Stem cell secretome attenuates acute rejection in rat lung allotransplant

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Received 24 July 2018; received in revised form 6 September 2018; accepted 23 September 2018

Abstract

OBJECTIVES: Stem cells secrete significant amounts of bioactive factors in their secretome that can be immunosuppressive. We studied the effect of the secretome obtained from bone marrow-derived mesenchymal stem cells (BMSC-sec) in combination with cyclosporine A following acute rejection of lung allografts in the rat.

METHODS: Lung allotransplants were performed from male Brown Norway donor rats to recipient male Fisher 344 rats. Rat BMSC-sec was introduced intratracheally in the recipient every day after the transplant until the day the animal was sacrificed. Group A (n = 5) received control medium and cyclosporine A (2.5 mg/kg body weight intraperitoneally) for 5 days post-transplant and group B (n = 5)

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received BMSC-sec and cyclosporine A. Blood gas analysis was performed to assess graft function at day 5 only from the graft, and the tissue was sampled for measurement of the wet/dry ratio and histological grading of rejection.

RESULTS: All control animals (group A) showed severe signs of rejection. At day 5 grafts in group B showed improved gas exchange (i.e. mean PaO₂ mmHg 237.9 ± 130 mmHg vs 24.9 ± 7.8 mmHg in group A). Histological examination according to the International Society of Heart and Lung Transplantation (ISHLT) revealed moderate to severe rejection in all animals in group A (III B) and a significant improvement in group B (I–IIA). The wet/dry ratio was also reduced in group B to 6.19 ± 0.6 compared to 9.36 ± 2 in group A. Furthermore, in vitro T-cell proliferation was reduced after treatment with BMSC-sec for CD 3 cells (69.55 ± 07 vs 73 ± 0.84), for CD 4 (24.95 ± 1.2 vs 27.75 ± 0.21) and for CD 8 cells (3.75 ± 0.2 vs 5.68 ± 0.02).

CONCLUSIONS: The BMSC-sec is a promising novel cell-based therapeutic option for acute rejection in a rat lung allograft model.

Keywords: The BMSC-sec is a promising novel cell-based therapeutic option for acute rejection in a rat lung allograft model.
study, a small number of animals were used. Left lung allotransplantation was performed in rats with a major full mismatch and 1 minor immunological mismatch [donor: Brown Norway rats, 220–240 g; recipient: Fisher 344 rats, 220–240 g] [10]. Two groups (n = 5 per group) were studied. The animals in group A received cyclosporine A daily [2.5 mg/kg/day intraperitoneally (i.p.)]; the control medium was introduced in the graft after implantation and then added intratracheally daily until the experiments were terminated. Group B (n = 5) received cyclosporine A daily (2.5 mg/kg/day i.p.) in combination with BMSC-cm introduced in the graft after implantation and then intratracheally daily until the experiments were terminated. A total of 500 μl of control medium or BMSC-cm was introduced each time. The animals were sacrificed on day 5 (Fig 1). During the course of the experiments, 3 recipients were sacrificed prematurely in group A due to technical issues and were excluded; these animals were replaced and from the subsequent analysis there were no deaths after the procedure. A total of 32 animals were used for this study.

Operative procedure

The surgical procedure was followed as explained in our previous studies [7, 10].

Donors

The animals were anaesthetized in a glass chamber by inhaling 4% isoflurane. Thiopental (Pentothal®; Abbott AG, Switzerland) at a dosage of 50 mg/kg b.w. was administered i.p. Heparin (Liquemin®, RochePharma, Switzerland) was administered by injection into the penile vein (500 IU/kg b.w.) [10]. The detailed procedure is explained in the Supplementary Material.

Recipients

The recipients were anaesthetized by breathing 4% isoflurane in the glass chamber. Intubation was carried out using a 14 guage catheter placed into the trachea. Anaesthesia was maintained with isoflurane at 2.5%. The recipient was ventilated with 1 cm water positive end-expiratory pressure, at a breathing frequency of 100/min and a tidal volume of 8 ml/kg b.w. using a rodent ventilator [10]. The animal was placed on a warm pad at 37°C throughout the procedure. The surgical procedure is explained in detail in the Supplementary Material.

Introduction of bone marrow-derived mesenchymal stem cells-conditioned medium or control medium

BMSC-cm or control medium was introduced to both lungs (native right lung and the implanted graft) intratracheally in a volume of 500 μl after graft reperfusion and repeated every day for the next 4 days.

Assessment

Graft function. Five days after the transplant, the recipient was preanaesthetized in a glass chamber inhaling 4% isoflurane, and thiopental (50 mg/kg b.w.) was administered i.p. The blood gas was measured as described in detail in the Supplementary Material.

Histological analysis

After explantation, the graft was isolated and the lung was cut into 3 pieces. The upper part was taken for histological analysis and fixed in 4% paraformaldehyde. The middle section was frozen for further analysis, and the lower portion was taken to determine the dry/wet ratio. After fixation in paraffin, the sections were cut. Routine haematoxylin and eosin staining was performed by deparaffinization by treatment with xylol followed by dehydration in serial concentrations of alcohol, the sections were stained and then rehydrated before mounting. The histological assessment was done by a trained lung pathologist in a blinded fashion according to the working Formulation for the Classification of Pulmonary Allograft Rejection of the International Society for Heart and Lung Transplantation [1].

Wet-to-dry lung weight ratio

The lung wet-to-dry weight ratio was measured as an index of the accumulation of water in the lung in the graft. To measure the total amount of water in the lung, the lung was measured immediately after its excision (wet weight). The lung tissue was then dried in an oven at 60°C for 48 h and reweighed as dry weight. The wet-to-dry weight ratio was calculated by dividing the wet by the dry weight; the result was represented as a ratio [7].

Mixed lymphoid reaction

The mixed lymphocyte reaction was performed using the recipient Fisher 344 splenocytes as responder cells and the donor Brown Norway splenocytes as stimulator cells. Three animals...
from each strain were used for this experiment. Responder cells were stained with 5 \(\mu\)M carboxyfluorescein succinimidyl ester (Thermo Fisher Scientific). Stimulator cell proliferation was blocked using 30 Gy gamma irradiation. After extensive washing, the responder and stimulator cells were mixed in 1:1 ratio and incubated in the presence of the BMSC-sec in Dulbecco’s modified Eagle medium, 10% FBS, 1% penicillin and streptomycin medium and 0.05 mM 2-mercaptoethanol. After 5 days of culture, the cells were collected, stained with anti-CD3 and anti-CD4 antibodies and analysed by flow cytometry. The proliferation index was determined using FlowJo software (FlowJo LLC, Ashland, OR, USA). T cells were stained for CD3-fluorescein isothiocyanate conjugate (Miltenyi Biotech, Bergisch Gladbach, Germany), CD4-phycocerythrin (BD Biosciences, Franklin Lakes, NJ, USA) and CD8-allophycocyanin (Thermo Fisher) for 20 min at 4°C. Appropriate isotype (Miltenyi Biotech) and compensation controls were also used.

### Statistical analysis

Data are presented as mean ± standard deviation. The unpaired \(t\)-test was performed to compare the 2 groups. A \(P\)-value less than 0.05 was considered significant. All the analyses were performed using Graphpad Prism 7 software (Graphpad Software, La Jolla, CA, USA).

### RESULTS

#### Adverse events during the study

During the course of the study, 3 recipients were sacrificed prematurely.

#### Arterial blood gas measurement

The PaO\(_2\) levels were significantly improved in BMSC-sec-treated animals (group B) compared to the control animals (group A) (237.9 ± 130 mmHg vs. 24.9 ± 7.8 mmHg, respectively) \((P = 0.017)\) (Fig. 2A).

#### Graft oedema measurement

Wet/dry ratio measurements showed that graft oedema was significantly reduced in the BMSC-cm-treated animals (group B, ratio 6.19 ± 0.6) compared to animals treated with control medium (group A, ratio 9.36 ± 2) \((P = 0.0079)\) (Fig. 2B).

#### Histological assessment of rejection score

The histopathological assessment demonstrated improvement in lung architecture in the BMSC-sec-treated animals compared to the control group as seen by the rejection score of I–II A in group B as compared to the III B rejection score in the control group A (Table 1, Fig. 3).

#### Mixed lymphoid reaction for T-cell activation

Stimulation of recipient splenocytes with \(\gamma\)-irradiated donor splenocytes in a mixed lymphocyte reaction showed reduced T-cell activation in the presence of BMSC-sec \textit{in vitro}. A decreased frequency of carboxyfluorescein succinimidyl ester in CD3\(^+\) cells was observed in BMSC-sec compared to control medium-treated culture (69.55 ± 0.07% vs. 73 ± 0.84%, respectively) as well as in CD4\(^+\) (24.95 ± 1.2% vs. 27.75 ± 0.21%, respectively \((P = 0.08)\) and CD8\(^+\) T cells (3.75 ± 0.20 vs. 5.68 ± 0.02, respectively, \(P = 0.005\); Fig. 4).

#### Proteomic analysis of the bone marrow-derived mesenchymal stem cells secretome

Gene set enrichment analysis of the BMSC-sec using the SetRank package (https://CRAN.R-project.org/package=SetRank) revealed...
several clusters of functionally related and intersecting protein sets. The biggest component of these contained protein sets was related to elastic fibre formation, collagen fibril formation and smooth muscle contraction (Supplementary Material, Fig. S1A). Visualisation and betweenness analysis of the protein-protein interaction network of the BMSC-sec showed that the signalling molecules transforming growth factor 2 and integrin subunit beta 2 possibly are key mediators of these fibre-forming factors (Supplementary Material, Fig. S1B).

**DISCUSSION**

The primary aim of the current study was to test the protective immunomodulatory effect of the BMSC-sec in the mitigation of acute rejection in the rat lung allotransplant model. We observed improved blood oxygenation and histological score as well as reduced oedema after intratracheal administration of BMSC-sec to the recipient. Furthermore, in vitro experiments demonstrated that the BMSC-sec reduced donor-specific T-cell activation, thereby confirming its immunosuppressive potential. Many clinical trials have shown the safety and efficiency of mesenchymal stem cells; however, the BMSC-sec still carries a potential risk such as transfer of infectious agents, limited lifespan in culture, and development to senescence [11]. Therefore, a therapy that can encase the beneficial effect of the BMSC-sec is the preferred choice for future therapeutic application. The BMSC-sec offers a viable cell-free approach for immunosuppression and immunomodulation. The stem cell secretome has been reported in various disease models, and possible mechanisms of action have been elucidated including that for lung disease [9, 12, 13]. There are studies in which the BMSC-sec was tested in acute injury models [14]; however, the immunosuppressive role of the BMSC-sec in solid organ acute rejection models has been poorly investigated. We introduced the BMSC-sec in the graft immediately after reperfusion and then every day for the next 5 days. The immunosuppressive properties of mesenchymal stem cells are well defined, and their suppressive effect on various immune cells like T cells, B cells and dendritic cells have been reported in the past [15]. However, whether the immunosuppressive effect can

**Figure 3:** Haematoxylin and eosin staining of histological sections of the graft on day 5 in the 2 groups. In the control group with cyclosporine A and control (International Society of Heart and Lung Transplantation, III B), marked alveolar oedema with inflammatory infiltrate and damaged epithelium is observed. Moreover, marked interstitial, perivascular and peribroncholar infiltration (arrow) is evident. The inset (higher magnification) shows an increased number of cellular infiltrate in the epithelium as indicated by arrow. In the bone marrow-derived mesenchymal stem cell-secretome group (International Society of Heart and Lung Transplantation, medium I–IIA), minimal perivascular mononuclear infiltrate is observed with minimal peribroncholar mononuclear cell infiltrate in the vicinity of the epithelium. The alveoli look very clear with minimal cellular infiltrate. Inset (higher magnification) shows clear alveolar space (representative picture for n = 5 in each group).

**Figure 4:** Cells were stained with CD3, CD4 and CD8 antibodies. T-cell activation was reduced after treatment of CD3 (A), CD4 (B) and CD8 (C) cells with the BMSC-sec (mean ± standard deviation, n = 2). BMSC-sec: marrow-derived mesenchymal stem cell-secretome.
also be achieved by the secretome is not known. To complement our in vivo results, we performed the in vitro study and showed that the BMSC-sec reduced T-cell activation, thus exerting an immunosuppressive effect in line with the earlier report in which bone marrow-derived stem cells were used [16]. We further performed a detailed proteomic study of the BMSC-cm to identify the mediators that could potentially play an essential role in reducing T-cell activation and immunosuppression. Although numerous factors that are anti-inflammatory and have immunosuppressive properties were identified in the BMSC-sec, we did not investigate further to identify the specific factor because we think that the secretome comprises natural secretions from the cells and that every component in the soup plays a critical role in exerting the therapeutic benefit. In the in vivo study, we did see improved histology, reduced cellular infiltrate, significant reduction in oedema and improved blood oxygenation. All of these beneficial effects can be partially explained by the protein set enrichment analysis using SetRank analysis, which revealed very interesting pathways that are involved. The most interesting pathways that are highlighted are the c-Jun N-terminal pathway (JNK); JNK phosphorylation and extracellular matrix structural constituents both are known to play an essential role in acute rejection [17]. It has been reported that the JNK is activated in response to inflammation and oxidative stress and is involved in various cellular activities such as cell growth, differentiation, survival and apoptosis. Moreover, expression of JNK is also involved in DNA damage repair [18]. JNK activation leads to phosphorylation of SIRT6 that recruits PARP1, which play a crucial role in the repair of a break in the double-stranded DNA [19]. Moreover, disruption in the components of the extracellular matrix has been associated with the development of acute allograft rejection in the transplant of solid organs [20]. The population-specific expression analysis revealed the presence in the secretome of basement membrane-organizing, collagen-forming and laminin-binding factors that reorganize the extracellular matrix. The presence of factors that act on these 2 essential pathways make the BMSC-sec a very interesting option for attenuation of acute rejection. Further studies to validate its potential are warranted. Furthermore, the most prominent of the interacting proteins shown are transforming growth factor b, integrin subunit beta 2, heat shock protein 90, Ras-related C3 botulinum toxin substrate 1, Actin 2 and various collagens. All these factors play an essential role in cytoskeletal reorganization and remodeling. The presence of these factors and their synergistic beneficial role makes the BMSC-sec an interesting option to further test in clinical application for attenuation of acute lung rejection.

Limitations

We demonstrated in a rat allograft model that BMSC-sec is a promising approach for treatment of acute graft rejection. We also showed the effect of BMSC-sec on T-cell proliferation in vitro. However, the major limitation is the daily intratracheal administration of BMSC-sec, which, when translated to the clinical setting, would be technically difficult to achieve. Therefore, other efficient routes of administration have to be investigated. Moreover, we still do not have a detailed mechanistic proof of the beneficial effect. A more elaborate study with more readout would help us to understand the possible mechanism, such as performing a global gene expression profile, a detailed cytokine array, and a detailed interactome using advanced bioinformatics tools. Also, a crucial limiting factor is the limited volume that could be introduced in the graft after implantation. Because the volume of 500 µl is just sufficient to introduce in the rat lung, we did not exceed this limit; moreover a better option would be to concentrate the BMSC-sec and administer lower volumes. Although the current study does offer a good model in which to establish the proof of concept, an additional preclinical study using a larger animal model would be of great benefit for optimization of the concept before clinical translation.

CONCLUSION

In conclusion, the BMSC-sec consists of essential immunomodulatory agents that attenuate acute lung rejection. BMSC can be easily isolated and cultured, and the secretome can be collected under standard culture conditions. It offers a cell-free option, thus bypassing the possible adverse effects of using cells. In addition, regulatory issues related to the use of mesenchymal cells have been agreed upon for facilitating their use in clinical studies [21]; therefore, further attempts to introduce the BMSC-sec for clinical application would be easy. However, further investigations are required to understand the detailed immunoregulatory mechanisms.

SUPPLEMENTARY MATERIAL

Supplementary material is available at ICVTS online.

ACKNOWLEDGEMENTS

We thank Mrs Anna-Barbara Tschirren, Department of Pulmonary Medicine, University Hospital Bern for excellent technical support.

Funding

The work was supported by the Department of Thoracic Surgery University Hospital Bern.

Conflict of interest: none declared.

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