A possible stem cell therapy for diabetes mellitus

Doctoral theses

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Introduction

Diabetes mellitus is a leading cause of morbidity and mortality in industrialized countries. It is caused by absolute insulin deficiency due to autoimmune destruction of insulin secreting pancreatic β-cells (type-1 diabetes) or by relative insulin deficiency due to decreased insulin sensitivity (type-2 diabetes). In both types of the disease, an inadequate mass of functional β-cells is the major determinant for the onset of hyperglycaemia and development of overt disease.

Curative therapy for diabetes mellitus mainly implies replacement of missing insulin-producing pancreatic β-cells, with pancreas or islet-cell transplants. The limited supply of available donor islets for transplantation, however determines that researchers must explore alternative sources of graft material or otherwise restore β-cell functioning. Generating surrogate β-cells from embryonic or adult tissue stem cells is one of the possible solutions of stem cell therapy. Besides, intense research has been focused on possible mechanisms to promote the expansion of existing pancreatic β-cells even with contribution of endogenous or exogenous progenitors or stem cells.

Some authors report that in streptozotocin (STZ)-induced diabetes bone marrow (BM) transplantation may result in temporary or definite normalization of blood sugar level and increased islet mass. Others reported that repeated transplantation of human BM-derived mesenchymal stem cells (MSCs) induced repair of pancreatic islets in NOD/scid mice suffering from STZ-induced diabetes. MSCs represent one of the most promising adult stem cell types due to their availability and the relatively simple requirements for in vitro expansion. They have not only the capacity to differentiate into several tissues in vitro, but also have regenerative, immunomodulatory and anti-inflammatory effects in vivo. In addition, they evoke only little immune reactivity in allogeneic recipient. Both regenerative and immunosuppressive properties of MSCs are particularly noteworthy during exploration of therapeutic prospects of a disorder like type-1 diabetes, that caused by autoimmune destruction.

Although, many studies support strongly that BM-derived haematopoietic and/or stromal cells can promote islet regeneration in diabetic animals, the mechanisms of the regenerative process and the appropriate conditions for using these cells for therapy are still very poorly understood. The aim of the present work is to characterize the function of BMCs and BM-derived MSCs in healing diabetes in mice.
**Aim**

Results of mouse experiments focusing on bone marrow derived cells as possible source of surrogate β-cells or of factors that promote expansion of existing β-cells seem to be confusing and conflicting. Mechanisms standing behind occasional recoveries also need to be elucidated. Our work aims to characterize the function of freshly prepared nucleated bone marrow cells (BMCs) and BM-derived mesenchymal stem cells (MSCs) in healing diabetes in mice.

1. **Induction of experimental diabetes**

In streptozotocin (STZ)-induced diabetic mice development of diabetes is followed by continuous monitoring of body weight, blood glucose, and serum insulin. Destruction of pancreatic islets and the decrease of insulin secretion are also verified by histological examinations by immunochemistry performed on sections prepared form paraffin embedded tissue samples.

2. **Influence of bone marrow transplantation on progression of the disease**

The effect of freshly prepared, bone marrow-derived nucleated cells (BMCs) on the progression of the disease and the potency of BMCs for regenerating destructed endocrine pancreas is studied. Furthermore, we determine the smallest but effective dose of total body irradiation (TBI) that is needed to be applied before transplantation, the ideal time for transplantation considered, and the therapeutically most reasonable number of cells has to be applied.

3. **Influence of in vitro expanded mesenchymal stem cell (MSC) co-transplantation on progression of the disease**

The effects of BMCs co-transplanted with previously in vitro expanded bone marrow-derived MSCs on the progression of the disease and the influence of MSCs administered alone is assessed. These cells are characterized in a flow cytometer by cell surface markers. Their plasticity is showed by making the MSCs differentiate into adipocytes and osteoblasts. Additionally, we also assess the number of cells considered to be the most effective for healing.

4. **Explore the mechanism behind observations**

The fate of administered BM-derived graft cells is monitored by histological examination through applying immunochemistry methods as well as fluorescence in
situ hybridisation (FISH) on sections. We aim to verify the BMCs’ homing to recipient’s bone marrow causing transitional chimerism. Another goal is to study the MSCs’ immunosuppressive effect by T-cell proliferation experiments, and to follow the MSCs’ fate, including their adherence.
Materials and Methods

Induction of diabetes
Type-1 diabetes was induced with multiple low doses of streptozotocin (STZ). 8-10 week old female C57Bl/6 mice were injected intraperitoneally (i.p.) with 50 mg/kg body weight STZ daily for 5 consecutive days. Body weight was followed and blood glucose was also measured twice weekly with a glucometer. Serum insulin was quantified with an ELISA Kit specific for rat/mouse insulin.

Intraperitoneal glucose tolerance test
Mice fasted for 4 hours were injected i.p. with 2 g/kg body weight of glucose. Glucose disposal was analyzed by measuring blood glucose at different time points.

Cell separation and transplantation of hyperglycaemic mice
Syngeneic bone marrow cells from male C57Bl/6 mice were extracted from the femurs and tibias and transplanted by tail vein injection into lethally (900 cGy) or sublethally (450, 250, or 150 cGy) irradiated diabetic female recipients on day 15 following STZ induction of diabetes. Syngeneic, semiallogeneic or allogenic mesenchymal stem cells (MSCs) from male C57Bl/6, (C57Bl/6xDBA/2)F1 or CD1 mice respectively, were isolated by their adherence to surface of plastic tissue culture flasks and serially passed in vitro at least 8 times before injection (see detailed in next point). Culture-expanded MSCs were co-infused with BM grafts in 0.2 ml of serum-free medium.

Isolation and culture of mouse mesenchymal stem cells (MSCs)
MSCs were isolated from bone marrow and expanded in vitro. Bone marrow was collected from 8-10 weeks old male mice by flushing femurs and tibias. Cells were then plated in a 25-cm² flask at a density of 2-5×10⁶ cells/cm² in complete medium and cultured in a humidified 5% CO₂ incubator at 37°C for 72 hours. Non-adherent cells were removed by sequential changes of the medium twice a week. Confluent primary cultures were lifted by incubation with trypsin/EDTA at 37°C for 5 minutes. Cells were then seeded into a 75-cm² flask. Subsequent passages were performed similarly. Cultures were contaminated with hematopoietic cells until passage 6 or 7.
Characterization of MSCs

MSCs were retrieved by trypsin digestion and aliquots of 5x10^5 cells were labelled in the dark for 30 minutes at room temperature with fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies developed against typical markers of haematopoietic cell lines CD3, CD45R/B220, TER-119, GR-1, 11b and also against Sca-1, that is typical marker of MSCs. Stained cells were washed with PBS and analyzed immediately on a FACScan flow cytometer.

Osteogenic differentiation was induced by culturing confluent MSCs for 2 weeks in complete medium supplemented with dexamethasone (10^{-8} M), β-glycerophosphate (10 mM), and ascorbic acid (0.3 mM). To observe calcium deposition, cultures were stained with Alizarin Red stain.

To induce adipogenic differentiation, confluent MSCs were cultured for 2 weeks in complete medium supplemented with dexamethasone (10^{-7} M) and 3-isobutyl-1-methylxanthine (0.5 mM). The cells were then fixed with 10% formalin and stained with Oil Red O or Giemsa and analyzed with microscopy.

Antigen-specific T-cell proliferation assay

Antigen-presenting cells (APCs) were enriched from spleens of untreated and STZ-treated (on day 8) female C57Bl/6 mice. Homogenized spleen cells were incubated on Petri dishes for 4 hours at 37ºC. Afterwards, nonadherent cells were removed by multiple washes in cold PBS and adherent cells were lifted by scraping. The APC enriched population was irradiated (15 Gy) before use.

T-lymphocytes were prepared from the pancreas of STZ-treated and control animals. Each pancreas was mechanically separated into single cell suspension. Cells were washed and T-cells were enriched by magnetic beads of a T-cell isolation kit.

APCs (5x10^4 cells/well) and T-cells (2x10^5 cells/well) were incubated together on 96-well plates for 72 hours at 37ºC. Eighteen hours before harvest, the cultures were pulsed with 1 μCi ³H-thymidine and incorporated ³H-thymidine was measured in liquid scintillation counter.

Histology and immunohistochemistry

Pieces of pancreas, liver, and lung were fixed in 4% neutral-buffered formalin for 3 hours, embedded in paraffin wax. Sections of 5 μm were stained with haematoxylin-eosin. For the purpose of immunohistochemistry sections were dewaxed, rehydrated, incubated in hydrogen peroxide solution for 5 min to block endogenous
peroxidases. The monoclonal anti-insulin antibody was applied to the sections, followed by biotinylated anti-mouse IgG and streptavidin-peroxidase conjugate.

**Combined immunohistochemistry and Y chromosome in situ hybridisation**

For detection of insulin containing male-derived donor cells in the tissues of STZ-treated female recipients we combined insulin specific immunohistochemistry with Y chromosome specific fluorescent in situ hybridization (FISH).

Sections from formalin-fixed paraffin embedded tissues were dewaxed, rehydrated and blocked with Tris buffered non-fat milk for 1 hour. After washing with TBS, monoclonal anti-insulin antibody was applied, followed by anti-mouse IgG-alkaline phosphatase antibody for 1-1 hours. Alkaline phosphatase activity was visualized by Fast Red stain.

The subsequent detection of Y chromosomes was carried out with mouse Y chromosome probe labelled with fluorescein isothiocyanate. The specificity of reaction was checked by comparing pancreatic slices from healthy control male and female mice.

**Monitoring of bone marrow chimism**

Y chromosome specific FISH analysis was performed on bone marrow cells prepared by standard cytogenetic techniques including hypotonic KCl-incubation (37°C for 20 min) and fixation steps (acetic acid/methanol 1:3). At least 50 nuclei were counted in each slides.

**Statistical analysis**

Student’s t test was used for p values. The difference was significant when p value was less than 0.05. The Kaplan-Meier method was used to calculate the survival data. Outcomes of treatments were compared using the Mann-Whitney U test.
Results

1. STZ-induced type-1 diabetes mellitus in mice
To evaluate the potential capacity of bone marrow-derived stem/progenitor cells to restore tissue function after pancreatic damage, destructive diabetes was induced with STZ in adult female C57Bl/6 mice. Morning-non-fast blood glucose level increased 4-5-fold in diabetic animals compared to that of untreated mice (5.71±0.42 mM and 24.91±3.93 mM, respectively) at day 35, resulting in severe hyperglycaemic condition. Elevation of blood glucose concentration starting at day 7 and continuing till day 35 correlated with gradual loss of body weight. Comparative histological study of healthy and diabetic pancreas sections prepared on at day 21, showed dramatic decrease of size of pancreatic islets containing cells with pyknotic nuclei in tissue from diabetic mice. Immunohistochemical staining of insulin producing cells detected presence of very few insulin-positive cells in diabetic mice compared to healthy control animals. Those animals were considered diabetic whose blood glucose level exceeded 10 mM at day 14 and 15. Without administration of insulin the sick mice ceased at 4-6 weeks.

2. Transplantation of freshly prepared nucleated bone marrow cells (BMCs) is not sufficient to treat STZ-induced diabetes
To study whether bone marrow cells were able to restore pancreas functions, 1 million nucleated BMCs, freshly isolated from adult male C57Bl/6 donors, were injected intravenously (i.v.) into diabetic mice after total-body irradiation (TBI) on day 15. Different groups of animals received various doses (900, 450, 250, or 150 cGy) of TBI. The highest dose of TBI (lethal dose of 900 cGy) resulted in total loss of diabetic, BM transplanted animals within a few days, whereas non-diabetic, BM transplanted control mice survived up to 180 days. Examination of blood glucose level showed no difference in its elevation between non-irradiated, non-transplanted and lowest dose-irradiated (150 cGy), transplanted diabetic animals. Elevation of blood glucose level was transiently delayed when diabetic mice were preconditioned with medium dose (250 or 450 cGy) irradiation followed by BMC transplantation. Therefore 250cGy was considered as the least but effective dose of TBI and was used henceforth. However normoglycemia was not restored since after 2-3 weeks the blood sugar concentration rapidly increased and all mice died between 8 and 10 weeks after transplantation. Thus, transplantation
of BM cells after a moderate sublethal irradiation (450 or 250 cGy) transiently reduces blood glucose levels in STZ-treated mice, but is not sufficient to treat the disease.

3. Mesenchymal stem cells (MSCs) co-operate with bone marrow cells (BMCs) in successful therapy of diabetes

To determine whether or not MSCs alone or together with unfractionated BM cells contribute to repair pancreatic functions, we injected syngeneic BM cells and/or different amount MSCs intravenously into STZ-treated animals after sublethal irradiation at a dose of 250 cGy. Phenotype of culture expanded MSCs was analyzed: these cells were strongly positive for the specific surface antigen, Sca-1 and negative for differentiation markers of other cell lineages and were able to differentiate into the osteoblast and adipocyte lineages in vitro, verifying MSC phenotype.

Blood glucose level (BG) and serum insulin (SI) concentration of diabetic animals rapidly returned to normal levels when received 10⁶ BM cells and 10⁵ MSCs on day 15 and these mice survived more than 180 days. Decreasing the number of MSCs to 5x10⁴ or 2.5x10⁴/animal greatly reduced the reversing effect concerning BG and SI levels. Similarly, a minimum number of 5x10⁵ nucleated marrow cells was required for successful treatment. Neither MSCs (2x10⁵) nor BMCs (10⁶) alone affected high BG and low SI levels and viability.

Glucose tolerance test and immunhistochemical analysis of insulin-positive cells in pancreatic sections also proved the successful cooperation between MSCs and BMCs in treatment of diabetes, since glucose tolerance and mass of insulin producing cells became comparable to that of healthy controls using optimal combination of transplants.

Similar results were obtained when syngeneic, semiallogenic or allogeneic MSCs were used for transplantation. Timing of transplantation severely affected the outcome of the disease: 6 of 9 animals survived longer than 20 weeks and returned to normal blood-glucose level when transplantation was carried out on day 8 after the beginning of STZ treatment; all, 9/9 animals survived and became normoglycemic at transplantation of day 15; 2 out of 9 mice survived when diabetic recipients were transplanted at day 22; whereas none of the animals survived if transplanted at day 29.
4.1. The mechanism: recovery from diabetes is not due to differentiation of donor BM cells or MSCs into insulin producing β-cells

Newly formed insulin-positive cells could be found only in recipient pancreas and did not appear in other organs such as liver, spleen, lung and bone marrow.

Nevertheless, Y chromosome-carrying cells localized within the BM, indicating that the engraftment of donor-derived BM cells and/or MSCs homed into the recipient’s marrow. The level of BM chimerism peaked at 10 days after transplantation (6-12% donor-derived cells in the recipient’s bone marrow) then rapidly declined, meanwhile the total number of nucleated cells in the bone marrow gradually increased.

Thorough examination of liver, spleen, stomach, intestine, lung and bone marrow of mice transplanted with BM cells and syngeneic or allogeneic MSCs did not revealed any signs of tumorogenic transformation in the above organs.

The new pancreatic β-cells in mice, recovered from diabetes may arise from endogenous or donor-derived sources. To determine whether donor bone marrow and/or MSCs can give rise to new insulin-producing cells a Y chromosome-specific FISH analysis combined with immunostaining for insulin on pancreatic sections was performed. The assay was validated firstly by using pancreatic sections from healthy control male mice. Punctate staining for Y chromosome was evident in the nuclei of both acinar and islet cells. FISH analysis, carried out at day 2 or day 84 following transplantation of BMcs and MSCs from matched male donors, failed to give positive signal in the nuclei of acinar and islet cells of female diabetic mice. Accordingly, using mismatched EGFPTg-CD1 transgenic mice as an MSC source, green fluorescence cells were absent in the recipient pancreas.

These results clearly shows that the new β-cells did not rise from donor-derived sources and that the reappearance of pancreatic insulin-secreting cells and normalization of blood sugar and serum insulin level was a result of an endogenous regenerative process activated in the host.

4.2. The mechanism: MSCs suppress β-cell specific T-cell response

To check immunosuppressive effect of MSCs, T-cells were isolated from the pancreas of diabetic mice transplanted with BMcs or MSCs alone, with BMcs and MSCs in combination or left non-transplanted. Splenic antigen presenting cells (APCs) isolated from diabetic mice induced intensive proliferation of autoreactive T lymphocytes prepared from animals non-transplanted or transplanted with BMcs
alone. In contrast, MSC transplantation fully diminished T cell proliferation either it was injected alone or in combination with BMCs.

These findings strongly suggest that MSCs are able to suppress the β-cell specific T lymphocyte response. MSCs therefore seem to play dual role: they may help tissue repair in cooperation with BMCs and inhibit β-cell specific T-cell response therefore promote survival of newly formed β-cells.
Conclusions

1. Our work demonstrated that co-transplantation of syngeneic unfractionated bone marrow derived cells (5x10^5 - 10^6 BMC/mouse iv.) and syngeneic, semiallogeneic or allogeneic culture-expanded mesenchymal stem cells (5x10^4 – 10^5 MSC/mouse iv.) can reverse streptozotocin (STZ) induced type-1 diabetes in mice. This treatment of hyperglycaemic and islet-destructed mice results in the reappearance of functional pancreatic insulin-secreting cells and normoglycaemia. Transplantation with neither BMCs nor MSCs alone is sufficient for treatment of diabetes, which indicates that these cells act in concert during islet regeneration.

2. We were able to find transplanted cells among recipients’ tissues as all of diabetic recipients were female while BMCs and MSCs were originated from male – in some cases from EGFP transgenic male – donors. The combination of Y chromosome specific fluorescent in situ hybridisation (FISH) analysis and insulin specific immunohistochemistry proved that neither Y chromosome-carrying nor EGFP positive donor derived insulin-producing cells are detected in the regenerated islets. Since new β-cells did not arise from donor-derived sources, therefore we concluded that bone marrow derived cells had been indirectly activating an endogenous regenerative process.

3. The appearance of β-cell specific autoreactive T-lymphocytes was detected in the pancreas at the onset of diabetes. This T-cell response became more pronounced close to the death of animals treated only with BMCs since they were alive some weeks longer than non-transplanted diabetic mice. These results clearly show that autoreactive T-cells have been continuously destroying new β-cells generated due to the autoregenerative processes that were induced by BMCs administered, so autoimmune response impedes healing of diabetic animals.

4. In contrast, MSCs administered alone or in combination with BMCs caused the disappearance of β-cell-specific T lymphocytes from diabetic pancreas. These results suggest that two parallel aspects of this successful treatment regimen operate synergistically in our model. First, BMCs and MSCs induce the regeneration of recipient-derived pancreatic insulin-secreting cells. Second, MSCs
inhibit T-cell-mediated immune response against newly formed β-cells, which, in turn, are able to survive in this altered immunological milieu.

In conclusion our work offers a novel potential therapeutical protocol for type-1 diabetes. The protocol we offer to treat diabetes is superior to others published so far:

a) Non-lethal, low dose irradiation results in minimal tissue damage, that able to regenerate spontaneously.

b) One step transplantation event can be carried out with a single intravenous injection of the graft without the risk of embolia caused by cell aggregation.

c) Transplanted MSC does not require genetic identity, so there is no need for specific donor. MSCs can be prepared in advance and always disposable for either of recipients.

d) No additional immunosuppression has to be applied.

Thus, the application of this therapy in human patients suffering from diabetes and/or other tissue destructive autoimmune diseases may be feasible.
Publications connected with theses:

**Journal articles:**


**Lectures and posters:**


9. **Urbán V.S**, Kiss J, Kovács J, Vas V, Monostori É, Uher F: *Cooperativity between bone marrow cells and culture-expanded mesenchymal stem cells during reversal of type-1 diabetes. Magyar Őssejtkutatás Szimpózium (ESTOOL), Budapest, 2008 (poster)
