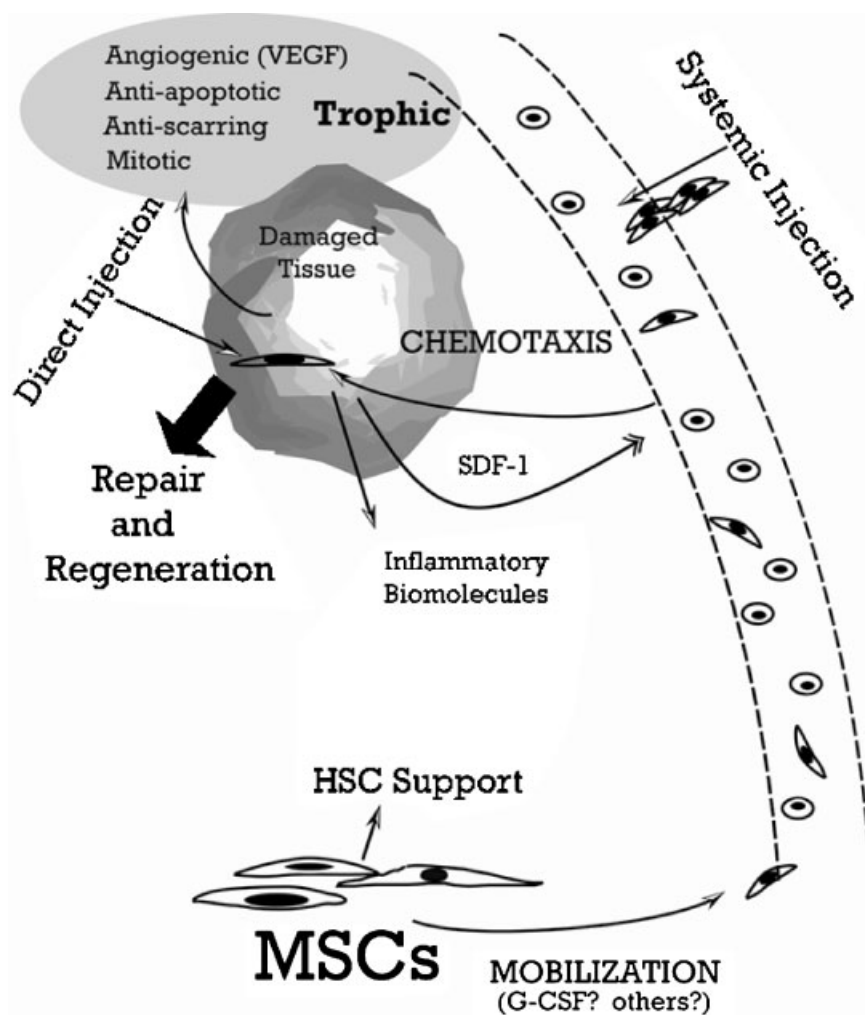
Fig. 3.   Trophic mechanism of tissue repair: MSCs have the capacity to trophically mediate hematopoiesis as a natural part of homeostasis. After tissue damage, MSCs can contribute to tissue repair by entering the damaged region either as a consequence of local chemo-attractants (i.e., SDF-1) or by systemic mobilization. MSCs expanded in culture can be introduced by direct injection into the damaged tissue or systemically by intravenous injection. Once in residence in the damage site, MSCs produce an array of bioactive factors that contribute to positive repair outcomes.

The details of the mechanism(s) for repair or abrogated damage in ischemic models are complex, as shown in Figure 3. For example, stromal-cell-derived factor 1 (SDF-1) has been shown to induce stem cell homing to injured myocardium immediately post-infarction, and it has also been shown that this homing effect can be mimicked by implantation of cardiac fibroblasts stably expressing SDF-1 [Askari et al., 2003]. This study showed an increase in CD117-positive cells, which indicates an increase in endothelial progenitor cells, in agreement with results published later by De Falco et al. [2004]. In another study [Kawada et al., 2004], granulocyte colony-stimulating factor (G-CSF) was shown to be a mobilizing factor in MSCs that have been implanted directly into the bone marrow of mice that were later injected with G-CSF, which promoted migration of the marrow MSCs into the heart after myocardial infarction. From these data, G-CSF is implicated as a general mobilization factor and SDF-1 as a chemotactic factor. What remains unclear is what the MSCs which land in the damaged tissue actually do to effect repair. Additional complexities arise in light of recent evidence that cardiac tissue may itself contain stem cells [Beltrami et al., 2003] which could be the targets of ischemia-induced signaling, such as SDF-1, or are the targets of MSC-

derived signals. These experiments do not conclusively demonstrate the mechanism of the therapeutic effect of MSCs on the restoration of heart function, but clearly establish that there is an MSC-mediated positive effect. The data indicate that MSCs exert a significant trophic effect on heart repair with the detailed mechanism(s) to be established.

MENISCUS

The meniscus is a fibro-cartilaginous structure that provides guidance and cushioning in the knee as the femoral condyle slides across the tibial plateau. The highly vascularized thickened base of the meniscus connects to the synovial-lining wall and progressively becomes thinner toward the femoral-tibial contact area. The distal third, which is progressively thinner, is avascular with the meniscal cells nurtured by the compressive–decompressive effects of the movements of the knee. Tears, rips, or other defects in this avascular distal third of the meniscus do not repair themselves and attempts to stimulate such repair have not proven successful [Arnoczky et al., 1988; Gao and Messner, 1996]. Current surgical therapy involves cutting out the damaged sector or historically completely removing the entire meniscus (partial or complete meniscectomy, respectively). The absence of the meniscus results in joint instability and eventually erosion of the knee cartilage, osteophyte formation, and progressive osteoarthritis.

Studies by Frank Barry and colleagues detail the use of an adult goat model to document the MSC-mediated regeneration of meniscus [Murphy et al., 2003]. Iliac marrow aspirates of adult goats were obtained, and the MSCs were isolated and expanded in culture. The goats were then subjected to knee surgery where the entire medial meniscus was removed and in some cases the anterior cruciate ligament (ACL) was severed, which renders the knee joint highly unstable. At 2 weeks following surgery, the goats were exercised daily for 1 hour on a treadmill. At 4 weeks following the initiation of treadmill exercise, the goats were given an injection of 10 million autologous or allogeneic MSCs in a hyaluronan delivery vehicle or, as a control, given an injection of the hyaluronan delivery vehicle without cells. The animals are sacrificed at 3 and 6 months after their original meniscectomy surgery [Murphy et al., 2003].

In the case of the control injections of hyaluronan alone, which is used clinically as a chondroprotective agent, massive cartilage erosion and osteophyte formation were noted with no obvious repair or regeneration of the missing meniscus. In animals where complete meniscal regeneration was observed, no cartilage damage was observed. The regenerated meniscus was morphologically, histologically, and immunocytochemically indistinguishable from normal meniscus [Murphy et al., 2003]. Although a detailed temporal sequence has not been established, it is probable that the injected MSCs or their secreted bioactive agents inhibited scarring and apoptosis at the cut surface, stimulated angiogenesis, and stimulated the host-derived reparative cells to proliferate and fabricate a new meniscus. Some pre-labeled MSCs could be observed in the new meniscus, but too few to account for the massive regeneration of this new tissue (unpublished observation, F. Barry).

We infer that the MSCs trophically enhanced the regeneration of the meniscus by mechanisms similar to those outlined above for heart and stroke models. It is further inferred that the capacity of MSCs to suppress the immune system and the fact that they do not express co-stimulatory molecules allows *allogeneic* MSCs to be as effective in meniscal regeneration as *autologous* MSCs.

CONCLUSIONS

MSCs can provide replacement cells for those cells that expire and can account for the support of turnover dynamics in young adults. It may be that the reduction of MSC numbers as individuals age is responsible for the loss of tissue mass such as that observed with age-related bone mass loss. The MSCs also secrete a number of bioactive factors with some of these factors acting to directly suppress immune recognition and/or the expansion of B and T cells and also provide a microenvironment that is supportive of long-term survival of HSCs and for the expansion of their progeny during hematopoiesis. In the case of severe tissue ischemia or damage, MSCs can be attracted to the damage site wherein they secrete bioactive factors that function to trophically assist the repair and regeneration process. As depicted in Figure 3, the damage site produces inflammatory signals, such as SDF-1, that may attract MSCs to the

injury site. The MSCs could be mobilized from the marrow or other depots or can be culture-expanded MSCs that are delivered to the damage site either by direct or systemic injection. Once at the site of injury, MSCs produce factors that inhibit scarring (fibrosis) and apoptosis, promote angiogenesis, and stimulate host progenitors to divide and differentiate into functional regenerative units. In this light, MSCs must now be thought of as multi-drug delivery vehicles that are injury-site sensitive and/or responsive. In this regard, the trophic effects of MSCs may have profound clinical use.

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