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Stem cells in the spleen: Therapeutic potential for Sjogren's syndrome, type I diabetes, and other disorders

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Abstract

The view of the spleen as an unnecessary organ has been shattered. The evidence shows the spleen to be a source of naturally-occurring multipotent stem cells with possibly pluripotent potential. The stem cells are sequestered in the spleen of not only of animals but also of normal human adults. The reservoir of cells is set for differentiation and they need not be manipulated *in vitro* or *ex vivo* before autologous or heterologous use. Splenic stem cells, of Hox11 lineage, have been found in disease or injury to differentiate into pancreatic islets, salivary epithelial cells and osteoblast-like cells, cranial neurons, cochlea, lymphocytes, and more differentiated immune cells that repair injured heart cells. Injury or disease in target tissues induces these stem cells, still in the spleen, to upregulate the same embryonic transcription factors artificially introduced into induced pluripotent stem cells (iPS). Splenic stem cells may have broad pluripotent potential, but unlike iPS cells, possess low oncogenic risk.

Keywords

Spleen; Stem cells; Regeneration; Sjogrens; Type 1 diabetes

1. Introduction

Long relegated to obscurity as an unnecessary organ, the spleen is gaining allure for harboring a unique population of multipotent, and possibly pluripotent, stem cells. In disease or injury, splenic stem cells are capable of self-renewal, differentiation, restoration and/or changes in function of a broad range of tissues, e.g., pancreatic islets (Kodama et al., 2003; Robertson et al., 2008; Park et al., 2009; Yin et al., 2006), salivary epithelial cells (Tran et al., 2007), osteoblast-like cells (Macias et al., 2001), cranial neurons (Lonyai et al., 2008a,b), inner ear structures (Lonyai et al., 2008a,b), lymphocytes (Kodama et al., 2003), and more differentiated immune cells (novel dendritic cells and macrophages) that repair damaged heart cells after ischemic injury or extended lymphocyte lineages (Swirski et al.,

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2009; Tan et al., 2010). In several of these studies, the newly differentiated stem cells contribute to full and stable functional restoration.

Most strides have been made with two autoimmune diseases, Type 1 diabetes and Sjogren's syndrome, which are the focus of this review. Type 1 diabetes (TID) is a debilitating disease marked by immune destruction of the insulin-secreting beta islet cells of the pancreas. The disease features the same constellation of multiple-organ morbidities as does Type II diabetes, namely neuropathy, renal failure, stroke, and blindness. Formerly seen only as a store-house for hematopoietic cells, the spleen is now also seen as a possible source for cellular replacement therapies for new beta islet cells of the pancreas for TID (Kodama et al., 2005a,b,c). Harvesting splenic cells from a healthy donor animal and injecting them into a diabetic recipient with advanced TID is an effective therapy for two reasons. The freshly harvested and matched donor splenocytes are administered with a second treatment (an inducer of the cytokine Tumor Necrosis Factor) that halted autoimmune attack by destroying the host's rare autoreactive lymphocytes responsible for killing islet cells (Ryu et al., 2001;Kodamaet al., 2003). The matched splenocytes directly and indirectly contributed to regeneration of pancreatic islets and return of blood glucose levels to normal (Ryu et al., 2001; Kodama et al., 2003; Yin et al., 2006).

The splenic donor cells that contributed to islet regeneration did not bear the typical phenotype of a lymphocyte, the most abundant cell type in the spleen. While most splenic cells bear the cell surface marker for lymphoid cells, CD45+, the splenocytes contributing to islet regeneration bore no such markings, rendering them CD45−. Using two types of lineage tracing techniques, the donor CD45− splenocytes were found to have differentiated into islet cells in the recipient (Ryu et al., 2001; Kodama et al., 2003). Only the CD45− cells were found to have contributed to regenerating islets. The source of stem cells was in the splenic capsule and not found diffusely throughout the pulp of the spleen. The newly differentiated donor stem cells functioned effectively as long as the underlying immune attack was held at bay. These studies were conducted in mice with end-stage diabetes, the most intractable type to treat. A recent study found that cultured mesenchymal stem cells (MSCs), extracted originally from bone marrow, prevent onset of diabetes in NOD mice via induction of CD4 T regulatory cells (Madec et al., 2009). MSCs are derived from the mesoderm of embryos and they differentiate into connective tissues and hematopoietic cells. We have yet to study the molecular relationship between MSCs and splenic stem cells except for the obvious distinctions in cell type (hematopoietic vs non-hematopoietic) and origin (cell lines derived from embryos vs fresh tissue), respectively.

The next application of splenic stem cells *in vivo* was in Sjogren's syndrome, an autoimmune disease affecting both the salivary glands and lachrymal glands as well as other organs. Sjogren's syndrome was a logical next step. First, like humans, the animal model (i.e., the NOD mouse) can manifest both type 1 diabetes and Sjogren's syndrome. The two diseases in the NOD mouse share overlapping pathology as a result of similar genetic and protein abnormalities (Hayashi and Faustman, 1999, 2000). Further, the salivary glands and the pancreas are developmentally related: they derive from the same embryonic region that spawns the spleen (Lonyai et al., 2008b; Kodama et al., 2003, 2005a,b; Robertson et al., 2008; Park et al., 2009; Yin et al., 2006; Ryu et al., 2001; Nishio et al., 2006; Suri et al.,

2006). In animals with Sjogren's syndrome, donor splenocytes both directly and indirectly regenerate the salivary glands (Tran et al., 2006). Mice receiving splenocytes display restoration of salivary flow during 40 days of active treatment. Further, the animals do not develop type 1 diabetes. This data was unique in not only showing the halt of disease or common data of many autoimmune interventions that prevent progression of disease, but data showing the actual reversal of disease by restored salivary flow back to normal levels after advanced disease.

Like the diabetes studies, the Sjogren's experiments were unique in restoring function, rather than slowing or delaying the decline in function. The hallmarks of the transformation were disease removal, regeneration, and restoration of either salivary flow or glucose levels to those of normal animals. The research demonstrated that CD45− spleen cells differentiated into healthy cells of more than one lineage. Their differentiation into at least two types of newly functional cells – beta islet cells of the pancreas and epithelial cells of the salivary gland—fulfill the criteria for being *multi-lineage* stem cells. Those criteria are robustness, durability, and assumption of function in more than one tissue (Anderson, 2001). These studies are among the first to demonstrate that splenic stem cells can succeed therapeutically over the long-term. One of two components of the therapy we devised was targeted removal of the autoimmune cells with an immunomodulatory agent. That was essential to enable regeneration of tissues with the stem cell infusion, the second component of the therapy. Otherwise, differentiating stem cells and the remaining islets would be subject to immune cell destruction. Other research has found that stem cells endogenous to the pancreas also contribute to regeneration of the pancreas in type 1 diabetes.

2. Cell origin and plasticity: splenic stem cells express Hox11, migrate and undergo differentiation in multiple target organs

The versatility of CD45− splenic stem cells and their implications for regenerative medicine may trace back to the spleen's embryonic origins in the aorta-gonad-mesonephros (AGM), an embryonic region that is the first site of intraembryonic hematopoiesis. During human embryogenesis, the spleen is responsible for hematopoiesis from 6 weeks gestation to the fifth gestational month in humans, a period prior to bone marrow hematopoiesis. Thereafter the bone marrow assumes hematopoiesis for the remainder of the life span (Chadburn, 2000). Nevertheless, the spleen continues to play a backup role when the bone marrow cannot meet the body's full demand—usually in development or disease (Chadburn, 2000). Taken together, it suggests that the spleen harbors stem cells with even greater multi-lineage potential beyond traditional hematopoietic lineages although little data exists to support these cells for a direct role in modulation of the autoimmune response.

The multi-lineage capacity of CD45− splenic stem cells may be explained by their expression of the well-known embryonic protein Hox11 (Kodama et al., 2005a,b,c). Hox11 is a highly conserved transcription factor contributing to embryonic development. It controls expression of other genes responsible for spatial patterning, cell fate, cell differentiation and/or regeneration (Dear et al., 1995; Raju et al., 1993). Deletion of the Hox11 gene (Hox11−/−) in mice leaves the animal's spleen missing (Roberts et al., 1994, 1995). Finding the Hox11 gene expressed in splenic cells of adult mice was unusual because Hox11

expression was thought to halt at birth. Further, Hox11 was not found in adult bone marrow, kidney, liver, and salivary gland (Kodamaet al., 2005a) although during fetal development Hox11 cells play a role in the development of these tissues. The expression of Hox11 in splenic stem cells suggests that the spleen harbors into adulthood a previously unknown reservoir of stem cells that may descend back to embryonic life. Strikingly, the same reservoir of Hox11 stem cells is found in the spleen of normal human adults (Dieguez-Acuna et al., 2007). Post-mortem analysis of human organs being harvested for donation revealed that Hox11 stem cells were only located in the spleen, as opposed to other organs, and were found in the same general capsular location seen in mice. It similarly found that the supply of Hox11 stem cells in the spleen is plentiful, suggesting that expansion in culture would not be necessary for applications in harvesting, transplantation, or infusion.

The target tissues for Hox11+ stem cell migration normally include the pancreas, salivary glands, tongue, blood, certain cranial nerves, parts of the hindbrain and cochlea, and lachrymal glands (Raju et al., 1993; Roberts et al., 1994; Lonyai et al., 2008a). After disease or injury, the infusion or transplantation of splenic stem cells contributes to regeneration or restoration of the same tissues to which Hox11 stem cells normally migrate during the process of embryogenesis: pancreatic islets, osteoblast-like bone cells, T lymphocytes, salivary epithelial cells, cochlea, and heart (Yin et al., 2006; Kodama et al., 2003; Lonyai et al., 2008a; Tran et al., 2007; Macias et al., 2001; Khaldoyanidi et al., 2003; Park et al., 2009; Swirski et al., 2009). In the study of monocytes from the spleen migrating to the heart for wound healing (Swirski et al., 2009), the authors used methods directly to demonstrate that the monocytes originally resided in the spleen: 41% of monocytes responsible for wound healing had migrated from the spleen (Swirski et al., 2009). The fact that disease or injury in certain tissues summons splenic stem cells – likely by signaling molecules yet to be identified in the same tissues and organs of embryonic migration – lends even greater weight to a stem cell reservoir in the spleen. The reservoir tapped by Swirski and colleagues was from the subcapsular region of the red pulp. Although these investigators did not assay their stem cells for Hox11 expression, the location they report is identical to the one from which our group's stem cells were extracted and express Hox 11 (Kodama et al., 2005a,b,c). In sum, the embryological formation of the AGM may account for the location of the reservoir of Hox11 undifferentiated cells in the spleen and their versatility, especially in light of research revealing that AGM tissues differentiate into cells and tissues outside of the hematopoietic system (Yao et al., 2007).

3. Functions: splenic stem cells' proteome reveals multipotency and possible pluripotentcy

Proteomic analysis is one of the best techniques to determine the multi-lineage or pluripotent capacities of cells. Pluripotentcy refers to stem cells that can develop into nearly all cell types in the body except for the placenta. Proteomic analysis identifies expression of proteins, such as key developmental transcription factors, in an effort to determine the cell's capacity for lineage commitment and replication. By virtue of the breadth of target tissues to which CD45− splenic stem cells migrate, differentiate, and often function appropriately, it is clear that they are multi-lineage stem cells. In a series of experiments, we asked which

expressed proteins are associated splenic stem cells' multi-lineage capacity. The second-order question was "how early" are the proteins expressed along the commitment trail? Cells that are too early along the lineage trail may be too unstable and thus pose a risk of carcinogenesis, whereas cells that are too far along in the commitment process might be safe but do not have enough versatility to be stem cells beyond one or two tissues. How did our expressed proteins compare with studies of pluripotent stem cells, or iPS cells. iPS cells are not naturally-occurring cells: they are produced by reprogramming into embryonic-like stem cells a well-recognized set of protein transcription factors and signaling proteins known to be active during embryogenesis. The transcription factors are not expressed in a differentiated cell, insofar as they must be introduced by retrovirus that integrates pluripotent transcription factors into the host genome. As a result of the use of retrovirus and other procedures to introduce genes and otherwise manipulate the cell, it is well-known that induced pluripotent stem cells (iPS) can induce tumors (Takahashi and Yamanaka, 2006). So our final question was whether splenic stem cells represented a risk of oncogenesis.

The CD45− splenic stem cell proteome was analyzed to determine its wider multipotentcy and possible pluripotency, on the one hand, and, on the other, its protection from malignant transformation (Dieguez-Acuna et al., 2009). With state-of-the-art proteomics and *in vivo* testing, we performed functional analyses of proteins unique to the CD45− cell fraction after we had subtracted out proteins derived from the CD45+ cell fraction. CD45− stem cells uniquely expressed proteins that coincided with those in iPS, including OCT3/4, SOX2, KLF4, c-MYC and NANOG. These proteins are known to be essential to induce pluripotency. Also expressed are Hox11, Gli3, Wnt2, and Adam12, all of which are transcription factors identical to those found in embryonic stem cells. These transcription factors were functional because their mRNA was upregulated in the spleen in association with concurrent damage to the pancreas and salivary glands—organs to which they normally contribute stem cells. Analysis of the likelihood of malignant transformation was found to be low when the splenic cell proteome was compared with that of Hox11+ cancer cells unrelated to lymphocyte traits. The analyses demonstrated that CD45− splenic stem cells are one of the first naturally-occurring candidates for a population of multi-lineage and possibly pluripotent stem cells with low oncogenic risk.

4. Associated pathologies: splenic stem cells and applications to multiple diseases

Splenic stem cells are strikingly poised for translational research. There is strong rationale because these naturally-occurring stem cells found in the mouse spleen are also found in humans. In animal models, they have been found to treat TID, Sjogren's syndrome, hearing defects, cranial nerve abnormalities and possible acceleration of heart healing after infarction. The breadth of even more possibilities, which have yet to be tested, arises from these stem cell advantages: their identification in the spleen of *humans;* their origin in a non-essential organ; their abundance, which precludes the need for *ex vivo* or *in vitro* manipulation (suggesting a lower safety profile); their potential for autologous or heterologous use, rather than the more controversial use of embryonic stem cells; and their

stability and low risk of transformation. Finally, in contrast to hematopoietic stem cells, *Hox11+* splenic stem cells may exert an even greater range of therapeutic applications.

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Cell facts

- Stem cells of the adult spleen or their predecessors are located in an embryonic region known as the aorta-gonad mesonephros are naturally-occurring cells that contribute during fetal development to formation of the salivary glands, pancreas and other tissues as diverse as bone, tongue, blood, cranial nerves, hindbrain and cochlea, and heart, among others.
- Stem cells of the spleen are newly discovered to persist into adulthood and retain their phenotype as multipotent and possibly pluripotent cells. They express the same protein transcription factors as do induced pluripotent stem cells. But splenic stem cells possess low oncogenic risk, a distinct advantage over induced pluripotent stem cells, which need to be manipulated in culture.
- Stem cells of the adult spleen of mice, when harvested and transplanted, have been shown to regenerate salivary cells in Sjogren's syndrome and speed the reversal of type I diabetes in an animal model. New research shows how these cells succeed: they begin by upregulating developmental transcription factors in response to injury or disease of the organs or tissues to which they normally contribute during development. The stem cells are likely to have a broader role in regenerative medicine, especially for treating diseases of lineages into which they normally differentiate.