TGF- β Engineered Mesenchymal Stem Cells (TGF- β /MSCs) for Treatment of Type 1 Diabetes (T1D) Mice Model

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ABSTRACT:

Objective: Mesenchymal stem cells (MSCs) are advantageous candidates for cell therapy of Type 1 diabetes (T1D). Considering immunomodulatory effect of MSC, in this study, we engineered MSCs with TGF- β gene to increase MSC potency for T1D therapy in mouse model.

Materials and Methods: Two plans were designed for prevention and treatment of diabetes, respectively. In both of them, MSCs were injected i.v. and then, the diabetes features including serum insulin, blood glucose, glucose tolerance, splenocytes proliferation, and IL-4/IFN- γ production were evaluated.

Results: TGF- β /MSCs treatment program resulted in the restoration of serum glucose after 3 weeks, while prevention program could delay diabetes progression for two weeks. TGF- β /MSCs treatment elevated the levels of serum insulin and Th2 cytokine shift on 5th week after start of treatment. TGF- β /MSCs (and MSCs alone) could also diminish body weight and enhance mice survival comparing to untreated diabetic mice.

Conclusion: Engineered TGF- β /MSCs could restore some T1D features, including the regulation of adverse immune responses and could be potent tools for cell therapy of T1D comparing MSCs alone.

Key words: Type 1 Diabetes, Mesenchymal Stem Cell, TGF- β , Cell Therapy

INTRODUCTION

According to the recent WHO report, diabetes is one of the most important leading causes of death worldwide (1). Type 1 diabetes (T1D) is caused by autoimmune destruction of the pancreatic β -cells that leads to loss of insulin secretion and absolute insulin deficiency; while, type 2 diabetes (T2D) is defined by insulin resistance or insulin deficiency (2). Etiology of T1D is complicated and a combination of environmental, genetic risk factors and autoimmune-mediated processes contribute to T1D etiology (3). Immunopathogenesis of T1D indicates that macrophages and dendritic cells (DCs) first infiltrate into the islets and then CD4 and CD8 T lymphocytes, as well as natural killer (NK) cells, and B lymphocytes penetrate (4, 5). Secretion of some cytokines such as interleukin (IL)-12 by macrophages and DCs activate Th1 cells, as Th1 cytokines (e.g. IL-2, IFN- γ , TNF- α , and IL-1 β) enhance the activation of CD8 T cells they also can lead to β -cell death via apoptosis in the islets (6). Receptor-mediated interactions (e.g., Fas-Fas ligand, CD40-CD40 ligand, and TNF-TNF receptor), proinflammatory cytokines, ROS, granzymes, and perforin from cytotoxic effector T cells mediate T-cell-mediated β -cell destruction (7). On the other hand, regulatory T cells (Treg cells) and suppressor mechanisms are crucial to predict the fate of disease (8). Accordingly, since, the major problems in T1D are autoimmune responses and destruction of β -cell, the goal of clinical interventions for T1D is to prevent or arrest the onset and autoimmunity progression, reverse β -cells, destruction, as well as restoring glycometabolic and immune homeostasis. Mesenchymal stem cell (MSC) based cell therapy is one of the best suggestions, as a novel and potential cures for T1D (9, 10). Not only have self-renewal potential, MSCs can make transdifferentiation into various cell lineages and show well-characterized immunomodulatory effects. So, these features make MSCs attractive for treating T1D (9, 10). Previous studies showed the beneficial uses of MSCs in T1D cell therapy (11, 12). For instance it has been shown that MSCs could effectively produce β cells in vitro (11). In experimental mouse models, intravenously infused MSCs are capable to migrate to pancreatic islets (12). Their administration to diabetic mouse models showed the delayed onset of diabetes, improved glycemic levels, reduced pancreatic insulinitis, and pancreatic tissue regeneration (13). Also, the induction of regulatory T-cells (Tregs) and prevention of autoimmune destruction of β -cells have been demonstrated (14). But, for treatment of T1D, the applications of MSCs alone could not yield to appropriate and completely successful results.

Unfavorable immune responses may destroy the engrafted cells. So, advanced immunoregulation will be desirable. Transforming growth factor (TGF- β 1) is a pleiotropic cytokine, secreted by immune and nonhematopoietic cells. TGF- β involves in many other mechanisms to modulate immune responses. Through different ways, it maintains immune homeostasis and acts as a potent immune suppressor by inhibition of proliferation, differentiation, activation, and effector function of immune cells (15). According to these facts, in this study we engineered a form of MSCs with TGF- β gene to evaluate their ability to regenerate β cells islet, diabetes features, and modulation of immune responses in STZ-induced diabetic mouse model in two systems including diabetes prevention capacity and treatment schedule.

MATERIALS AND METHODS

Mice

Five- to six-week-old BALB/c and C57BL/6 mice were purchased from Razi Institute (Shiraz, Iran). All mice were inbred and maintained under specific pathogen free and standard conditions. All of the experimental procedures for animals handling were approved by the animal ethical committee of Shiraz University of Medical Sciences.

Construction of vector and engineered lentivirus production:

pCR4-Topo vectors carrying TGF- β (GenBank: BC013738.1) mouse genes were purchased (ImaGene, USA). The genes were successfully sub-cloned into p240 (pLOX-EWgfp modified vector) lentivirus transfer vector (Addgene, USA), using SmaI and BamHI enzymes, and confirmed by electrophoresis and sequencing. P240-TGF- β vector recombinant lentiviruses (LVs) were then produced in HEK293T cell line (NCBI code: C497, cell bank- Pasteur institute of Iran). LVs constructed with plox-MD2 (Addgene, USA) and plox-PAX2 (Addgene, USA) vectors. The supernatant of infected HEK293T cells was then concentrated by ultracentrifugation. Expression of GFP (i.e. green fluorescent cells aka GFP+) was used for monitoring the transduced cells by both immunofluorescence microscopy and titration of LVs by flow cytometry.

Isolation and Culture of Bone Marrow Derived Mesenchymal Stem Cells:

MSCs isolation (routine lab protocol) from 6-8 weeks old inbred BALB/c mice bone marrow (n=5) was done by sequential culture method (16). The procedures were performed according to "Guide for the Care and Use of Laboratory Animals". In brief, bone marrow cells were isolated from femurs and tibiae of BALB/c mice. Isolated cells were cultured in 25 cm² flasks in low glucose Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), sodium pyruvate (1%), glutamine (1%), and penicillin-streptomycin (100 µg/ml) in a CO₂ incubator at 37° C. After 48 hours, non-adherent cells were removed. Adherent cells were grown to 80-90% confluency, then trypsinized and passaged (16). In passage 6, the cells purification by means of CD31, CD34, CD11b, CD45, CD135, CD44, and Thy1.2 (CD90), markers through flow cytometry method.

In vitro Multilineage Differentiation Studies:

For adipogenesis assay, BM-MSCs were cultured in DMEM supplemented with 1 µM dexamethasone, 0.5 µM ascorbic phosphate, 200 µM indomethacin and 10% FCS for 14 days. Osteogenesis was evaluated in the appropriate inducing media including 1 µM dexamethasone, 10 mM β- glycerolphosphate, 0.5 µM ascorbic phosphate, and 10% FCS in DMEM for 14 days. The differentiation phenotype was documented using oil red O staining for adipocytes, and alizarin for osteocytes.

Transduction of Mesenchymal stem cells:

Prior to transduction by 16 multiplicities of infection (MOI) of concentrated TGF-β LVs, 1.5×10⁶ MSCs/ml were cultured for 24 h in a 6-well plate containing DMEM. TGF-β transduced MSCs was considered as test groups, while the control groups included empty vector-MSCs and untransduced MSCs. Efficacy of MSCs transduction was evaluated by GFP transduction and flowcytometry. TGF-β in the supernatant of MSCs-TGF-β was assessed by enzyme-linked immune sorbent assay (ELISA) according to the manufacturer's kit instruction (eBiosciences, USA).

Diabetic mouse model induction and in vivo treatment (prevention and Treatment):

Streptozotocin (STZ: Sigma, St. Louis, MO) (a single dose of 200 mg/kg body weight in 0.1 M citrate buffer) was daily injected intra peritoneally to 6-8 weeks old male BALB/c mice, to induce type 1 diabetes. Just the buffer was injected to control mice. Blood glucose levels were examined 7 days after the final STZ injection by obtaining blood from the lateral saphenous vein and measuring glucose concentration with a glucometer (Accu-Check; Roche, Basel, Switzerland). Mice with blood glucose levels greater than 300 mg/dl were considered as diabetic. Study protocol was designed for two series of both diabetes prevention and treatment groups (N=5 per group). In diabetes prevention groups, mice received MSCs and 7 days later they were STZ injected. In treatment groups mice became diabetic and on day 7 after diabetes induction, 5×10^5 MSCs were injected into the tail of different treatment groups (MSCs-TGF- β , MSCs-LV, MSCs).

ELISA for Measuring Serum Insulin:

Serum insulin was quantified by mouse insulin-specific ELISA kit (ALPCO, Salem, NH, USA). Upon euthanasia, whole blood was collected from retro-orbital vein. After clotting the blood samples at room temperature for 10 min, they were centrifuged for 10 min at 4,000 rpm and the sera were isolated. Insulin levels were measured according to the manufacturer's instructions.

Blood Glucose Monitoring:

Using a glucometer, Blood glucose levels were determined by blood samples from the tail tip weekly (Accu-Check; Roche, Basel, Switzerland).

Intraperitoneal Glucose Tolerance Test (IGT):

For glucose tolerance assessment, mice were fasted overnight for at least 14 h and then 2 g/kg body weight glucose were injected intraperitoneally (i.p). Blood glucose was checked every 0, 10, 30, 60, and 120 min after glucose injection.

Mice body weight and survival:

Mice body weight were measured weekly after STZ injection for 5 weeks. Mice survival (5 mice) was recorded until day 80, and the differences were studied using Kaplan-Meier method.

Splenocyte isolation, proliferation and cytokine assays:

On day 35 post diabetes induction, the mice were scarified. The splenocytes were then isolated in aseptic condition, and 4×10^5 cells/well were cultured in 96 well plates containing RPMI-1640 complete medium. For stimulation, 5 μ g/ml PHA was added to each well. 48 hrs later, the splenocytes proliferation was assessed by MTT assay kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Furthermore, the concentrations of IL-4 and IFN- γ in splenocytes supernatant were analyzed by ELISA kit (eBiosciences, Germany), according to the manufacturer's instructions. Tests were done in triplicate manner.

Statistical analysis:

Statistical analysis of data was performed by one-way ANOVA followed by Tukey's test. Kaplan- Meier test was used for survival, and the comparison of groups was determined by Log-rank. Data are presented as mean $\pm$ SD and P value < 0.05 was considered as statistical significance.

RESULTS

Induction of experimental diabetes:

In this study, we expanded a high-dose STZ diabetes model. The concentration of blood glucose was more than 220 mg/dl during 1 week after STZ treatment (Fig. 1A).

Serum Glucose monitoring:

Monitoring of serum glucose was done during 5 weeks after diabetes induction (Fig. 1). Results analysis showed a significant difference between TGF- β /MSCs treated mice including 3-5 weeks for treatment group and 1-2 weeks for both prevention group and diabetic mice with no treatment, (P<0.05). The enhancement of Blood glucose by injecting of MSCs alone or by MSCs transduced lenvti vectors was not statistically significant (P>0.05).

Intraperitoneal Glucose Tolerance (IGT) changes:

In order to detect the clearance of an intraperitoneally (IP) injected glucose load from blood; glucose tolerance test was executed on week five after diabetes induction (Fig. 2). At 30, 60, and 120 min after intraperitoneally glucose injection in treatment group, TGF- β /MSCs treated mice showed a significant increase in the glucose tolerance test compared to untreated diabetic control group ($p < 0.05$). In diabetes prevention group, the clearance of glucose in 60 and 120 min after glucose injection were significantly different in TGF/MSC treated mice comparing untreated diabetic mice ($p < 0.05$).

Serum insulin levels:

On day 35 after diabetes induction, serum insulin levels were evaluated in different mice groups (Fig. 3 A). Serum insulin differences between untreated diabetic mice and all MSCs treated groups were statistically significant ($P < 0.05$). More detailed analysis between groups showed that in both treatment and prevention groups, serum insulin of TGF- β /MSCs treated mice was higher than MSCs/linetiviral vector or MSCs alone injected mice ($P < 0.05$).

Mouse model splenocytes proliferation and cytokines release

On day 35 after diabetes induction, mice splenocytes were harvested and cultured. After 48 hr, the splenocytes proliferation and IFN- γ /IL-4 release were measured (Fig. 3 B-D). Both treatment and prevention groups showed the same pattern of splenocytes responses, while the treatment group showed the higher level of them. In proliferation analysis, although lower proliferation level was observed in untreated diabetic mice comparing the normal mice ($P < 0.05$), the level of proliferation between MSCs/TGF- β and MSCs-LV and MSCs groups were not significantly different ($P > 0.05$). Also, differences between untreated diabetic mice and different groups of MSCs injection were not significant ($P > 0.05$). Untreated diabetic mice released higher levels of IFN- γ and lower levels of IL-4 comparing normal mice ($P < 0.05$). In all MSCs groups (MSCs/TGF- β , MSCs-LV and MSCs), splenocytes produced lower levels of IFN- γ and higher levels of IL-4 comparing untreated diabetic mice ($P < 0.05$). Potency of MSCs/TGF- β treated mice in suppression of IFN- γ release and enhancement of IL-4 production were higher than what was seen in two other MSCs treatment groups (MSCs-LV and MSCs) ($P < 0.05$).

Mice body weight change and survival:

The changes in mice body weight models were monitored during 35 days (Fig. 4) and the survival rates of treated mouse model were studied during 80 days (Fig. 5). Comparing non treated diabetic mice, the body weight of TGF- β /MSCs treated groups was significantly increased from 2nd week in prevention mouse model group and from 3rd week in treatment group ($P < 0.05$). The survival of mouse model was enhanced by TGF- β /MSCs treatment comparing diabetic mice with no treatment ($P < 0.05$).

DISCUSSION

MSCs application for T1D cell therapy is based on self-renewing, transdifferentiation and immunomodulatory properties; while complete and appropriate responses haven't explained yet. To increase the therapeutic potency of MSCs, we engineered MSCs which could over produce TGF- β and then used these cells for T1D prevention or treatment in mouse models. T1D mouse model was produced by large dose of streptozotocin (STZ) injection. STZ is a naturally occurring chemical that is particularly toxic for the insulin-producing beta cells of the pancreas in mammals and thus, is used to produce animal model of T1D in large dose and T2D with multiple low doses (17). Depending on MSCs transfer and STZ injection, the study groups were divided in to two categories: prevention and treatment groups. In prevention group the mice received MSCs and 7 days later STZ was injected, while treatment groups received STZ injection and 7 days later MSCs. Increased levels of blood glucose in MSCs untreated mouse model after 7 days indicated the appropriate induction of T1D in mouse models. More than 300 g/dl blood glucose levels were considered as positive for induced diabetes. Monitoring the blood glucose during 5 weeks after diabetes induction in different study groups showed the significant differences between TGF- β /MSCs treated mice and non treated diabetic mice. These differences were in weeks 1-2 in prevention group and weeks 3-5 in treatment group. In prevention schedule series no blood glucose increase in the first 2 weeks of diabetes induction by TGF- β /MSCs injection indicates the protective ability of these engineered MSCs against diabetes. This prevention ability was not strong, effective and permanent; so that, blood glucose level increased from week 3 and it was not statistically different comparing untreated diabetic mice. In treatment group, blood glucose level diminished slowly after 3rd weeks in TGF- β /MSCs treated mice.

Indicating the potency of engineered MSCs to overcome the induced T1D. These results were completely compatible with goal and logic of study design. After 5th week of diabetes induction, treated mice were evaluated for diabetes symptoms and immune responses changes. Serum insulin levels and intra peritoneal glucose tolerance (IGT) analysis showed the ability of TGF- β /MSCs treatment to return the ability of pancreas for insulin production and clearance of injected glucose from the blood. This ability was more compatible with treatment, whereas preventive effect of engineered MSCs to maintain insulin production was moderate; such that serum insulin levels was significantly lower and the clearance of glucose completed after 60 min post glucose injection. In addition to blood glucose and insulin restoration by TGF- β /MSCs treatment, two common symptoms of T1D including body weight loss and diminished survival were also influenced by this treatment. Protection against body weight loss and increased survival possibility were compatible with previous studies. We evaluated splenocytes proliferation potency and IFN- γ /IL-4 cytokines secretion as cytokine markers of Th1/Th2 immune responses. Overall, the comparison between diabetic mice and normal mice showed the reduced proliferation potency of splenocytes accompanied by higher levels of IFN- γ and lower amount of IL-4 secretion in T1D mice. It is the nature of T1D to suppress the lymphocytes proliferation capacities of induced Th1 (18). Also, increased levels of IFN- γ and suppression of IL-4 production in diabetes have been reported (19). Splenocytes proliferation rate between diabetic mice and MSCs injected mice were not statistically different. Studying the cytokine production showed that, IL-4 production by TGF- β /MSCs transferred mice was higher than MSCs/linetiviral vector or MSCs received groups. Also, release of IFN- γ was dropped by TGF- β /MSCs injection. Both treatment and prevention groups showed the same pattern of splenocytes responses but treatment groups have shown the stronger effects. So our results mentioned the immune regulatory of TGF- β /MSCs. Previous studies showed that MSCs are capable of suppressing the immune responses by inhibiting the maturation of DCs and suppressing the proliferation/function of T cells, B cells, and NK cells (20, 21). Moreover, MSCs studies have showed their ability to induce the expansion of CD4⁺CD25⁺Foxp3⁺ Tregs (22). On the other hand, it has been well described that TGF- β regulates both systemic immune suppression and local immune regulation. TGF- β cooperates with IL-21 to induce CD4⁺ CD25⁺ regulatory Treg cells that counterbalance the effect of IL6, which promotes proinflammatory IL-17-producing T

cells. Furthermore, TGF- β inhibits NK cell function through the modulation of CD4⁺ CD25⁺ regulatory T cells (23). In other studies, transgenic expression of porcine TGF- β 1 active form in pancreatic insulin-secreting β cells, and glucagon-secreting cells could protect NOD mice against diabetes (24, 25). The cooperative functions of MSCs and TGF- β to suppress immune responses are completely compatible with our results. A study showed that TGF- β reduce inflammation to protect MSCs from apoptosis and increase MSCs capacity for nerve regeneration (26). It has been shown that adenovirus-TGF- β transfected MSCs could enhance rabbit articular cartilage defects (27). Also, it was reported that using nonviral vector TGF- β 1-gene engineered MSCs, could induce cartilage regeneration in vivo (28). Here we need to remind that beside beneficial uses of MSCs there have been some concerns about possibility of anutoimmunity induction when using autologous MSCs, possibility of tumor incidence and adverse effects of gene manipulations on MSCs and also bystander cells or cellular connections. Although these concerns are logic thoughts and always should be kept in mind, but several studies examined and demonstrated that there have not been any sign of these adverse effects on their study subjects (29, 30). Also we used lentiviral transduction method as an effective method for engineering but for human and clinical applications more safe methods of engineering should be considered.

Moreover adverse effects of TGF- β such as diabetic nephropathy (DN) effects or atherosclerosis acceleration in diabetes have been reported (31). But in other hands TGF- β signaling stopped by neutralizing antibodies, anti-sense oligonucleotides, and soluble receptors resulted in limited signs of diabetic nephropathy, while importance of TGF- β in immunoregulation remains crucial (32). Also, it has been mentioned that differences in TGF- β effects on diabetic nephropathy is related to sex hormones (33). Such reports remind us to contemplate the adverse effects of TGF- β on diabetes treatments. In case of fate of injected MSCs will be considered based on two aspects of efficacy and safety. Route, dosage and frequency of injections should provide a balance between appropriate distribution and survival and effective function of MSCs and safety issues as immediate concerns as entrapment in the lung or spleen and long term resistance of engineered MSCs (30). Detection of MSCs on spleen and bone marrow after 3 and 7 days after injection and effective immunoregulatory of injected MSCs indicate appropriate administration while long term adverse effects should be monitored in our next steps of studies.

In conclusion, our study showed the cooperative functions of MSCs and TGF- β treatment of T1D. TGF- β /MSCs could restore insulin production and also suppress adverse immune responses. While engineered TGF- β /MSCs was more capable than MSCs alone. Increasing potency of the MSCs by immunoregulatory molecules may be the key of MSCs autoimmune cell therapies effectiveness. By the way probable adverse effects should be evaluated by further applications.

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Figure Legends:

Figure 1: Blood glucose was monitored in MSCs treated and control mice model for 5 weeks in two series of prevention (A) and treatment (B). Blood glucose between normal mice and diabetic mice were significantly different ($P<0.05$). TGF- β /MSCs treated mice blood sugar was significantly diminished since week 3 in treatment mice model series ($P<0.05$), in prevention schedule series in weeks 1-2 for post diabetes induction serum glucose was lower than untreated diabetic mice ($P<0.05$). N=5 mice per group.

Figure 2: Intraperitoneal (IP) Glucose Tolerance (IGT) was monitored in MSCs treated and control mice model on day 35 after diabetes induction both series of prevention (A) and treatment (B). Blood glucose return between normal mice and diabetic mice were significantly different ($P<0.05$). Blood glucose return to baseline after 30 min of IP glucose injection were significantly observed in TGF- β /MSCs treated mice compared with diabetic mice in treatment model series and after 60 min in prevention treatment schedule ($P<0.05$). N=5 mice per group.

Figure 3: Serum insulin levels (A) and mice model splenocytes proliferation (B) and IFN-g (C) and IL-4 (D) cytokines release were determined in MSCs treated and control mice model on day 35 after diabetes induction both series of prevention and treatment. All factors between normal mice and diabetic mice were significantly different ($P<0.05$). To determine function of TGF- β engineering of MSCs, comparison between TGF- β /MSCs and MSCs/linetiviral vector or MSCs alone showed that in both series of Prevention and Treatment schedules production of insulin and IL-4 by TGF- β /MSCs was higher than MSCs/linetiviral vector or MSCs groups ($P<0.05$) and in the same pattern release of IFN-g was lowered ($P<0.05$). Mentioned effects in treatment series were stronger than prevention series ($P<0.05$). N=5 mice per group.

Figure 4: Mice body weight changes were monitored in MSCs treated and control mice model within 5 weeks after diabetes induction both series of Prevention (A) and Treatment (B). Mice body weight of TGF- β /MSCs treated groups were significantly increased since week 2 for Prevention mice model series schedule in compare to non-treated diabetic mice and since week 3 for treatment schedule ($P<0.05$). N=5 mice per group.

Figure 5: Mice model survival was monitored in MSCs treated and control mice model for 80 days after diabetes induction both series of Prevention (A) and Treatment (B). Mice model survival extended by TGF- β /MSCs treatment in compare to diabetic mice with no treatment in both prevention and treatment schedules ($P<0.05$). N=5 mice per group.

A

B

Figure 1:

Serum Glucose monitoring

Figure 2:

Intraperitoneal Glucose Tolerance changes (IGT)

Figure 3:

Serum insulin levels and Mice model splenocytes proliferation and cytokines release

A

B

Figure 4:

Mice body weight change

A

B

Figure 5:

Mice model survival