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Original article

Uterine-derived progenitor cells are immunoprivileged and effectively improve cardiac regeneration when used for cell therapy

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ABSTRACT

Cell therapy to prevent cardiac dysfunction after myocardial infarction (MI) is less effective in aged patients because aged cells have decreased regenerative capacity. Allogeneic transplanted stem cells (SCs) from young donors are usually rejected. Maintaining transplanted SC immunoprivilege may dramatically improve regenerative outcomes. The uterus has distinct immune characteristics, and we showed that reparative uterine SCs home to the myocardium post-MI. Here, we identify immunoprivileged uterine SCs and assess their effects on cardiac regeneration after allogeneic transplantation. We found more than 20% of cells in the mouse uterus have undetectable MHC I expression by flow cytometry. Uterine MHC $I^{(neg)}$ and MHC $I^{(pos)}$ cells were separated by magnetic cell sorting. The MHC $I^{(neg)}$ population expressed the SC markers CD34, Sca-1 and CD90, but did not express MHC II or c-kit. *In vitro*, MHC $I^{(neg)}$ and $^{(pos)}$ SCs show colony formation and endothelial differentiation capacity. In mixed leukocyte co-culture, MHC $I^{(neg)}$ cells showed reduced cell death and leukocyte proliferation compared to MHC $I^{(pos)}$ cells. MHC $I^{(neg)}$ and $^{(pos)}$ cells had significantly greater angiogenic capacity than mesenchymal stem cells. The benefits of intramyocardial injection of allogeneic MHC $I^{(neg)}$ cells after MI were comparable to syngeneic bone marrow cell transplantation, with engraftment in cardiac tissue and limited recruitment of CD4 and CD8 cells up to 21 days post-MI. MHC $I^{(neg)}$ cells preserved cardiac function, decreased infarct size and improved regeneration post–MI. This new source of immunoprivileged cells can induce neovascularization and could be used as allogeneic cell therapy for regenerative medicine.

1. Introduction

In response to an ischemic injury to the heart, the recruitment of regenerative cells from both the bone marrow and the heart is essential for recovery [1,2]. Currently, bone marrow stem cells are used in the clinic as cell therapy for the treatment of heart disease [3,4]. However, recipients of autologous cells received only marginal benefits [5] in comparison to the extensive regeneration seen in pre-clinical animal studies [4]. The advanced age of the cardiac patients and age-related stem cell dysfunction have been suggested to play a major role in the difference between these preclinical and clinical findings [5,6]. To overcome this limitation, allogeneic cells from younger donors have been tested. Allogeneic mesenchymal stem cells (MSCs), embryonic stem cells and induced pluripotent stem (iPS) cells have promising regenerative properties, however their suitability as cell therapy agents remains an ongoing question because of the host immune response generated after transplantation [7,8]. Cell rejection remains a major clinical concern when using cell therapy. Strategies to maintain immunoprivilege in stem cell transplantation may directly improve the outcomes of cell therapy.

Research on immunoprivileged stem cells remains an ongoing area of investigation and new sources of allogeneic stem cells are being studied. Uterine stem cells are of interest for two reasons. The uterus is a unique organ with distinct immune characteristics allowing the presence of a (semi)allogeneic fetus within an exceptional tolerogenic environment [9]. Thus, regenerative uterine stem cells may also preserve some unique immunoprivileged properties. Additionally, the endometrium is a rare site of physiological angiogenesis in the post-development adult body, with regenerative cells that cyclically create and shed decidual tissue without scarring. In the context of heart regeneration after ischemic injury, angiogenesis is a crucial process to rescue cardiomyocyte death and restore blood flow. Therefore, uterine stem cells are an attractive candidate for testing.

Our group has previously demonstrated that the uterus is a source of potent progenitor cells which induce angiogenesis when injected into the infarcted heart [10]. Furthermore, females may have the advantage

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Table 1
Real-time PCR primer sets.

Gene	Forward	Reverse
MMP2	ACCAGAACACCATCGAGACC	CCATCAGCGTTCCCATACTT
MMP9	CGTCGTGATCCCCACTTACT	AGAGTACTGCTTGCCCAGGA
TIMP1	GCATCTGGCATCCTCTTGTT	CTCAGAGTACGCCAGGGAAC
TIMP2	AAGCAGTGAGCGAGA AGGAG	GGGTCCTCGATGTCAAGAAA
TIMP3	CCGAGGCTTCAGTAAGATGC	TTGCTGATGCTCTTGTCTGG
TIMP4	ACTTCTGCCACTCGGCTCTA	ACATGGCACTGCATAGCAAG
IL6	CCGGAGAGGAGACTTCACAG	GGAAATTGGGGTAGGAAGGA
IL10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
TGFb2	TGGCTTCACCACAAAGACAG	TTCGATCTTGGGCGTATTTC
AKT1	GCAGGAAGAAGAGACGATGG	GTCGTGGGTCTGGAATGAGT
ANGPT1	CAGTGGCTGCAAAAACTTGA	TCTGCACAGTCTCGAAATGG
VEGFC	CAAGGCTTTTGAAGGCAAAG	TTAGCTGCCTGACACTGTGG
TNFα	CCCCAAAGGG ATGAGAAGTT	CTCCTCCACTTGGTGGTTTG
INFgamma	GCGTCATTGAATCACACCTG	TGAGCTCATTGAATGCTTGG
PIGF	TGCTGGTCATGAAGCTGTTC	ACCCCACACTTCGTTGAAAG

of a utero-cardiovascular axis in support of cardiac healing, as we have reported that hysterectomized rats without oorphorectomy showed progressive cardiac dilatation and heart failure following myocardial infarction (MI) which was comparable to males [11]. The transplantation of a green fluorescent protein (GFP^+) uterus complete with vascular anastomosis in a hysterectomized wild-type recipient animal resulted in GFP^+ cell mobilization to and engraftment into the heart, rescuing ventricular dysfunction after MI. GFP^+ cells were found around blood vessels supporting angiogenesis in these recipient animals [11]. Intravenous injection of uterine cells following hysterectomy and MI also enhanced tissue repair and prevented hysterectomy-related cardiac dysfunction [11]. These data support the notion that a functional uterus serves as a reservoir of highly regenerative cells that are mobilized in response to injury to function in cardiac regeneration.

These highly angiogenic uterine stem cells with distinct immunoprivileged characteristics could represent an excellent source of cells able to overcome the key challenges impairing the efficacy of currently used cell therapy for ischemic heart disease, such as dysfunctional aged cells and rejection. We identified a unique population of immunoprivileged, highly angiogenic uterine progenitor cells and have demonstrated their potential as an allogeneic cell therapy in regenerative medicine, particularly addressing cardiac regeneration post-ischemic injury.

2. Methods

For detailed methods, see the online Supplemental Information.

2.1. Experimental animals

For *in vitro* studies, C57BL/6N mice were used for uterine cell isolation and allogeneic leukocytes were isolated from FVB mice. For *in vivo* studies, female FVB mice of mouse major histocompatibility complex (MHC) haplotype 2, q variant ($H2^q$) were used as cell recipients, while female C57BL/6N mice of mouse MHC haplotype 2, b variant ($H2^b$) with green fluorescent protein (GFP) were used as cell donors. All animal procedures were approved by the Animal Care Committee of the University Health Network, and all animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (8th edition, NIH, 2011).

2.2. Uterine cell isolation and characterization

Mice were sacrificed, their uteri dissected, and the uterine cells processed to create a single cell suspension. Uterine cells were sorted into MHC $I^{(pos)}$ and MHC $I^{(neg)}$ populations by allophycocyanin-targeted magnetic isolation and their purity analyzed by flow cytometry. Both uterine cell populations were characterized with respect to the expression of the following markers: Sca-1 and c-kit (considered hematopoietic lineage markers [12]), CD34 (identifies early hematopoietic and endothelial stem cells [13,14]), CD90 (a cell surface marker associated with differentiation potential in uterine stromal cells [15]), and MHC I and II.

2.3. In vitro assays

To quantify leukocyte-mediated cytotoxicity and cytotoxic T cell activation, mixed spleen leukocytes (5×10^5) from FVB mice were isolated and co-cultured with MSCs or MHC $I^{(neg)}$ and MHC $I^{(pos)}$ uterine cells. Leukocyte-mediated cytotoxicity was evaluated by lactate dehydrogenase (LDH) release from the damaged cells after 5 days of co-culture and by proliferation of cytotoxic leukocytes measured using 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) staining.

Colony-forming unit (CFU) assays were performed on MHC $I^{(neg)}$ or MHC $I^{(pos)}$ uterine cells to assess their fibroblast (CFU-F) and hematopoietic (CFU-GM) progenitor potential. Endothelial differentiation potential was assessed by flow cytometry and immunostaining for expression of von Willebrand factor (vWF).

The angiogenic potential of MHC $I^{(neg)}$ or MHC $I^{(pos)}$ uterine cells was assayed *in vitro* by a scratch wound healing assay and by endothelial cord formation and compared to MSCs.

2.4. In vivo experiments

The angiogenic potential of the MHC $I^{(neg)}$ and MHC $I^{(pos)}$ uterine cells was compared with MSCs *in vivo* by an abdominal subcutaneous Matrigel implantation assay. Cells mixed with Matrigel were implanted into male mice. Seven days later, the nodules were excised. The vessel network in the nodule was photographed and assessed by hematoxylin and eosin (H&E) staining. Arteriole and capillary density was assessed and leukocyte recruitment was visualized by staining for CD45.

To assess the functional benefits of uterine cell implantation after ischemia, the left coronary artery of female FVB mice was permanently ligated and 0.5×10^6 allogeneic MSCs (at passage 4), or MHC $I^{(neg)}$ or MHC $I^{(pos)}$ uterine cells from C57BL/6 N GFP^+ mice were injected into the border zone. Uterine cells were freshly isolated on the day of transplantation. As each uterus contains 25–30% MHC $I^{(neg)}$ cells, approximately 1.5×10^6 MHC $I^{(neg)}$ cells could be isolated from each donor animal. 0.5×10^6 freshly isolated syngeneic GFP^+ bone marrow mononuclear cells (BMMCs) were injected into positive control animals and media injection alone was used as a negative control. Cardiac function was measured by echocardiography at baseline and at 7, 14 and 21 days post-MI to assess percent fractional shortening, left ventricular internal systolic dimension (LVIDS), left ventricular internal diastolic dimension (LVIDD), percent fractional change, left ventricular external systolic area (LVESA) and left ventricular external diastolic area (LVEDA).

Flow cytometry was used to quantify engraftment of the GFP^+ transplanted cells and infiltration of $CD4^+$ and $CD8^+$ T cells at day 1 (baseline), and at days 7 and 21. These markers were also assessed by immunohistochemistry on heart frozen sections.

To determine other humoral effects of cell transplantation on ischemic hearts, mice were sacrificed and cardiac tissue was collected 5 days after MI. Total RNA was isolated from heart tissue (scar and border zone) of the MHC $I^{(neg)}$, MHC $I^{(pos)}$ and media control groups. RT-qPCR was performed to measure the level of gene expression of various angiogenic molecules, growth factors, proteases and matrix proteins, cytokines and genes involved in cell survival.

2.5. Staining and immnohistochemistry

Masson's trichrome staining was performed to depict scar thickness. Heart sections were immunolabeled with antibodies against GFP, Sca-1, α-smooth muscle actin (α-SMA), isolectin, vWF and CD45. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cultured cells were