CHAPTER 6

Embryonic Stem Cell Immunogenicity Increases upon Differentiation After Transplantation into Ischemic Myocardium


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Chapter 6

ABSTRACT

Background: We investigated whether differentiation of embryonic stem cells (ESCs) in ischemic myocardium enhances their immunogenicity, thereby increasing their chance for rejection.

Methods: In one series, 129/SvJ-derived mouse ESCs (ES-D3 line) were transplanted by direct myocardial injection (1x10⁶ cells) into murine hearts of both allogeneic (BALB/c, n=20) and syngeneic (129/SvJ, n=12) recipients after LAD ligation. Hearts were procured at 1, 2, 4 and 8 weeks after ESC transplantation and analyzed by immunohistochemistry to assess immune cell infiltration (CD3, CD4, CD8, B220, CD11c, Mac-1, Gr-1) and ESC differentiation (H&E). In a second series (allogeneic n = 5, sham n = 3), ESC transplantation was performed similarly, however after 2 weeks, LAD-ligated and ESC-injected hearts were heterotopically transplanted into naive BALB/c recipients. After an additional 2 weeks, donor hearts were procured and analyzed by immunohistochemistry.

Results: In the first series, the size of all ESC grafts remained stable and there was no evidence of ESC differentiation 2 weeks post-transplantation; however, after 4 weeks both allogeneic and syngeneic ESC grafts showed the presence of teratoma. By 8 weeks, surviving ESCs could be detected in the syngeneic but not in the allogeneic group. Mild inflammatory cellular infiltrates were found in allogeneic recipients at 1 and 2 weeks post-transplantation, progressing into vigorous infiltration at 4 and 8 weeks. The second series demonstrated similar vigorous infiltration of immune cells as early as 2 weeks after heterotopic transplantation.

Conclusion: In vivo differentiated ESCs elicit an accelerated immune response as compared to undifferentiated ESCs. These data imply that clinical transplantation of allogeneic ESCs or ESC derivatives for treatment of cardiac failure might require immunosuppressive therapy.
INTRODUCTION

Stem cell transplantation has recently emerged as a novel approach for replacement of injured myocardium. The potential of both hematopoietic stem cells (HSCs), harvested from the adult bone marrow and embryonic stem cells (ESCs), harvested from the inner cell mass of embryos at the blastocyst stage, to develop into viable heart muscle cells in the damaged heart is now being explored. Despite early encouraging results with HSC transplantation, the ability of these cells to transdifferentiate into cardiomyocytes in vivo and to functionally repair ischemic myocardium has recently been seriously questioned. Instead, previous studies have reported successful differentiation of ESCs into cardiomyocytes, both in vitro and in vivo. This prompted us to investigate the potential of ESCs as a source of cell transplantation for myocardial restoration.

Pluripotent ESCs are capable of spontaneous differentiation into cells of all three different germ layers. Due to their early stage of development, ESCs are considered “immune privileged”, i.e. unrecognizable by the recipient immune defences. This assumption is bolstered by observations in neonatal tolerance. An embryo, consisting of 50% foreign material derived from the father, is usually not rejected by the maternal host. However, recent evidence suggests that even in their undifferentiated state, human ESCs express low levels of HLA class I antigen, that moderately increase as the cells differentiate. In addition, rat embryonic stem cell-like cells (RESCs) also have been shown to express minimal, but detectable, levels of MHC class I molecules. The presence of distinct MHC antigens, suggests that developing ESCs can be at risk for immune rejection when introduced in vivo across histocompatibility barriers. However, no progressive studies analyzing the immune fate of ESCs during their development in vivo have been reported so far.

In this study, we tested the hypothesis that intramyocardially transplanted ESCs elicit an immune response that results in rejection of the transplanted cells. In addition, we investigated whether the state of cell differentiation affects their immunogenicity and the intensity of the recipient immune response.

METHODS

Animals.

Six- to ten-week-old female BALB/c (H-2d) and 129/SvJ (H-2b) mice (20-25 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at no more than five per cage in our American Association for Accreditation of Laboratory Animal Care-approved facility with 12:12-h light-dark cycles and free access to standard rodent chow and water. All animal procedures were approved by the Animal Care and Use Committee of Stanford University.
**Cell culture.**

ES-D3 embryonic stem cells, a line developed from the 129/SvJ mouse strain, were kindly provided by Dr. I.L Weissman (Department of Pathology and Developmental Biology, Stanford University School of Medicine). Undifferentiated ES-D3 cells were maintained on gelatinized tissue culture dishes in Dulbecco modified Eagle medium (Specialty Media, Phillipsburg, NJ) supplemented with 15% fetal bovine serum (HyClone, Logan, UT), 100μg/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 x non essential amino acids, 0.1 mM 2-mercaptoethanol (all Gibco, Frankfurt, Germany) and 10³ U/ml ESGRO™ (Chemicon, Temecula, CA). Cells were maintained at 37˚C in a humidified atmosphere of 5% CO₂. Monolayers were passaged by trypsinization at confluence of 70-80%. At the day of transplantation, ESC were harvested and aliquoted in culture medium.

**Left coronary ligation and ESC transplantation.**

Mice were premedicated with ketamine (100 mg/kg IP) and anesthetized with inhaled isoflurane (2-3%). Mice were intubated and ventilated with a mouse respirator (model 687, Harvard Apparatus, Inc., Holliston, MA) and anesthesia was maintained with inhaled isoflurane (1-2.5%). A left thoracotomy was performed in the 5th intercostal space, the left lung retracted and the pericardium opened. The left anterior descending (LAD) artery was permanently ligated with a 9-0 Ethilon suture just distal to the level of the left atrium. Infarction was visually confirmed by blanching of the antero-lateral region of the left ventricle along with dyskinesia. After five minutes, 1.0 x 10⁶ ES cells were transplanted by injection into the injured myocardium (volume = 25 μL) of a series of allogeneic (BALB/c, n=20) and syngeneic (129/SvJ, n=12) recipients. Similar surgical procedure, with injection of cell free culture medium, was performed on sham control animals (BALB/c, n=8). A thoracostomy tube was placed and lungs were re-expanded using positive pressure at end expiration. The chest cavity was closed in layers with 5.0 Vicryl suture, and the animal was gradually weaned from the respirator. Once spontaneous respiration resumed, the endotracheal and thoracostomy tubes were removed, and the animal was placed in a temperature controlled chamber until they resumed full alertness and motility.

**Heterotopic transplantation of LAD-ligated and ESC-injected hearts.**

To study the immune response against in vivo matured ESC, a second series of animals (allogeneic n=5, sham controls n=3) underwent LAD ligation and ESC transplantation, however after 2 weeks, their hearts were explanted and heterotopically transplanted into the abdomen of naive syngeneic BALB/c recipients. Heterotopic cardiac transplantation was performed according to the method of Corry et al 14 with some modifications. Anaesthesia was induced and maintained as described above. Cardiac graft viability was assessed daily by abdominal palpitation.
Tissue collection, immunofluorescent and histological analysis.
Subsets of allogeneic, syngeneic and sham operated animals from the first series were sacrificed at 1, 2, 4 and 8 weeks after ESC transplantation. Animals from the second series were sacrificed at 2 weeks after heterotopic heart transplantation. Hearts were perfused with cold saline and rapidly excised. They were fixed in 2% paraformaldehyde for 2 hours at room temperature and cryoprotected in 30% sucrose overnight at 4°C. Tissue was frozen in optimum cutting temperature compound (OCT compound, Sakura Finetek USA, Inc. Torrance, CA) and sectioned at 5 μm on a cryostat. To evaluate inflammatory cell infiltration, immunostaining was performed with a panel of hematolymphoid antibodies. Serial sections were blocked and incubated with hamster anti-CD3 (clone G4.18), rat anti-CD4 (H129.19), rat anti-CD8 (53-6.7), rat anti-CD45R/B220 (RA3-6B2), hamster anti-CD11c (HL3), rat anti-Mac-1 (M1/70) or rat anti-Gr-1 (RB6-8C5) antibody (BD Pharmingen, San Jose, CA) for 1 hour at room temperature. Primary antibodies were detected by incubation of the slides with goat anti-hamster Texas Red (Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-rat Alexa 488 or goat anti-rat Alexa 594 (Molecular Probes, Eugene, CA) for 45 minutes at room temperature. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and analyzed with a Leica DMRB fluorescent microscope (Leica Microsystems, Frankfurt, Germany). Images were acquired with a SPOT RT Color camera and electronically merged with SPOT RT software (Diagnostic Instruments, Sterling Heights, MI). To detect differentiated structures within the ESC grafts and to evaluate morphological changes of the left ventricular wall, sections were stained with Hematoxylin and Eosin, Masson’s trichrome and Mucin stain (all Sigma-Aldrich Corp., St. Louis, MO) according to established protocols and carefully analyzed by a blinded pathologist (H.V.).

Histological evaluation.
Shortly after immunofluorescent histology, sections were evaluated and graded a score for degree of inflammatory cell infiltration by three independent observers (R.J.S., M.T., F.G.). Scores related to the following descriptions: -, absent, no infiltration; +/-, trace, few infiltrating cells; +, mild, scattered infiltrate or focal accumulation; ++, moderate, modest infiltrate progressing into diffuse; ++++, severe, intense and diffuse cell infiltration.

RESULTS

Transplantation of undifferentiated embryonic stem cells elicits progressive inflammatory graft infiltration.
Following successful LAD ligation and ESC injection, ESCs were detected in all transplanted animals at 1 week post-injection. (H&E staining, Figure 1) Masson’s trichrome staining confirmed location of ESCs within the infarcted left ventricular wall. (Figure 2A) Immunofluorescent histological analysis demonstrated that allogeneic ES-D3 cell transplantation elicited
progressive graft infiltration of various types of immune cells, involved in both adaptive and innate types of immunity. As shown in figure 2B, immune cells strictly infiltrated the area of infarction and ESC transplantation. Table 1 summarizes the immunohistology data obtained over the 8-weeks time course.

Table 1. Cellular composition of graft infiltrates over time after intra-myocardial ESC injection. Cell surface markers of T lymphocytes (CD3), T helper cells (CD4), Cytotoxic T cells (CD8), B lymphocytes (B220), Dendritic cells (CD11c), Macrophages (Mac-1) and Granulocytes (Gr-1). Degree of infiltration: -, absent; +/−, trace; +, mild; ++, moderate; ++++, severe.

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*Time after LAD ligation and ESC injection
†Time after heterotopic heart transplantation (post-htx) of LAD-ligated and ESC-injected hearts

At 1 week post-injection, allogeneic ESC grafts displayed mild CD3⁺ T lymphocyte infiltration within the ESC allograft, which was composed predominantly of CD4⁺ T helper cells. At that time point, sparse CD4⁺ T cell clusters could be seen at the border of the graft area, whereas at weeks 4 and 8 after transplantation massive infiltration of both CD4⁺ and CD8⁺ T cells was observed throughout ESC graft including the inner area. (Figure 2C) Limited numbers of infiltrating T lymphocytes were present in syngeneic ESC grafts (Figure 2D) as well as in sham control hearts over the whole tested time period. These results show that
over time, progressive T lymphocyte graft infiltration occurs following injection of allogeneic ESCs. Intensity of T cell infiltration depends on MHC incompatibility between donor ESCs and recipient. These data suggest that alloantigens presented by developing ESCs are recognized by allospecific host T cells.
Dendritic cell (DC) infiltration was absent in sham control hearts showing that, at least at the time points tested, the performed surgical procedures did not cause non antigen-specific DCs activation/migration. Also, no or minimal numbers of infiltrating DCs were found in syngeneic hosts demonstrating that MHC disparity is required for DC migration into the area of transplanted ESC grafts. Similar to alloreactive T cells, the numbers of graft infiltrating DCs increased progressively over time in full-mismatched hosts, and peaked at 4 and 8 weeks after ESC transplantation. On the overall, small numbers of ESC graft-infiltrating B cells were detected as compared to T cells.

Inflammatory cells mediating innate immunity, including macrophages and granulocytes, were also detected in the ESC graft area. Although more profound in the experimental (ESC injection) hearts, we found that macrophage and granulocyte cell infiltrates were also present in sham control hearts. This indicates that post-transplantation, innate response was not exclusively related to ESC antigens but could also be due to surgical trauma after transplantation (LAD ligation and/or medium injection procedures).

**Embryonic stem cells differentiate into teratomas in ischemic myocardium.**

In both allogeneic and syngeneic ESC grafts, tumor formation was observed. (Figure 3) From 1 to 4 weeks after transplantation, Masson’s trichrome staining of infarcted left ventricular

![Figure 3. Transplantation of ESCs causes tumor formation.](image)

Tumors were found extending from the left ventricular wall after both syngeneic transplantation (A, white arrow), and allogeneic transplantation (B, black arrow). In sham operated hearts (C), as expected, no tumors were detected.
walls showed the intramural ESC grafts similarly increasing in size in both allogeneic and syngeneic hearts. (Figure 4) H&E staining revealed no evidence of ESC differentiation at 1 and 2 weeks post-transplantation. However, at 4 weeks, both in the allogeneic and syngeneic ESC grafts, differentiated structures originating from all three different germ layers could be observed. (Figure 5A-D) At this time point, tumors could be characterized as teratomas. At 8 weeks, apart from little intramural cartilage, no differentiated or undifferentiated ESCs were found in the allogeneic hearts, whereas in the syngeneic hearts both cell types were still present. (Figure 4) This finding suggested that at 8 weeks following transplantation the allogeneic ESC grafts had been destroyed as a result of the host inflammatory alloimmune response.

Embryonic stem cell immunogenicity increases upon differentiation.

In the present study, progressive host anti-ESC graft immunity associated with ESC differentiation. Thus, differentiated structures could be found within the ESC grafts at 4 weeks post-transplantation. At the same time, severe ESC graft infiltration of inflammatory cells was observed. Taken together, these findings suggest that once ESCs reach a more differentiated state, they can be more effectively recognized and rejected by the recipient immune system.

Figure 4. Morphological changes of left ventricular wall over time. Masson’s trichrome staining of heart sections at different time points after allogeneic (allo) and syngeneic (syn) ESC transplantation revealed intramural tumors over time increasing in size. Note that at 8 weeks after allogeneic transplantation, the allogeneic ESC grafts had been destroyed as a result of the host inflammatory alloimmune response. The left ventricular walls of the sham control hearts are shown in the lower panels.
To further investigate this hypothesis, we designed a model to study the immune response against *in vivo* matured ESCs. A group of BALB/c mice underwent similar procedure of myocardial infarction and allogeneic ESC transplantation. However, in this case, after 2 weeks, their hearts were explanted and heterotopically transplanted into naive syngeneic BALB/c recipients. Daily assessment of heterotopic cardiac grafts by abdominal palpation confirmed viability of the grafts throughout the study period. Interestingly, as early as 2 weeks after heterotopic transplantation of the isografts containing partly developed allogeneic ESC grafts, we found severe graft infiltration of various types of immune cells, including T and B-lymphocytes. (Figure 6 and Table 1, right column) Therefore, ESCs that have matured and differentiated *in vivo* for 2 weeks elicit more potent and immediate alloimmune response as compared to undifferentiated ESCs. These data indicate that the immunogenicity of ESC transplants increases upon *in vivo* differentiation.

**Figure 5. Intramyocardially transplanted undifferentiated ESCs differentiate into teratomas.** Four weeks after ESC transplantation structures originating from all three different germ layers were found in both allogenic and syngenic recipient hearts. H&E staining shows formation of intramyocardial cartilage (A, mesodermal derivative, arrowheads, 200x), squamous epithelium (B, ectodermal derivative, 400x) and glandular epithelium (C, endodermal derivative, 400x). Secretion of mucus (pink) by the intramyocardial glands was confirmed by mucin staining (D, 400x).
DISCUSSION

This study was designed to investigate whether ESCs elicit an immune response when transplanted into genetically identical or full MHC-mismatched ischemic myocardium. These data demonstrate, that there is progressive infiltration by various types of inflammatory cells within the ESC graft following transplantation across histocompatibility barriers. Severe cellular invasion was observed 4 weeks after intra-myocardial injection, followed by disappearance of the ESC allografts between 4 and 8 weeks after transplantation, presumably due to a robust alloimmune response. When transplanted into a naive recipient after 2 weeks of in vivo maturation, ESCs triggered an accelerated infiltration of immune cells, indicating that the immune response towards developing ESCs in allotransplant settings increases over time.

Figure 6. Graft infiltration of immune cells after transplantation of in vivo differentiated ESCs. Representative images of ESC graft infiltration of T- (red) and B-(green) lymphocytes at 2 weeks following undifferentiated ESC transplantation (A) versus 2 weeks following in vivo differentiated ESC transplantation through heterotopic transplantation of the LAD-ligated and ESC-injected hearts (B). In vivo differentiated ESCs elicited a vigorous and more immediate immune response as compared to undifferentiated ESCs. Counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI, blue) (Original magnification: 400x)
In contrast to tissue allografts, ESC transplants are devoid of highly immunogenic mature dendritic cells, or any other type of professional antigen presenting cells. Thus, the transplanted cells do not express MHC class II molecules, required for effective priming of CD4+ alloreactive T cells through direct recognition\textsuperscript{10,15}. Therefore, the direct pathway of alloresponse is likely to be dominated by MHC class I-reactive CD8+ T cells, whereas the indirect alloreactive pathway (presentation of processed allogeneic peptides by host APCs) would engage both CD4+ and CD8+ T cells\textsuperscript{15}. In the present study, similar amounts of both CD8+ and CD4+ infiltrating T cells were detected within the ESC grafts. Similarly, the numbers of graft infiltrating DCs increased progressively over time. Co-localization of antigen presenting DCs and T lymphocytes within the ESC graft points at ongoing T cell-APC interaction, and suggest that both direct and indirect allorecognition were involved in immune reactions against allogeneic ESC antigens. Furthermore, ESC graft infiltration of B cells was observed following allogeneic ESC transplantation. CD4+ T cells primed by indirect allorecognition could provide contact-dependent help for B cells to produce alloantibody by a classical cognate T cell-B cell interaction. Alloantibody might mediate further graft damage by various mechanisms, including complement-dependent target-cell injury.\textsuperscript{13}

ESC transplantation is being widely investigated as a potential novel approach to regenerate infarcted myocardium. However, a review of the current literature on transplantation of ESCs, revealed a surprising lack of concern for potential immunological conflict\textsuperscript{13}. The issue of immune recognition by the host is often circumvented by syngeneic transplantation\textsuperscript{16}, immunosuppressive therapy\textsuperscript{17}, or transplantation into an immune privileged site\textsuperscript{18}. Moreover, previous studies in xenogeneic transplant models provide no evidence for immune rejection of engrafted ESCs\textsuperscript{6,7}. This may create an impression that the problem of immune rejection does not apply to ESC transplantation. The present study designed to address this question, does not support this notion. Thus, we detected potent progressive inflammatory ESC graft infiltration, indicating efficient recognition of ESC alloantigens by the host immune system.

One could argue, that ESC immunogenicity could be due to inflammation of the host heart tissue caused by LAD-associated ischemia injury. However, histological analysis following syngeneic ESC transplantation revealed no progressive infiltration, demonstrating that observed ESC immunogenicity in vivo could not be attributed to ischemia-induced inflammation of the recipient heart. In addition, it has been reported that transfection of ESCs with a genetic marker such as green fluorescent protein (GFP) could alter their immunogenicity.\textsuperscript{19,20} Meanwhile, GFP transfection of ESCs is frequently used in quantitative analysis of ESC survival and differentiation. To exclude potential immune reactivity towards exogenous proteins, we used a non-manipulated ESC line.

It is known that undifferentiated ESCs may form teratomas when transplanted under the skin of immunodeficient SCID mice\textsuperscript{8}. In addition, teratoma formation in the host retroperitoneum has been observed following ectopic transplantation of allogeneic ESC-derived cardiomyocytes\textsuperscript{21}. On the other hand, it has been suggested that the infarcted heart pos-
sesses paracrine signaling pathways that are capable of directing in vivo differentiation of transplanted ESCs into functional cardiomyocytes. In the present study, intramyocardial teratomas were observed in both allogeneic and syngeneic hearts at 4 weeks after ESC transplantation. Although, this study was not designed to evaluate cardiomyocyte differentiation parameters, detection of ESC derivatives originating from various germ layers demonstrates that, at least under current experimental conditions, the host environment could not guide differentiation of all transplanted cells into the cardiomyocyte lineage. Careful removal of undifferentiated elements from ESC derivatives prior to transplantation may possibly help to circumvent formation of intracardiac teratomas.

In conclusion, these data provide a clear demonstration of the induction of an alloimmune response against developing ESCs in an experimental animal model. Furthermore, it offers a novel experimental approach, in which immunogenicity of partly differentiated ESCs can be assessed following heterotopic transplantation of previously LAD-ligated and ESC-injected hearts into naïve syngeneic hosts. This model could provide further insight into the characterization of anti-ESC immunity in animal models. In the present study, immunogenicity of ESCs was analyzed by immunohistologic evaluation of graft infiltration by host immune cells. Intragraft migration and infiltration represents an important step in the sequence of immune reactions. It indicates that other systemic immune events, such as peripheral lymphocyte activation in the spleen and/or lymph nodes, cytokine release and production of circulating alloantibodies, likely are present in this model. Experiments evaluating these parameters are in progress in our laboratory.

In summary, we report that ESCs elicit alloimmune response after transplantation into MHC-mismatched ischemic myocardium. Upon in vivo differentiation, ESC immunogenicity increases, resulting in efficient recognition of ESC antigens by the host immune system, and alloimmune rejection. These results imply that clinical transplantation of ESCs or ESC derivatives harvested from allogeneic donors for treatment of cardiac failure might require immunosuppressive therapy.
REFERENCES


