Early Cell–Cell Coupling Contributes to the Loss of Stem Cell Retention in Co-Culture with Hypoxic Cardiac Myocytes

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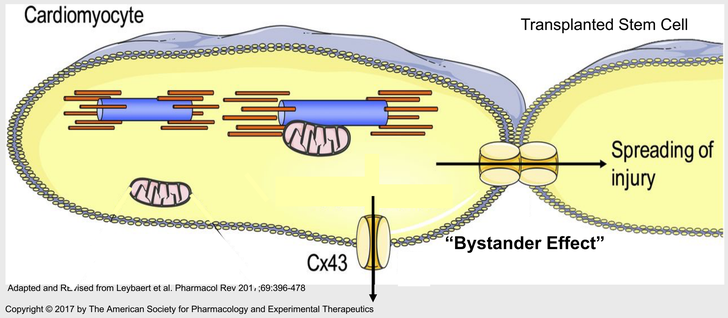
Background

There is a significant loss of cardiac cells that occurs within hours after a heart attack. Implanting stem cells into the damaged area of the heart can prevent further loss of at risk tissue and potentially regenerate new tissue to replace the dying cells. Implanted stem cells are placed into a microenvironment exposed to disrupted blood flow, limited oxygen availability and inflammation. Consequently, many of these stem cells are lost during the first few hours after implantation and this represents one of the key issues facing cardiac stem cell therapy. Our preliminary research shows that cardiac myocytes and stem cells form connections or channels called gap junctions (GJ), composed of connexin proteins, within hours after co-culture. Gap junctions and hemichannels provide a conduit for signaling molecules to migrate between cells and potentially impact the survival of implanted cells. This has been previously reported in other cell types and is known as a ”bystander effect” (Figure 1). Thus, we hypothesized that early coupling between implanted stem cells and hypoxic cardiac myocytes may play a detrimental role in stem cell retention in the acute phase of cardiac cell therapy. Furthermore, disruption of the coupling mechanism may improve implanted cell viability.

Figure 1. Proposed Role of Cx43 in Cell-to-Cell Coupling Between Cardiac Myocytes and Bone Marrow Mesenchymal Stem Cells



Adult Human Bone Marrow-Derived Stem Cells

To assess the presence of connexin43 protein in stem cells, adult human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were obtained from StemCell Technologies (Vancouver; Ca. # 70071; Lot # 1904240229). Approximately 98% of the cells were positive for the mesenchymal stem markers CD73, CD90, and CD105, while only 1% of the cells tested positive for the hematopoietic stem cell markers CD14, CD34, and CD45. Before the cells were plated, the bottom of the culture dish was coated with animal component-free cell attachment substrate (1:300 dilution in DPBS). We initially plated the cells at a density ≥ 25,000 cells/cm^2 recommended by StemCell Technologies. The cells were cultured in complete MesenCult™-ACF Plus Medium and incubated at 37^o C and 5% CO_2. When the cells reached 80-95% confluence, they were passaged and re-plated at the recommended density. Cells were intermittently lifted and stored in liquid nitrogen to preserve them at lower passage numbers.

Murine Cardiac and Bone Marrow Stem Cell Co-Cultures

Murine HL-1 cardiac myocytes were cultured under normoxic or hypoxic conditions for 4 hours. Subsequently, murine bone marrow-derived mesenchymal stem cells (mBM-MSC) were seeded on the HL-1 cell monolayer and the co-cultures were returned to the cell incubation chamber. One co-culture group continued under normoxic conditions and a second group continued under hypoxic conditions. In a third group the hypoxic media was replaced with normoxic media and the cells continued under normoxic conditions. The non-selective GJ inhibitor, carbenoxolone (CBX, 100 μ*M*) was added to the mBM-MSC in half of the wells from each of the three groups immediately prior to and during co-culture of the HL-1 and mBM-MSC. Cells remained in co-culture for an additional 2 hours after which time they were lifted and labeled with tagged antibodies for fluorescence activated cell sorting (FACS). Co-cultured cells were labeled for stem cell antigen-1 (Sca-1), Pacific Blue Annexin V and Sytox Red. The latter two antibodies were used to determine the percent of cells in co-culture that were undergoing early and late apoptosis, and cell death.

Results

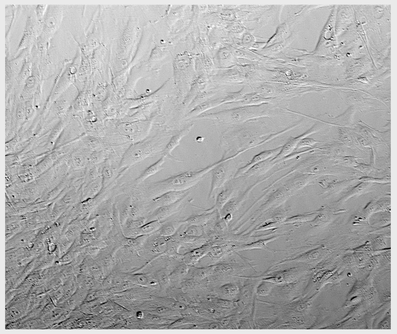


Figure 2. Human bone marrow-derived mesenchymal stem cells in culture at 80% confluency. Cells were cultured on animal component-free substrate at 25,000 cells per cm^2 for up to 4 days at which time they were passaged. Cells were detached from the wells with an enzyme solution and replated on sterilized class cover slips for immunofluorescent analysis (shown below).

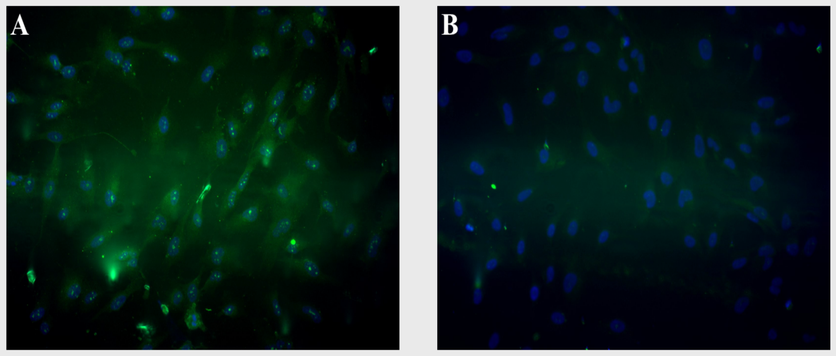


Figure 3. Human bone marrow-derived mesenchymal stem cells express connexin43 protein. hBM-MSCs were fixed in acetone for 10 mins and permeabilized with 1% Triton-X 100 in PBS for 1 hour followed by blocking with a 1% casein solution. The primary antibody was Connexin 43 Polyclonal Antibody (Invitrogen) at a 1:50 dilution. An Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen) secondary antibody was applied at a 1:500 dilution. On some of the coverslips with cells, we omitted the primary antibody only. These coverslips served as our negative controls. (A) Connexin43 expression (green) in the hBM-MSC, (B) Negative controls. DAPI nuclear stain is represented in blue for both A and B.

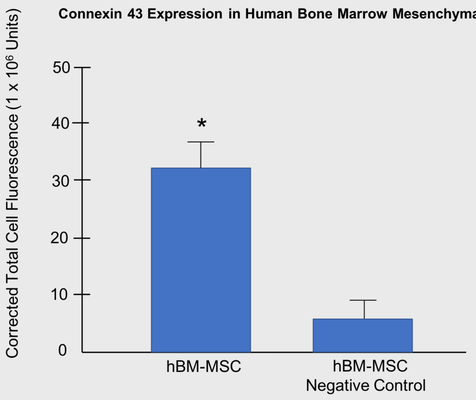


Figure 4. Aggregate data from hBM-MSCs in Figure 3. To quantify the fluorescence, we used the public software program, ImageJ. We documented the mean gray value, integrated pixel density, and the selected area of the intended cell and multiple background readings. CTCF = Integrated Density – (Area of selected cell x mean fluorescence of background reading). *P < 0.05 vs. Negative Control.

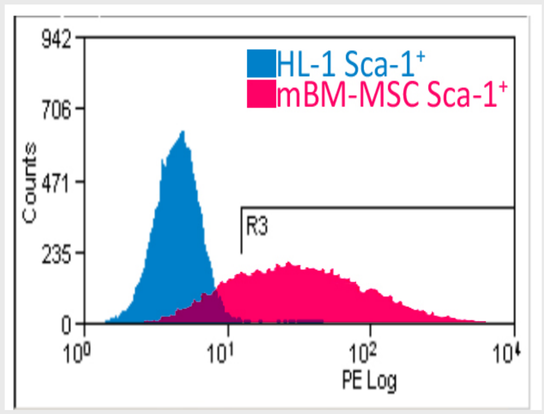


Figure 5. Murine bone marrow-derived mesenchymal stem cells express the membrane antigen Sca-1 while Sca-1 is absent in cardiac myocytes. During FACS analysis of the cells following co-culture, mBM-MSC were gated on the stem cell membrane antigen Sca-1 (> 80% positive), to distinguish them from HL-1 cells (Sca-1 negative). This allowed us to determine the percentage of stem cells that were undergoing early apoptosis and cell death when co-cultured with the hypoxic cardiac myocytes. Cell sorting results displayed in the figure demonstrates that the majority of Sca-1 positive cells were mBM-MSC.

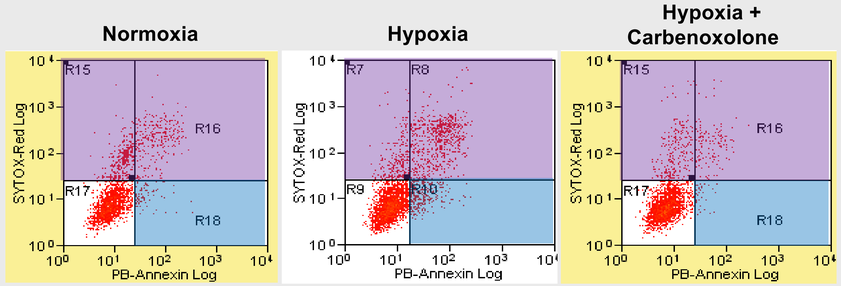


Figure 6. Hypoxia resulted in a greater degree of apoptosis and cell death compared to normoxia. The GJ inhibitor, Carbenoxolone, attenuated the degree of early apoptosis and cell death. Representative FACS for Sca-1 gated mBM-MSCs from one co-culture in each of the Normoxia, Hypoxia, and Hypoxia+Carbenoxolone groups. Each dot on the figure represents a single cell and the level of either Sytox-Red (late apoptosis/cell death) or Pacific Blue (early apoptosis) stain. Cells in the white lower left quadrant above were viable cells that were not undergoing apoptosis. Cells in the upper right purple quadrant were cells displaying both late apoptosis and cell death.

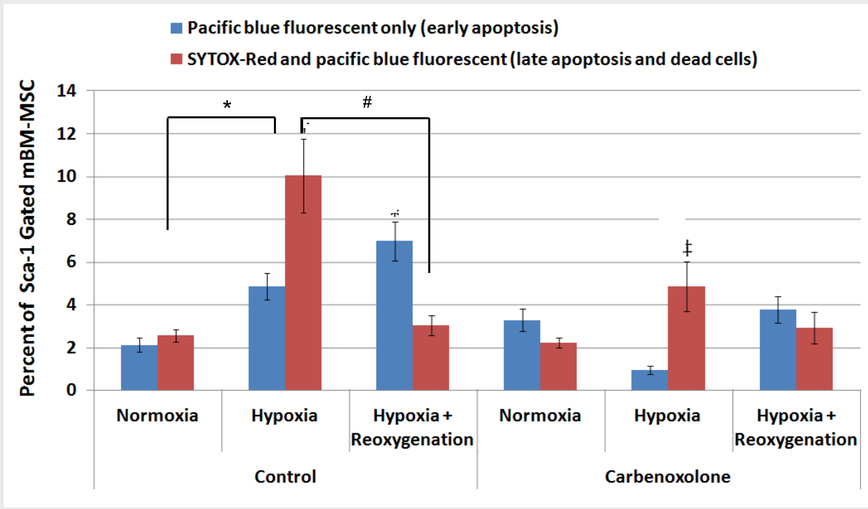


Figure 7. Gap junction inhibition reduces apoptosis and cell death in mBM-MSC when co-cultured with hypoxic cardiac myocytes. Aggregate data for the Normoxia, Hypoxia and Reoxygenated cells in both Control and Carbenoxolone studies are represented in the figure above. Hypoxia induced a greater proportion of dead and apoptotic mBM-MSCs over the 2 hour co-culture period compared to normoxia in the Control group (blue and red bars *P < 0.05). Reoxygenation following hypoxia resulted in fewer late apoptotic and dead mBM-MSC (red bars; #P < 0.05) but early apoptosis (blue bar) was significantly higher compared to normoxia (blue bar #P < 0.05). The addition of the gap junction inhibitor, carbenoxolone, significantly reduced mBM-MSC apoptosis compared to the control group when added prior to co-culture with the hypoxic cardiac myocytes. Data represent the mean ± standard deviation; n=7 culture wells per each of the media conditions.

Summary and Conclusions

Hypoxia induced a greater proportion of dead and apoptotic mBM-MSC during co-culture with cardiac myocytes compared to normoxia.

The addition of the GJ inhibitor, carbenoxolone, attenuated early apoptosis and reduced cell death in mBM-MSC when co-cultured with cardiac myocytes exposed to hypoxia

While GJ formation is an important component in long-term cell therapy, our data suggest that early gap junction formation may represent a novel paradigm whereby hypoxic cardiomyocytes create a “bystander effect” with coupled stem cells and thus impair retention and impact potential long-term benefits.

Acknowledgments

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