Mesenchymal Stem Cell-Based Immunomodulation in Allogeneic Heterotopic Heart-Lung Transplantation

Biancamaria Longoni1,*, Erzsébet Szilágyi1, Lorenza Puviani2, Benedetta Mazzanti1, Giacomo Timoteo Paoli1, Serena Urbani1, Paola Quaranta1, Sara Antonini1, Sergio Tripodi1, Marcella Cintorino1, Riccardo Saccard1, Bruno Nardo2 and Franco Mosca1

1Department of Oncology, Transplantation and New Medicine, University of Pisa, Pisa, Italy
2Department of Surgery, Bologna University, St. Orsola Hospital, Bologna, Italy
3Bone Marrow Transplant Unit, Hematology Unit, Careggi Hospital, Florence, Italy
4Department of Human Pathology and Oncology, Pathology Section, University of Siena, Siena, Italy

Abstract

Mesenchymal stem cells are able to differentiate in various cell lineages and they have shown immunomodulatory properties in vitro, altering the cytokine secretion profile of T helper, T effector and dendritic cells and stimulating natural killer cells towards an anti-inflammatory and tolerant phenotype. In vivo they prolong skin allograft survival and may decrease graft-versus-host disease after hematopoietic stem cell transplants. In this work we studied the effects of mesenchymal stem cell treatment in an allogeneic heterotopic heart-lung transplant model.

The following experimental groups were formed: A) Control B) Immunosuppressive therapy (Cyclosporine A) C) Mesenchymal stem-cell intravenous infusion D) Mesenchymal stem-cell infusion plus immunosuppressive treatment.

The infusion of mesenchymal stem cells improved the mean graft survival up to 14.5±3.7 days with respect to the control group (2±0.8 days). Treatment with Cyclosporine A plus mesenchymal stem cells (group D) produced a mean survival time of 18.25±4.9 days, and was not significantly different to the results for group B (21.75±3.5 days). Furthermore, in the immunosuppressive treatment and the mesenchymal stem cell treatment, histological analysis revealed a reduction in the grade of rejection in heart and lung grafts. This decrease was most significant in group D.

In conclusion, mesenchymal stem cells alone or in combination with Cyclosporine A were able to prolong graft survival time. These data suggest that, in vivo, mesenchymal stem cells retain their ability, already shown in vitro, to suppress lymphocyte activation and proliferation.

Keywords: Immunosuppression; Graft; Rejection; Organ Transplant; Cyclosporine A

Abbreviations: 7-ADD: 7-Amino-actinomycin D; ANOVA: Analysis Of Variance; CyA: Cyclosporine A; DMEM: Dulbecco’s Modified Eagle Medium; FACS: Fluorescence-Activated Cell Sorting; FBS: Fetal Bovine Serum; FITC: Fluorescein Isothiocyanate; GVHD: Graft-Versus-Host-Disease; IL: Interleukine; IM: Intramuscular; IFN-γ: Interferon Gamma; IP: Intraperitoneal; MLR: Mixed-Lymphocyte Reaction; MSCs: Mesenchymal Stem Cells; PBS: Phosphate Buffered Saline Solution; PE: Phycoeritin; S.E.M.: Standard Error of the Mean

Introduction

Immunomodulation in clinical transplantation should prevent rejection of the allograft as well as the toxicity of long-term immunosuppressive drug treatment. In recent years attention has focused on the therapeutic application of mesenchymal stem cells in an attempt to improve graft survival and achieve immunological tolerance [1] without pharmacological immunosuppression.

Mesenchymal Stem Cells (MSCs) are bone-marrow-derived pluripotent cells, capable of differentiating into multiple lineages [2,3]. Although the frequency of these cells in the bone marrow is very low, they can be isolated by simple procedures and expanded, maintaining their ability to differentiate into multiple lineages. MSCs do not express the hematopoietic markers CD34, CD45 or CD14, and can be identified by positive staining for a number of non-specific surface adhesion molecules such as CD29, CD44, CD73 and CD1059 [3]. Human MSCs do not express costimulatory and MHC class II molecules, which might be why they escape the immune system in vitro [4] and are considered good candidates for cellular therapy in an allogeneic setting [5]. MSCs are able to affect immune response both in vitro and in vivo; recent studies indicate that they suppress T-cell-mediated responses involved in tissue rejection [4,6-9].

Many in vitro studies demonstrate that MSCs from various species can exert an immunoregulatory function and suppress T-cell proliferation, in autologous and allogeneic conditions, in response to various stimuli with cell contact-dependent and -independent mechanisms [4,10]. Emerging data indicate that MSCs act through interaction with a broad range of immune cells: T-lymphocytes, B-lymphocytes, natural killer cells and dendritic cells [11-14]. Moreover, MSCs suppress T-lymphocyte proliferation when added to a Mixed-Lymphocyte Reaction (MLR) [15].

Further support for their in vivo immunomodulatory features are the observations that allogeneic MSCs may prolong skin allograft survival in immunocompetent baboons [15], prevent the rejection of allogeneic B16 mouse melanoma cells in immunocompetent mice [16], and attenuate Graft-Versus-Host Disease (GVHD) in mice and humans [17,18].

Given the ability of MSCs to act through multiple mechanisms, they have been used to prevent rejection in solid organ transplantation. Indeed MSCs can simultaneously exert antiinflammatory, vascular...
and regenerative effects in transplantation, like most conventional pharmacological immunosuppressants.

MSCs have been successfully employed in several clinical trials, and in many animal models of transplantation have been shown to prolong graft survival [19]; nevertheless, in vivo studies on preclinical organ transplantation models [15, 20-26] have yielded conflicting results.

The aim of our work is to study the effect of MSCs on the rejection control of solid organ transplantation on a preclinical model of heterotopic heart-lung transplantation in rats. This experimental model investigated the effect of intravenous MSC infusion and compared it with immunosuppression induced by Cyclosporine A (CyA).

Methods

Animals

In this study male Wistar rats were utilized as the heart and bone-marrow donors, and male Sprague-Dawley rats as the recipients. They weighed between 250 and 275 g. The animals were purchased from Charles River Laboratories, Italy, fed standard rodent chow (Rieper, Italy) and water both given ad libitum under a 12 h light/dark cycle. All the animal procedures were carried out with the approval of the University of Pisa’s Ethical Committee for Animal Use and Care.

Experimental design

We performed a study to assess the effect of intravenous administration of MSCs in rats undergoing heterotopic heart-lung allograft transplantation. The experimental design comprised four groups, which underwent heterotopic heart-lung allograft transplantation as follows: control animals receiving heterotopic heart-lung transplantation alone (group A, n=15); animals receiving heterotopic heart-lung transplantation plus intravenous administration of MSCs (group B, n=25); animals receiving heterotopic heart-lung transplantation plus CyA immunosuppressive treatment (group C, n=13); and animals receiving heterotopic heart-lung transplantation plus immunosuppressive treatment combined with intravenous MSC administration (group D, n=13).

MSC and CyA administration protocol

The Sprague-Dawley rats of groups B and D received Wistar MSCs in the tail vein on transplantation day. The administered MSC dose was 20 x 10⁶/kg in 500 μl of PBS-1x solution (SIGMA-ALDRICH, Italy) at day 0.

The animals in groups C and D received intramuscular CyA (Sandimmun®; Novartis, Novartis Pharma S.p.A., Varese, Italy) administration at a dose of 5 mg/kg/day 24 hours before transplantation.

Results

MSC characterization

Bone marrow cells were collected from the male Wistar rats using Dobson’s procedure [27]. They were then re-suspended in culture medium (DMEM-Low Glucose, Eurobio, Italy), supplemented with 10% FBS (Eurobio, Italy), counted with a haemocytometer and seeded at 24 x 10⁶ flask. Subsequently, the cells were incubated (37°C in a humidified atmosphere containing 95% air and 5% CO₂), expanded and characterized as previously described [26]. In vitro differentiation was performed by incubating MSCs in osteogenic and adipogenic medium. At the fourth or fifth passage, the morphologically homogeneous population of MSCs were analysed for the expression of particular cell-surface molecules (CD45-CyChrome™, CD11b-FITC, CD90-PE, CD106-PE, CD73-PE, CD54-FITC, CD44-FITC, BD Pharmingen, San Diego, CA, USA) using FACSCalibur (BD).

Heterotopic heart-lung transplantation

The recipients were anesthetized with Zoletil 100 (10 mg/Kg, Virbac s.r.l., ip), while the donors were anesthetized with Penthotal Sodium (75 mg/Kg, ip, Gellini International); heterotopic heart-lung transplantation was performed as previously described [28,29]. The systemic injection of MSCs (20 x 10⁶/kg diluted in 0.5 ml PBS-1X solution, via the tail vein) was performed immediately after the heart-lung transplantation. CyA was diluted in PBS-1X solution and injected i.m. at a dosage of 5 mg/kg/d 24 hours before transplantation. Heart grafts were checked daily and graft rejection time was defined as the day on which no cardiac contractions were detectable by abdominal palpation and direct laparotomic visualization of the allograft.

Histological examination

Animals were sacrificed at different time points after transplantation. The grafted heart and lung were excised, fixed in 4% formaldehyde solution and embedded in paraffin. For both organs, 5-μm sections were stained with haematoxylin-eosin for histological examination. A pathologist performed a blinded analysis to assess the intensity of rejection, and each sample was graded according to a coring system described by Billingham’s standardized working formulation [30].

Statistical analysis

All the data were uploaded into a statistical analysis program (GraphPad Prism5.0, GraphPad software). Values are reported as mean ± standard error of the mean (S.E.M.). Statistical analysis was conducted with the unpaired t-test; difference with p values <0.05 were considered significant. Kaplan-Meier analysis (Origin version 5.0 Professional [OriginLab, Northampton, MA]) was used to determine allograft survival in the MSC- and CyA-treated groups, and a comparison was made with the control group.

Effect of MSC administration on graft survival

To study the effect of MSC administration on graft rejection in solid organ transplantation, we used a rat model of heterotopic heart-lung transplantation. In our model we performed heart-lung transplantation either just with MSCs or in association with CyA, and compared the effects with data obtained with pharmacological CyA treatment alone. In the control group (rats receiving heterotopic heart-lung transplantation alone), the mean allograft survival was 3.05 ± 0.6 days, because of acute rejection (Figure 2a). Systemic MSC administration (20 x 10⁶/kg, via the tail vein) prolonged graft survival up to 14.5±3.7 days (Figure 2a); however, the treatment with MSCs resulted in a high post-infusion mortality rate (~50%) due to respiratory failure caused...
by pulmonary edema. The immunosuppressive therapy with CyA (5mg/kg, i.m.) prolonged graft survival up to 21.75±3.5 days after transplantation, while the combined therapy (CyA + MSCs) increased graft survival to 18.25±4.9 days (Figure 2a).

These results show that all the above-mentioned treatments were able to improve graft survival compared to the control groups, though CyA treatment proved to be the most efficacious. The Kaplan-Meier survival curve illustrated in Figure 2b shows the loss of functionality over a period of 30 days. It is clear that acute rejection appeared in the first 5 days: in the control group, 100% of functionality was lost in this period, while in the group treated either with MSCs or CyA plus MSCs, survival increased by up to 60%, with no significant statistical differences between these groups. It is worth noting that the treatment with CyA alone improved graft survival by up to 85% (Figure 2b).

Figure 1: Characterization of MSCs. Differentiation into respective lineages was identified under specific conditions: deposits of hydroxyapatite intensely red stained with Alizarin S after culture in osteogenic medium (a), white lipid vacuoles of the cytoplasm of MSCs treated with adipogenic medium stained with Sudan Black (b). Flow cytometric analysis of surface adhesion molecules on rat MSCs. Cells positive to 7-AAD were discarded in order to exclude dead cells (c), isotype control IgG1 (d), cells were labelled with monoclonal antibodies specific for molecules indicated in each flow cytometric diagram: CD11b and CD45 antibodies showed no contamination of haematopoietic cells, while CD44, CD54, CD73, CD90 and CD106 were positive as classical mesenchymal markers (e-h).
Histological analysis

To evaluate the grade of rejection in this model, histological analysis was performed on the heart and lung allografts harvested 5 days after transplantation. Our results were expressed according to Billingham’s standardized working formulation for heart and lung rejection [30].

We found that the heart rejection grade of the control group was uniform and classifiable as 3B (Figure 3a); the histology revealed the presence of diffuse small lymphocyte infiltrates with myocytic damage and perivascular edema, as shown in Figure 4.1a. Both the treatments with CyA and MSC infusion caused a reduction of acute rejection to 2B (mean value, * p<0.05 vs. control, Figure 3a). Histological evaluation of CyA and MSCs are showed respectively in Figure 4.1b and 4.1c. High variability was observed in the MSC grafts, and in some animals the grade of rejection was 1A, with focal interstitial infiltrates without myocyte damage (Figure 4.1c). Finally, the combined therapy of MSCs plus CyA showed the lowest mean rejection level, 2/2A, with the highest statistically significant values (** p<0.001 vs. control). As can be seen in Figure 4.1d, the heart histology of a recipient treated with MSCs plus CyA showed a rejection grade of 1A, with solitary perivascular infiltrates and normal myocytes.

Lung histological analysis showed similar results to that for the heart. The lunglobe, without breathing function, acts as a blood reservoir and in the space of a few days undergoes complete hepatization. In the control group, the mean grade of rejection was A3/A4 (Figure 3b), with the presence of diffuse perivascular, interstitial and alveolar infiltration composed by mononuclear cells; prominent pneumocyte damage and neutrophils infiltration were also present. Necrotizing vasculitis and endobronchial exutation were evident (Figure 4.2a). In the group of animals treated with CyA there was a reduction to the A2/A3 level (Figure 3b). The histological evaluation showed a perivascular mononuclear infiltrate and expansion of the perivascular interstitium by inflammatory cells. Alveolar macrophage desquamation was also present (Figure 4.2b). Both immunosuppression with MSC infusion and the combined therapy (MSCs + CyA) reduced the rejection level to A2 compared to the controls (Figure 3b). The treatment with MSCs resulted in high variability and consequently the reduction was not statistically significant. The histology evidenced scattered mononuclear infiltrates and blood vessels cuffed by plasmacytoid cells.
and lymphocytes. In some cases, many alveolar spaces were devoid of cells, with a resulting A1 rejection grade (Figure 4.2c).

In the group treated with MSCs plus CyA the reduction of the rejection grade to A2 was statistically significant, as shown in Figure 3b (mean value, * p<0.05 vs. control). The histological data show that scattered mononuclear cells were present in the interalveolar septa; moreover, the alveolar morphology was well preserved as a consequence of minimal acute rejection (Figure 4.2d). In all the immunosuppressive treatments, lymphocyte infiltration in the parenchyma and inflammation were reduced.

Given the complete absence of histologic evidence of acute antibody-mediated rejection in all the cases (such as capillaritis in the lung and acute capillary injury with macrophages or neutrophils in heart capillaries), we did not evaluate the immunohistochemical expression of C4d according to the current protocols for graft evaluation. Furthermore, C4d antibody, a marker of antibody-mediated rejection in the kidney, may be of limited value in the lung because of the patchy nature and low sensitivity of C4d staining in the lung [31].

Discussion

MSCs have immunomodulatory effects and interact with the immune system. Because of these effects they are considered a promising option for the future development of clinical applications such as transplantation, in view of their role as immunosuppressants. Although the immunomodulatory effect of MSCs in vitro is well established, recent works have yielded conflicting results in in vivo studies on preclinical organ transplantation models [15,20-26]. In this work we studied the ability of MSCs to reduce acute rejection, either on their own or in combination with Cyclosporine A, in a model of heterotopic heart-lung transplantation.

Gao and colleagues have demonstrated that the distribution of mesenchymal stem cells in tissues is the same either by intravenous or intra-artery injection [32]. Consequently, we chose intravenous MSC administration. The dose used in our model, 20 x 10^6/kg, is equivalent to 5 x 10^6 MSCs per animal, which is the maximum dose usable without causing emboli in the lung [15] and which is able to prolong skin graft survival in a baboon model after in vitro suppression of mitogen response, in a dose-dependent manner [6]. Our results showed that MSCs were indeed able to significantly prolong graft survival (14.5±3.7 days), compared to the untreated animals (3.05 ± 0.6; control group). Nevertheless, a subtherapeutic dose of CyA (5 mg/kg) was more effective in prolonging graft survival (21.75±3.5), while MSCs in combination with CyA showed a survival time similar to the CyA group (18.25±4.9 days). However, histological analysis showed that the MSC and MSC plus CyA treatments were more effective in reducing heart and lung rejection (respectively from 3B to 2A/2B and from A4/A3 to A3/A2), (Figure 3a,3b).

According to the results of Ge et al. [33], our histological data showed that MSCs alone attenuate acute allograft rejection, but this treatment was not sufficient to completely inhibit the allogenic response; indeed, scattered infiltrates were present in both the heart and lung. Histological data showed that the MSC treatment was more efficacious in reducing lymphocytic infiltration, although this was not accompanied by a parallel increase in post-transplant graft survival when compared to CyA treatment. In the combined therapy of MSCs plus CyA, an increase of the effect was observed (Figure 2a).

These results are in contrast with the findings of Inoue [15], who reported that MSCs were not only ineffective in prolonging graft survival, but also that when they were administered in combination with CyA graft rejection was accelerated. It is important to underline that the protocols used by Inoue differed from ours. Indeed, a higher dose of MSCs (20 x 10^6/kg) was injected systemically (in the tail vein) in our experiments, while Inoue’s group used lower doses. Moreover, in the combined treatment we used a ten-fold higher dose of CyA (5 mg/kg), administered daily and beginning with the first dose 24 hours before transplantation, versus 0.5 mg/kg from day 5 to 9 in Inoue’s protocol.

It is our belief that the time gap in our protocol between the first injection of CyA and the MSC infusion is the ideal condition to enable...
MSCs to engraft and to exert their immunosuppressive function; this is similar to the effect found by Ge et al. [33] for the combined treatment MSCs plus Rapamycin.

Many works report that CyA inhibits IFN-γ production by T cells [34,35] and the activation of MSCs, thus preventing the immunomodulatory effect of MSCs [36-43]. Nevertheless, Kim observed that autologous MSCs plus CyA down-regulate immune response in a rat islet transplantation model by reducing the production of pro-inflammatory cytokines, IL-2, IFN-γ, and increasing anti-inflammatory cytokine production, especially IL-4 and IL-10 [19]. Similarly, in our previous work on islet transplantation, we observed a reduction of pro-inflammatory cytokines (IFN-γ, GM-CSF) in animals treated with MSCs [44]. We therefore hypothesize that an analogous decrease in pro-inflammatory cytokines may be part of the mechanism relating to the control of acute rejection in heterotopic heart-lung transplantation. MSCs induce a down-regulation of IFN-γ, the main cytokine secreted by CD4+ and involved in the recruitment and/or activation of other inflammatory cells in allograft rejection. The presence of high levels of IFN-γ is a necessary requirement for CD4+ T cell-mediated acute allograft rejection [45]. The role for CD4 T cells in acute heart allograft rejection is well established.

In conclusion, our data demonstrate that allogeneic MSCs are able to prolong graft survival in a rat heterotopic heart-lung transplantation model as observed in a rat islet transplantation model [44]. Moreover, MSCs administered in combination with CyA showed an increased efficacy compared to both cellular and pharmacological treatment alone.

The existence of conflicting data regarding the use of MSCs in solid organ transplantation is such that further studies are required to understand the underlying mechanism of action and the precise role of these cells in increasing graft survival. Enhanced knowledge of these mechanisms will be of enormous help in the development of future clinical applications.

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References


