

Blood Stem Cells and non-Hematological Clinical Practice: Pragmatics Before Therapeutics

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Abstract: There is considerable interest in biological sources for replacement, repair, as well as vascularization of tissue. The remarkable properties of blood stem cells encourage interest in their therapeutic potential. But what are these properties, and how do they influence their clinical potential and the advisability of stem cell use as a therapeutic resource? Rational assessment of the significance of *in vitro* and animal *in vivo* data should precede the rush from the bench to the bedside. Basic stem cell research is rife with examples where the truth of the subsequently demonstrated mechanism is stranger than the initial interpretation proved fiction. This review will assess tissue contribution by different blood related stem cells, differing possible mechanisms underlying observed repair phenomena, and consider the potency and pitfalls of stem cell therapeutics.

Key Words: Stem cell, hematopoietic, mesenchymal, progenitor, endothelial, somatic, clinical studies.

INTRODUCTION

The goal of this review is to exemplify through discussion of a number of significant studies how our understanding and interpretation of the observed phenomena associated with stem cells influences assessment of blood related stem cell therapeutic potential for non-hematological conditions. There is considerable interest in engineering tissue for replacement, repair, or vascularization. As will be developed in this essay observations made in stem cell research are in need of constant re-evaluation in light of new data and new ideas.

Somatic stem cells are undifferentiated, potential sources of maintenance and repair that are ostensibly dedicated to a particular organ or tissue. Although this is how a number of different stem cell populations with extraordinarily different properties and behaviors are classified, the term primarily demarks them as being different from embryonic stem or germ cells. However, the number of properties that are shared among such populations is dwarfed by the number of differences, as has been discussed elsewhere (e.g. [1,2]). There is general concordance in their ability to differentiate into one or more mature cell types that are associated with a particular organ or tissue. Less agreement is found on the issue of self-renewal, although this is an ostensibly necessary feature of hematopoietic stem cells (HSCs); the rapid replacement of which is so vital to the maintenance of life.

CORD BLOOD STEM CELLS AND THERAPY

Human umbilical cord blood was first properly examined as an alternative source of HSCs for transplant use seventeen

years ago, by Broxmeyer *et al.* [3]. Umbilical cord blood has been thought unviable for adult 'transplant' therapies because of the limited volume and therefore limited number of cells involved. However, there is clinical interest in their use in child transplants, and their purportedly higher proliferate and differential potential as well as their alleged lower immunogenicity has increased interest in their use in adults. In 2002, it was estimated that at least 2,000 cord blood transplants had been done worldwide [4]. Not least, because of the youth of the majority of recipients, there is a need for a better characterization of the vulnerability, the suitability and a greater understanding of the risk factors associated with cord blood samples. The successful therapeutic use of cord blood would be advanced by a more rigorous examination of which stem cells present in the cord actually contribute to the therapeutic effect. Unfortunately, numerous publications and therapeutic efforts refer to "cord blood stem cells" without actually delineating what type of stem cell(s) is present, nor which is responsible for any functional improvement. The blood-related stem cells, Fig. (1), could be categorized as: (1) HSCs; (2) Mesenchymal stem cells; and (3) Other stem cells that may not share the characteristics of either of the first two, but may contribute to functional recovery in a given model.

HEMATOPOIETIC STEM CELLS

HSCs, that *in vitro* are capable of a certain level of self-renewal, and can with appropriate culture be differentiated into the subclasses of terminally differentiated blood cell types that constitute the hematopoietic system, are the most researched somatic stem cell population. Their clinical exploitation combined with the elaborate characterization of their differentiation potential and pathways make them the gold standard of stem cells against which the properties and behaviors of all other somatic stem cells are compared. Successful exploitation of the salicylic marker CD34, allows the isolation of a cell population that has an impressive level of

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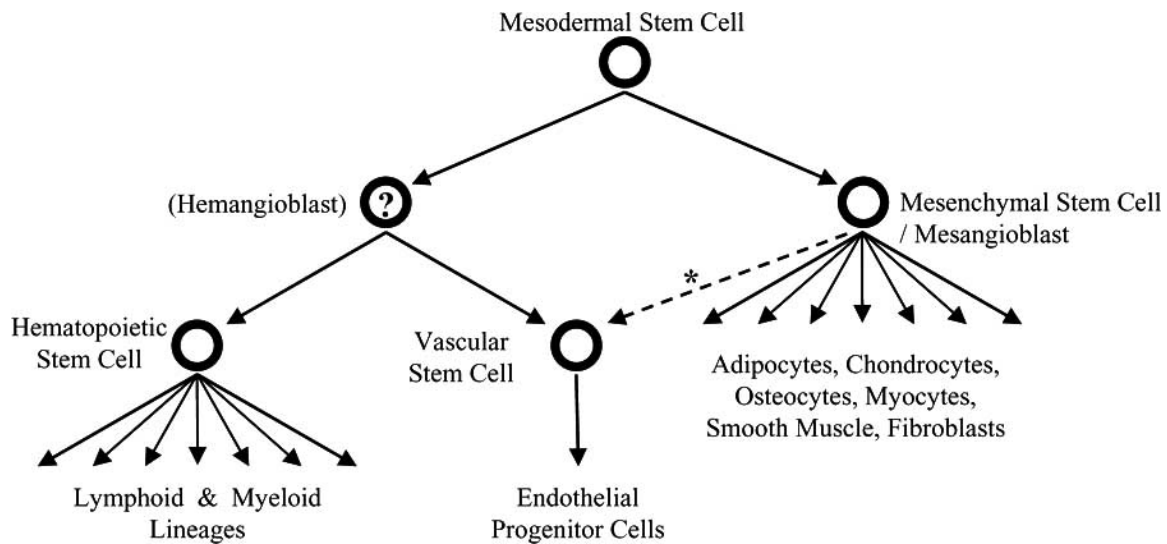


Fig. (1). A model of how blood-related stem cells may be related. *The author wishes to acknowledge the suggestion of Ralf Huss, MD, PhD.

purity, as assessed by the expression of this marker. However, there may also be cells that do not express CD34, but would be capable of differentiating into HSCs [5]. It therefore should be clarified that the majority of studies related to HSCs, are examining a specific sub-population of HSCs.

As of writing, HSCs are still the only demonstrably functional stem cell therapy. For many years bone marrow (BM) transplants have been performed, the success of which depends on the stem cells homing to the recipient's BM, engrafting and providing the blood cells required to maintain the recipient's life. Furthermore, donations can be cryopreserved for a number of years and still be reliably thawed and used. A number of conditions have thus been treated including leukemia; lymphoma and several inherited blood disorders, as well as the reconstitution of the blood supply of patients receiving radiation and other chemotherapies. Such success depends on the ability of the cells to engraft and begin self-renewal. However, if HSC are so good at self-renewing, why is it that the world's blood banks are in such crisis? Although many publications describe improvements and supporting data concerning our ability to culture and expand HSC *in vitro*, the fact remains that a given blood treatment from cultured or expanded source is not as efficient as an original donation, whether it be fresh or cryopreserved. Two avenues of research will hopefully address this problem: (1) the definitive characterization of the most primitive HSC; and (2) the improvement of our *in vitro* expansion of HSCs.

Towards the end of the 90's a number of publications started to appear that challenged a basic tenet of developmental biology; that once embryonic cells had developed into the three germ layers of the endoderm, mesoderm, and ectoderm, their specification to that germ layer was irreversible. Such publications suggested the possibility that somatic stem cells had the ability, the 'plasticity', to become mature cells associated with a different germ layer. Probably the most dramatic and seized upon example was that of converting HSCs into neural stem cells, or indeed straight to mature neural cells - a cell from the mesodermal germ layer

becoming a mature cell of ectodermal origin. The mooted ability to convert blood to brain holds such strong appeal for a number of reasons: (1) the relative ease of access to a patient's HSCs; (2) the ready replacement of the extracted HSCs; (3) extensive knowledge of culture and handling of HSCs; (4) the difficulty of isolating NSCs from a patient; and (5) the relative quiescence of the brain. Most of these advantages would also apply to the conversion of HSCs to numerous other somatic cell types.

The evolution of the interpretation of such data can best be illustrated by examples from the literature. In 2000 [6], the restoration of function in compromised murine liver was reported using BM-derived HSC; a cell from the mesoderm germ layer repairing function in an endodermal-derived organ. However, in 2003 [7] the same lab demonstrated that the alleles that were homozygous in the original HSCs were now heterozygous in the hepatocytes that repopulated the murine liver. Furthermore, karyotyping of the relevant gender mismatched donor cells demonstrated both diploid-to-diploid and diploid-to-tetraploid fusion events between donor HSCs and host liver cells. To many observers, the suggestion that functional recovery in the liver had resulted from one germ layer cell fusing with a cell of a different germ layer appeared even more fantastic than the original suggestion of transdifferentiation. Other authors have since noted that mobilization of BM increases such events [8] an observation also made with respect to fusion events between HSCs and skeletal muscle cells [9,10]. Additionally, cell fusion has been reported to occur between HSC and Pukinje neurons and cardiomyocytes [11,12]. Perhaps, then, the ability to fuse with, and restore or maintain function to a mature cell is a unique property associated with stem cells. However, a further surprise was in store. In 2004 [13], it was reported that macrophages derived from BM HSCs were also capable of fusing with hepatocytes to produce functional epithelial cells. Thus, an ostensibly committed mesodermal cell is capable of repairing an endodermal organ. This fascinating series of investigations suggests such a cell could be used which has no apparent potential for self-renewal. In specific clinical settings this would hold advantages over the

clinician having no control over the replicative behavior of the therapeutic agent.

The frequency of cell fusion events was described [13] as making the process 'well below the thresholds of therapeutic significance'. But either the animal model demonstrated functional recovery and cell fusion was wholly responsible, or an additional mechanism was also involved. However, once the phenomenon of cell fusion had been described, a number of publications appeared from authors in a range of journals declaring that all other mechanisms that might account for, or contribute to, functional recovery in a given model should be discounted. In spite of this, further evidence in support of alternative mechanisms derives from *in vitro* co-culture experiments. HSCs co-cultured with liver tissue expressed markers associated with terminally differentiated hepatocytes at the mRNA and protein level [14]. Karyotyping indicated cell fusion events had occurred, but only between HSCs, with no fusion events involving HSCs and hepatocytes. Using a similar co-culture system, our laboratory published evidence suggesting that HSC could become NSC and thence differentiate to a neural fate [15]. Such data have been dismissed by some as *in vitro* curiosities with little therapeutic implications for the *in vivo* setting [16,17], describing transdifferentiation as an 'extremely rare event, if it occurs at all'. However, reports supporting the occurrence of transdifferentiation of HSC still continue to be published [18]. A recent report examined post mortem brain tissue of female bone marrow transplant recipients from male donors [19]. Perhaps surprisingly, the authors observed no evidence of XXY or XXXY karyotyping indicative of fusion events, though this may be influenced by the time elapsed between marrow transplant and death. The authors did observe cells containing a Y chromosome that were immunohistochemically stained with antibodies characteristic of mature neural cell types: β -III tubulin for neurons, and GFAP for astrocytes. Furthermore, the cells displayed morphology consistent with the appropriate mature neural cell. However, there is nothing to indicate that the cells responsible were in fact HSC; the male donor cells responsible may have been MSC, and as we shall see below, these cells are capable of expressing mature neural cell markers.

What is also unaddressed is the functional significance of the apparent incorporation of marrow transplant cells into the neural tissue. That it occurs is of interest, but was any clinical benefit derived, and what implications are there for normal neural development? For if transplanted cells are capable of contributing to the neural architecture then surely our endogenous blood-related stem cells should also routinely contribute to the neural environment. Noteworthy is the reported level of representation: 1% of all neurons and up to 2% of glial cells. As a percentage of the oft-quoted figure of 100 billion (U.S.) neurons that the human brain contains, that makes a billion neurons that owe their existence to one BM transfusion!

MESENCHYMAL STEM CELLS

Mesenchymal stem cell (MSC) is arguably the most commonly used term for members of a population of cells that serve a number of developmental and maintenance functions. These cells can give rise to the populations of adipo-

cytes, osteoblasts, and chondrocytes that in turn develop into fat, bone and cartilage. They also provide the stromal cells that contribute to the hematopoietic environment that supports the maintenance, expansion and differentiation of HSC as well as endothelial cells. Numerous groups have isolated such cells with very similar properties from BM [20], skeletal muscle [21], fat sites [22], the umbilical cord [23], and from the cord blood itself [24]. MSC derived from these different sites share similar morphology, differential potential and substantially the same immunophenotypic profile. Unlike HSC, however, there is no single marker seen as being characteristic of MSC, but rather a list of markers that are expressed on a large percentage of an MSC population. The differential potential of these cells is the only discriminative assay of their identity. However, there does appear to be differences in the propensities of MSC derived from differing sources to differentiate into the different mesenchymal fates (e.g. [25]).

The literature concerning the use of MSC in tendon/ligament repair is thus far thick on review and sparse on original reports [26-28]. Out of 28 relevant articles, fully 13 were reviews. The animal model of choice is the rabbit wherein recent reports have emphasized the use of MSC less as a source of regeneration and replacement, but as an adjunct to autologous tendon autograft [29]. Treating the graft with a layer of MSC resulted in a surrounding layer of cartilage forming, a decrease in observed scarring around the tissue and an increase in mechanical performance of the graft. Other reports also observe functional improvement but with no obvious incorporation of MSC into the tendon itself [30].

Since 1988 [31], researchers have been examining MSC-derived osteogenesis and since the mid-90's have posited MSCs as a potential therapy for conditions such as osteogenesis imperfecta [32]. Animal studies using rats femurs implanted with ceramics bearing human MSCs were stronger than those implanted with only ceramics [33]. Children with osteogenesis imperfecta that received allogenic BM transplantation showed reduced incidence of bone fractures compared to controls [34]. The authors attributed the repair to engraftment of MSCs. Since then further studies have attempted to better characterize the contribution of human MSCs in animal models of bone injury [35], emphasizing in particular the acceleration of injury repair and the increased efficacy of MSC treatment combined with platelet-rich plasma [36] - such results raise the issue of whether the presence of MSC-derived osteoclasts might increase the efficiency of bone engraftment of the MSC. It also questions whether the improvement is directly due to stem cell-derived tissue contribution, or a secondary benefit derived from improved vascularization of the tissue. A 2004 human clinical report of treatment of distraction osteogenesis with MSC/platelet-rich plasma [37] emphasized the possibility of shortening treatment time, but as yet there have been no follow-up reports describing long-term improvement. A more dramatic intervention was performed in utero on a fetus diagnosed with osteogenesis imperfecta [38]. The allogenic transgenic fetal MSCs successfully engrafted in the immunocompetent and HLA-incompatible patient. The success of the engraftment was further confirmed by a median estimate of over 7% of bone biopsy cells being Y positive at 9

months old. This, once more, suggests a fantastic level of representation; although in this case an argument might be made that the engrafted cells are 'fitter' than the endogenous cell population. Autologous treatment with MSC has also been performed on a girl bearing multiple fractures of the skull that was otherwise resistant to treatment [39]. Adipose-derived MSC were mixed with fibrin glue and applied and retained under resorbable sheets. The prognosis was much improved with almost complete continuity of the skull surface. However, there is no way to assess the mechanism responsible: the MSC may have differentiated into osteocytes and thence contributed to the skull tissue; the transplant may have contributed in an alternative mechanism to bone resorption/re-formation; or endogenous repair may have been induced by cytokines released by the exogenous MSC, or elicited by their presence.

Recent reports suggest MSCs can differentiate towards a neural fate *in vitro* (for example, [40,41]) and thence effect *in vivo* functional improvement in the CNS [40,42]. Papers, such as [43], examined previously the potential for incorporation of human MSC into the rat ischemic brain and their subsequent expression of neural markers. The interpretations of such earlier reports are now complicated by the demonstrated ability of MSCs to constitutively express markers that are understood to be characteristic of, if not specific for, neural cells. Reports confirm the protein expression of nestin - formerly seen as indicative of neural stem cells but now regarded as a primitive neuroepithelial marker - and Tuj1 in MSCs [44]. But does the protein expression of tyrosine hydroxylase and GFAP in the same cells constitute, as the authors conclude, evidence that the MSCs have now differentiated into mature neural cells? Or does it reflect the expanding knowledge of MSC ability to express proteins associated with neural cells? Similar papers have also observed the expression of tyrosine hydroxylase by cells differentiated from fibroblast-like cells expressing not only fibroblast markers but also embryonic markers such as OCT3/4 and neuroectodermal markers nestin and β -III tubulin [45]. However, other reports are more wary to define the resulting cells as neural. Padovan *et al.* [46] also observed the expression of neural markers by MSC-derived cells, but found the cells incapable of producing fast sodium currents or responding to pharmacologic challenges consistent with receptor-mediated electrophysiological events. In contrast, a recent report claims that MSC-derived cells not only express neural markers, but are also capable of generating action potentials and are electrophysiologically responsive to GABA and glutamate [47]. The authors claim, though, that only nestin-positive MSC are capable of differentiating into the neuron-like cells. This is confusing, as to our knowledge the only way of determining expression of nestin renders the cell no longer viable. The attribution of properties to a population of cells based on the analysis of a subset appears reasonable, but not if the discriminandum is differentially expressed within that population. The problem is exacerbated when the population in question is as heterogeneous as MSC.

A model of stroke that involves the temporary occlusion of the rat middle cerebral artery has been used for over 20 years [48]. In 2000 [49], Chopp suggested BM cells combined with brain derived neurotrophic factor (BDNF) infusion would increase BM differentiation as well as functional

recovery following middle cerebral artery occlusion. They, and other authors, have questioned the mechanisms responsible for the functional improvement observed in animal models following treatment with stem cell populations. Such papers examined whether the contribution of SC to functional repair of tissue is mediated by cytokines or growth factors [50]. Further examination of this issue includes gene-modified MSCs, especially to express (BDNF) [50-53]. A number of reports have proposed BDNF as the actual treatment, with MSCs being simply the vehicle for delivery [50,52]. Furthermore, Kurozumi *et al.* [54] observed functional and cytoarchitectonic improvement in rats that received telomerized human MSCs transfected with BDNF and GDNF, but not CNTF or NT3. Over ten years ago, it was reported that pretreatment of rats with intraventricular bFGF significantly reduced infarct volume following temporary bilateral carotid occlusion and permanent occlusion of the distal middle cerebral artery in rats [55]. The Chopp laboratory has described [56] how rats subjected to middle cerebral artery occlusion showed functional improvement following intravenous injection with human MSCs. These cells were observed to migrate to the brain. However, there was a significant increase in neural stem cell proliferation in the neurogenic subventricular zone over controls, and an increase in newly formed neural cell migration to the ischemic area. There is no suggestion by the authors that the MSCs became neural, or even that their incorporation into the neural tissue directly contributed to any neural repair. Instead, the authors suggested a role for the observed MSC-induced increases in insulin-like growth factor-1 as being important to the observed functional recovery. Interestingly, more human cells were detected in the brain when assayed a number of weeks later. Presumably, the cells are either continuing to migrate to the brain from the periphery, or are proliferating centrally. Until recently it had been accepted that neurogenesis in the human brain ceases to occur after a certain age. This was disappointing from the point of view of repairing or replacing lost tissue in neurodegenerative conditions, but comforting in that the brain is hardly a tissue within which you would want spontaneous growth. However neurogenesis does now appear possible in the human adult brain [57]. The efforts to characterize which cytokines might be responsible for therapeutic improvements in animal models offer the possibility of therapeutic application of a stem cell-derived cytokine, obviating the need to introduce exogenous stem cells into the brain.

'OTHER' BLOOD STEM CELLS

So far, this review has been restricted to how stem cells might contribute either directly or indirectly to tissue repair and replacement. However, this is effectively only half the story. The second part of the problem is the provision of nutrients and removal of breakdown products. Vascularization hence provides a possible alternative explanation for observed functional recovery following stem cell treatment, regardless of demonstration of tissue incorporation, karyotyping or specific marker expression. This point becomes more acute when we now consider a population of cells relatively recently described which appear to derive from, or from a related source to, HSCs. Endothelial progenitor cells, and to a certain extent mature endothelial cells also express

CD34 [58], the archetypal HSC marker. Examples such as Taguchi *et al.* [59] propose that enhancement of neurogenesis is a result of improved angiogenesis following treatment with CD34+ cells. Neovascularization after administration of human cord blood-derived CD34+ cells is suggested to provide the necessary conditions for regeneration of the ischemic brain to occur [60]. The necessary reappraisal of existing data on blood stem cell contribution to neural repair is further advanced by the observation that nestin expression is upregulated in newly formed human blood vessels [61,62]. The further characterization of such cells (for a review, see [63]) and how they relate to existing stem cell populations is important, see Fig. (1), not only to general tissue replacement and repair, but to vascularization as an end in itself.

Persistent confusion exists as to what cell type is actually contributing to an observed effect. CD34+ cells are homogeneous for the expression of one surface marker, and even then only in that they express that marker, not in the level of expression. Beyond that marker, there are myriad surface markers and characteristics that will further define their status as a cell, HSC vs. EPC, early vs. late HSC, etc. When we isolated CD34 cells to explore their neuropoietic potential [15], the purity of each sample was between 93 and 97%. However, when the cells capable of expressing 'neural' markers account for only 5% of that initial population, the problem of interpretation becomes obvious.

In summary, there are a number of mechanisms whereby blood stem cells may be of non-hematological therapeutic use: (1) Support to existing cells may occur through cell fusion, possibly transdifferentiation, or by providing a matrix for repair; (2) Stem cells may release cytokines, or elicit an endogenous release of such factors, to signal a further cascade of tissue repair; (3) MSC and HSC-derived osteoclasts appear to prepare the engraftment site for subsequent repair or engraftment. Furthermore, MSC may have a role in denoting the target zone of injury, either in imaging techniques or in further exploitation of MSC as a drug/factor delivery system.

Finally, there are two unhealthy trends appearing in stem cell biology. The first, therapeutic, is the rush to exploit the untreatable with the unproven. 'Experimental therapy' can, and has, proceeded in instances where the rationale for the treatment choice had only a nebulous proposed mechanism and scant regard to the long-term sequelae of the intervention. The argument that a patient may have no other treatment options could only be tenable in situations where the patient has a life expectancy shorter than any reasonable expectation of a therapeutic breakthrough, and a body of animal model evidence exists in support of the rationale. However, when, for example, we are invited to applaud reports where one spinal injury patient shows improvement while ten remain unchanged or worse following treatment with MSCs we must question the responsibility of the clinical intervention. The second trend, scientific, is a rush to condemn novel observations or interpretations of result that run counter to current dogma. Hopefully, the research efforts celebrated in this review will have helped demonstrate to clinicians and researchers alike that stem cell research continues to generate phenomena that outpace our ability to definitively characterize such observations at time of publica-

tion. The hope is that good data relevant to the therapeutic application of stem cells will be available to the scientific and clinical community for subsequent necessary review and reinterpretation. Stem cell research is necessary to our understanding of normal human development and hence is of clinical use in understanding why sometimes development goes wrong. Non-hematological clinical practice benefits from the knowledge derived from stem cell research, but as yet such therapeutic application of blood-related stem cells receives the Scottish verdict of 'not proven'.

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