J. Biomedical Science and Engineering, 2014, 7, 526-532
Published Online June 2014 in SciRes. http://www.scirp.org/journal/jbise
http://dx.doi.org/10.4236/jbise.2014.78054

Why Mesenchymal Stem/Progenitor Cell Heterogeneity in Specific Environments?

—Implications for Tissue Engineering Applications Following Injury or Degeneration of Connective Tissues

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Received 12 April 2014; revised 27 May 2014; accepted 10 June 2014

Abstract

Mesenchymal stem/progenitor cells (MSC/MPC) from a variety of tissue sources (bone marrow, adipose tissue, fat pads, synovial membranes, synovial fluid, skin, muscle and periosteal tissue) have been widely applied for tissue engineering applications to generate replacements for injured or degenerated tissues. Alternatively, they have also been injected as free cells in an attempt to facilitate *in vivo* repair. Nearly all studies reported have used mixed cell populations of MSC/MPC, usually defined by cell surface phenotypes and/or functional ability to differentiate towards multiple cell lineages. Using more detailed cell surface phenotyping and limiting dilution approaches to isolate individual MSC/MPC clones have indicated that such mixed cell populations are very heterogeneous. In addition subsets of cells from different sources may have epigenetic modifications. While it is clear that MSC/MPC cells exhibit heterogeneity, the question of why this is the case has not been well addressed. This review will address some of these issues, as well as provide some insights into the implications when using such diverse cells for tissue engineering applications.

Keywords

Mesenchymal Stem/Progenitor Cells, Cell Heterogeneity, Tissue-Specific Heterogeneity, Tissue Engineering

1. Introduction

Adult stem cells, particularly mesenchymal stem cells (MSC), have been the subject of intense investigation

over the past several years. Sometimes also called mesenchymal progenitor cells (perhaps a more accurate designation), such cells can be isolated from a variety of tissues (bone marrow, fat/adipose tissue, skin, synovial membranes, synovial fluid; to name a few). A recent search of the literature revealed >25,000 articles when the term mesenchymal stem cells were used as the search term, and >19,000 when mesenchymal progenitor cells were used. Nearly all of the reports have used cell populations that are selected by physical properties of the cells, or they are selected based on expression/lack of expression of specific cell surface markers. Stemness or Progenitorness is usually based on the ability of such cell populations to differentiate towards at least three cell lineages, osteogenesis (bone), chondrogenesis (cartilage), or adipogenesis (adipose tissue) under the influence of appropriate differentiation media [discussed in 1]. Interestingly, cell populations derived from different tissue sources appear to be "biased" toward some lineages more than others [1]. Thus, MSC/MPC populations derived from bone marrow (BM) appear to prefer the osteogenesis route, while MSC derived from the synovial fluid (SF) or synovial membranes (SM) appear to prefer chondrogenesis. Overall, these cells have the intrinsic ability to proceed towards any of the three lineages under specific differentiation stimuli. However, as many of such studies use MSC populations rather than cloned cells, it is not possible to determine whether the same cells are proceeding to each of the lineages. Thus, in many of the studies reported, it is not clear whether subsets of MSC exist in populations of MSC.

Evidence for the existence of subsets of MSC has been reported in a study by Ando *et al*. [1] where MSC from sheep joints following an injury/inflammation were found to be partially compromised for proceeding to the chondrogenic lineage but not the osteogenic or adipogenic lineages. Exposure of normal sheep joint MSC to purified IL-1beta led to a decline in chondrogenesis similar to that seen with the MSC from the inflamed joints. Moreover, adding IL-1β in increasing doses led to a specific decline in chondrogenesis to approximately 60% of normal which then plateaued, indicating that a subset of MSC was being affected by this inflammatory cytokine.

Interestingly, injecting millions of MSC from any one of several sources into joints in an attempt to repair damaged menisci [2]; and many others], or into the circulation to repair damaged hearts reviewed in [3] [4] and many others, led to only a small number of the injected MSC remaining at the site of the injury, again indicating that only a subset of cells were able to home and localize to the damaged area. This pattern of minimal localization seems to be the norm rather than the exception. Where the remaining cells went, or why they failed to localize to an injury site remains unclear.

Results such as those identified above (and many more citations) have led investigators to address the issue of population heterogeneity by looking for subsets of MSC with unique phenotypes. An example of this approach is the work of Gullo and De Bari [5] who showed that they could identify a subpopulation of human synovial membrane MSC using CD39 positivity in a CD73(+) population. This CD29(+)CD73(+) subset of cells had higher endogenous levels of Sox9 and Runx2, and an enhanced chondro-osteogenic potency. Many other reports and reviews have also indicated that the populations usually isolated from diverse tissues and fluids are heterogeneous from a number of perspectives [6]-[15]. Thus, MSC populations are heterogeneous. Even in 2013, the issue is still being discussed.

Further evidence for MSC/MPC heterogeneity has come from limiting dilution analysis of cell populations. This approach leads to the isolation and propagation of single cells which can then be assessed for lineage potency. DeBari *et al*. [16] showed using human periosteal cells that regardless of donor age, clonal MSC could be propagated for at least 30 population doublings and such single cells could differentiate into chondrocyte, osteoblast, adipocyte or skeletal myocyte lineages. Some association with endogenous levels for markers such as Sox-9 and Runx2 in undifferentiated cells with differentiation potency was noted. Karystinou *et al*. [17], using human synovial membrane derived MPC, showed that the 50 clones analyzed varied widely in proliferation rates. They also varied with regard to adipogenic differentiation (30%), but all were capable of chondrogenic and osteogenic differentiation, with variable potency.

Recently, Ando *et al*. [18] reported that MSC clones derived from female porcine knee synovial fluid obtained by limiting dilution also vary extensively with regard to proliferation rates to 30 or 40 population doublings. Further analysis (JJ Kutcher, MSc Thesis, University of Calgary, 2012; Kutcher *et al*., in preparation) has revealed that these synovial fluid derived MSC clones also vary in multi-lineage differentiation potential. Many clones were capable of the three lineages assessed, osteogenesis, chondrogenesis and adipogenesis, but with variable potential. Interestingly, the chondrogenic potential was not associated with the basal levels for Sox-9. Furthermore, a unique phenotype for the chondrogenic-adipogenic lineages was detected.

The basis for the heterogeneity observed within and between cells from different locations has not been ad-

dressed in detail. Possibilities include age, sex, genetics, environmental factors (inflammation, nutrition, stress), replicative senescense [19], and potentially epigenetics. The latter factor has also been addressed by Noer *et al*. [20] and Schellenberg *et al*. [19], and shown to also vary within adipose-derived cells, and between bone marrow and adipose-derived cells, respectively. From the above discussion, it is clear that heterogeneity exists in and between MSC/MPC populations derived from different tissue sources. Therefore, the critical question is not whether it exists, but why it exists!

2. Potential Explanations for the Detected Heterogeneity

2.1. The Cell Surface Markers We Use to Define the Cell Surface Phenotype of MSC/MPC Are Too General and Do Not Really Define the Cells Well

Most studies of MSC/MPC use a battery of well excepted surface markers to phenotype what are called MSC/MPC. This can vary between species as for some species one has to use adherence to culture dishes and antibodies that cross-react with specific surface markers (e.g. CD90, CD105, CD73, CD39, etc). The, MSC/MPC from various tissues are usually phenotyped by being positive for some markers and negative for others. Therefore, the current battery of markers may define the general category of MSC/MPC, but this covers multiple subsets. Evidence for this concept comes from the Gullo and DeBari [5] studies cited above where they identified a subset of MSC using CD39+CD73+ cells to define a subpopulation exhibiting a specific set of features. It is likely that as the MSC/MPC field progresses, using both cell surface phenotyping and lineage-specific functional assays, better definition of MSC/MPC subsets will evolve. While the above will effectively recapitulate what was done for the field of hematopoietic stem cells decades ago, it will still not address the reasons for the within and between tissue heterogeneity.

2.2. The Lineages We Use to Define Pluripotency Are Not Reflective of Other Uses for These MSC/MPC Cells in a Specific Location (e.g. Synovial Fluid of the Knee). e.g. They Are Heterogeneous Because We Force Them into "Pathways"!

The term MSC/MPC is a default label we use to describe cell behavior that is convenient and has become accepted in the literature. It describes "potential", however, it is a term that may not reflect what the cells are really doing in the bone marrow, fat, synovial fluid, etc. Proliferation and differentiation are only two parameters. Perhaps we need to assess individual cell secretosomes to also assess the secretion of growth factors/regulators/modifiers ("nurse" cells) as this may be a function that assists other cells better become cartilage, bone, heart tissue, muscle, etc *in vivo* rather than artificially *in vitro*. Likely there are many other functions one could envision for such cells other than regeneration/repair of damaged tissues (normal turnover vs overt injury vs subclinical repair). One approach will be to assess *in vivo* functioning of isolated clones vs. mixed populations in a variety of circumstances/situations.

Another possibility to explain the heterogeneity of MSC populations is that it is reflective of a "mixing" of endogenously produced MSC in a specific tissue (e.g. fat pad of the knee or the synovium of the knee with cells that either circulate from other sites of production (e.g. bone marrow) and localize in the synovial fluid of the knee either through attraction via trophic factors, or via a stochastic process of localization and re-location. MSC/MPC produced in different locations may exhibit tissue-specific clonal "signatures" due to the microenvironment they are produced in (e.g. unique epigenetic signatures; [20]). Given this scenario, not only are the different microenvironments (e.g. bone marrow, skin, synovium, synovial fluid) different biologically, but also mechanically. For instance, synovial fluid contains high levels of hyaluronans and other lubricating molecules which can also have biological activities (e.g. hyaluronans and CD44 or RHAMM; [21] [22]). Thus, it may be naïve to conclude that MSC/MPC developing in the bone marrow, which is a unique environment biologically, will function optimally in the mechanical and biological environment of the knee. If we add in the genetic diversity of individuals, as well as their varied MSC "history", then one has a number of factors which could account for some of the observed diversity and heterogeneity.

In some respects, such a mixing of cells is analogous to what has been observed at delayed hypersensitivity sites, were <1 in 100 T-lymphocytes at the site are antigen-specific, and therefore, many cells localize to the site not because of the antigenic stimuli, but because of the response to the antigenic stimuli with the release of trophic factors that are not antigen-specific. However, the concept of "clonal signatures" for individual clones of MSC/MPC is somewhat attractive, and one that should be pursued going forward using both cell surface pheno-

typing and functional assessments. Certainly such signatures may not be the same as those for T- and B-lymphocytes (arising via recombination in a clonal manner), but there may be a parallel system for identifying unique subsets of MSC/MPC.

An important set of questions remain, and they are: **Is the heterogeneity "planned" or random, and is the diversity an intrinsic strength of the system, or are we over interpreting its significance?** The answers to such questions are important from the perspective of understanding the impact and regulation of MSC/MPC, but also from the perspective of using such cells for endogenous and exogenous tissue engineering applications.

Finally, one has to acknowledge that some of the heterogeneity may be an artifactual result of culturing MSC/MPC *in vitro* in artificial conditions (e.g. fetal bovine serum, non-optimized culture media, in plastic dishes, etc). Clearly, these are non-normal conditions for cells from unique biological and mechanical environments that likely can "skew" the outcomes of proliferation and differentiation assays. Relevant to this point is a recent set of studies by Dry *et al*. [23], indicating that the calcium concentration of synovial fluid is much higher than what is found in many common tissue culture media, and supplementing the media with additional calcium salts leads to an overt acceleration in proliferation rates. Therefore, the cells are cultured in media of convenience, rather than the most optimal conditions which mimic the *in vivo* environments. This concept of cells *in vitro* being conditioned by the microenvironment has also been discussed by Gregory *et al*. [24].

2.3. Irrespective of MSC/MPC Heterogeneity, Undifferentiated MSC/MPC Used for Generating an *in Vitro* Tissue Engineered Construct (TEC), Can Still Lead to Effective *in Vivo* Cartilage Repair

While the above discussion has raised issues regarding MSC/MPC cell heterogeneity, and the potential basis for such heterogeneity, it is clear that generating TEC *in vitro* with undifferentiated MSC/MPC leads to constructs that can adhere *in vivo* to defects in articular hyaline cartilage and adapt to become an effective repair tissue out to 1 year post-implantation in either adolescent or adult porcine models (discussed in [25]-[27]). Furthermore, osteo-TEC constructs also become integrated in both porcine and rabbit models [28] [29]. Thus, such MSC/MPC constructs are able to differentiate *in vivo* in the microenvironment (biology and biomechanics) of the knee to become a close to normal looking and functioning cartilage. However, it is not perfect as the extreme surface layer (lamina splendans) is not normal [30]. However, one current limitation is that it has not been clearly shown that the cells populating the implant are the same ones that were in the original TEC. It will be important to clarify this point in the future, as well as determine whether TEC generated from differentiated mixed populations or TEC generated with clonal MSC/MPC are as effective as the above discussed TEC for long term cartilage, meniscal [31], or bone repair (discussed in [32]).

In addition, other laboratories have reported that injection of MSC derived from synovium into rabbit knees can lead to significant repair of massive meniscal defects over a 6 month period post-injection [33]. Some (a small percentage) of the injected cells did home to the site of the damage and were detectable for 14 days [33], so even if only a small percentage of the isolated cells are effective, it may be sufficient. Similarly, other authors have used this approach to repair meniscal defects in other models [34]. However, if that small population could be identified and isolated, perhaps the long term outcomes could be improved yielding an engineered tissue which optimally adapts to the meniscal microenvironment.

2.4. Implication of MSC/MPC Heterogeneity for Tissue Engineering Applications

As heterogeneity in MSC populations/subpopulations may arise from genetic, epigenetic, localization-relocalization, site history, and likely sex/gender factors, this variability may be of concern for those applying such cell populations to generate tissue engineered replacement tissues. This is likely a concern for both autologous and allogeneic applications, but likely of more concern for allogeneic applications. Thus, using mixed populations with intrinsic variation with regard to the subpopulations present may lead to varied outcomes that may not be predictable. Therefore, using very well characterized clonal populations as the "starting material" for generating an engineered construct may be the preferred approach to minimize variation and risk.

Use of such clonal populations may also mitigate potential risk of unwanted complications associated with the use of mixed populations. While there is the general perception that MSC/MPC do not pose any safety risks, it is still not clear that all MSC that exhibit the ability to proliferate extensively, have epigenetic modifications that may influence gene regulation and responsiveness to stimuli, and retain the ability to differentiate along several

lineages, do not pose some risk until proven otherwise. Given the emerging literature regarding the role of tissue-specific stem cells in various cancers [35]-[37]; and many others), proof of no risk in this regard is essential and using well defined clonal populations may mitigate some of that risk.

3. Summary

Studies with MSC/MPC from multiple sources indicate that cells rising in or localized to specific environments are very heterogeneous and apparently influenced by their microenvironment. While there are likely several clues as to how such heterogeneity arises and is maintained, for what purpose is still unclear and needs further investigation. Such heterogeneity has many implications for those interested in using mixed populations for tissue engineering applications, but it remains to be better understood whether mixed populations or individual clones of MSC/MPC are the best starting cells for tissue engineering applications for the long term repair/regeneration of damaged or injured tissues. Finally, while it is assumed in some quarters that MSC/MPC pose no safety risk, many clinical trials are likely warranted to confirm there is no risk, or that risk can be mitigated by using well characterized clonal cells.

Acknowledgements

The author thanks many local, national and international colleagues and trainees who have participated in studies emanating in part from the author's laboratory, and Dr. Yamini Achari for reviewing the manuscript and providing input into the final version. The financial support of Alberta Innovates Health Solutions, the Canadian Arthritis Network, and the Canadian Institutes for Health Research is gratefully acknowledged.

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