Original Article

The effect of stem cell from human exfoliated deciduous teeth on T lymphocyte proliferation

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Abstract

Background: Mesenchymal stem cells (MSC), a specific type of adult tissue stem cell; have the immunosuppressive effects that make them valuable targets for regenerative medicine and treatment of many human illnesses. Hence, MSC have been the subject of numerous studies. The classical source of MSC is adult bone marrow (BM). Due to many shortcomings of harvesting MSC from BM, finding the alternative sources for MSC is an urgent. Stem cells from human exfoliated deciduous teeth (SHED) are relative new MSC populations that fulfill these criteria but their potential immunosuppressive effect has not been studied enough yet. Thus, in this work the effect of SHED on the proliferation of *in vitro* activated T lymphocytes were explored.

Materials and Methods: In this study, both mitogen and alloantigen activated T cells were cultured in the presence of different numbers of SHED. In some co-cultures, activated T cells were in direct contact to MSCs and in other co-cultures; they were separated from SHED by a permeable membrane. In all co-cultures, the proliferation of T cells was measured by ELISA Bromodeoxyuridine proliferation assay.

Results: In general, our results showed that SHED significantly suppress the proliferation of activated T cells in a dose-dependent manner. Moreover, the suppression was slightly stronger when MSCs were in physical contact to activated T cells.

Conclusion: This study showed that SHED likewise other MSC populations can suppress the activation of T lymphocytes, which can be used instead of BM derived MSCs in many investigational and clinical applications.

Key Words: Immunosuppression, mesenchymal stem cells, T lymphocytes

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INTRODUCTION

A specific group of adult stems cells which generated a great excitement in the field of Regenerative Medicine, Cell therapy and Tissue engineering is mesenchymal stem cells (MSC).[1,2]

For the first, MSCs were described as a population of adherent, fibroblast-like cells whit the ability of bone, cartilage and muscle differentiation in bone

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marrow (BM).[1,2] Until date MSCs have also been isolated from a variety of other human tissues, including connective tissue, umbilical cord blood, adipose tissue, blood, placenta and dental pulp.[2,3]

MSCs now are identified and isolated by a combination of morphological, phenotypic and functional properties *in vitro*. They are negative for the hematopoietic cell the markers CD45, CD14 and CD34, but they usually express some cell surface molecules such as CD29, CD73, CD90, CD105 and CD166.[3] MSC morphologically have fibroblast-like appearance and under proper conditions the ability to differentiate into various differentiated cells.[1,3,4]

The capacity of multipotent differentiation toward mesodermal tissues, the chemotactic ability[4] and high proliferation potential[5] propose several clinical applications for MSCs. Recent findings suggest that the differentiation range of MSCs expand to ectodermal (neurons) and endodermal (hepatocytes) lineages.[1,4]

Moreover, MSCs have unique immunoproperties that induce great interest for their application in immunosuppression; numerous studies have shown that MSCs evade from immune-recognition and also actively suppress immune reactions.[6] The immunoregulatory of MSCs[7,8] may have a major role in peripheral immunetolerance to autoantigens and allografts, prevention of autoimmunity, tumor immunoevasion as well as in mother-fetus interactions.[7] It has been shown that MSCs suppress the response of both naive and memory T lymphocytes whether stimulated by alloantigens or mitogens *in vitro* and *in vivo*.[6,8] MSCs can reduce the proliferation of B lymphocytes and antibody production of them[1,3] and inhibit the proliferation of NK cells.[6] Moreover, MSCs have effects on other immune cells and cytokine secretion.[2,6]

The mechanism(s) of immune-inhibitory of MSCs have not been known yet; some reports imply that the direct physical contact between MSCs and immune cells is necessary for immunosuppression, whereas others have shown that the immunosuppression depend on soluble factor secretion.[8-10] In general it seems that MSCs exert their immune-inhibitory by both cell to cell contact and soluble factors. Many inhibitory molecules, whether secreted from MSCs or from MSC-affected immune cells, have been reported in the Immunosuppressive setting of MSCs that the important ones including prostaglandin E2, interleukin-10, hepatic growth factor, interferon-γ, transforming growth factor-β and indoleamine 2 3 dioxygenase. However, there are conflicting data about the amount of participation of each factor in the immunosuppression, otherwise the elimination of none completely block the immune-inhibitory of MSCs.[11,12]

Regarding these properties, MSCs are hopeful candidate for various therapeutic applications such as tissue regeneration in autoimmune disease, prevention of graft-versus-host disease after BM transplantation and rejection after solid organ transplantation.[3,13] Therefore MSCs, mostly isolated from BM, have been the subject of many basic and preclinical studies. However, MSCs are in a few numbers in BM and it is not always possible to obtain enough numbers of them, especially in aged or malignant patients.[4,12] Furthermore harvesting MSCs from BM is an invasive and severe process that followed by many pains and difficulties.[2,3] Thus, finding alternative tissue, such as human exfoliated deciduous teeth which is useless and the isolation process inflicts no pain and side-effect, for obtaining MSCs is necessary.

Stem cells from human exfoliated deciduous teeth (SHED) are relatively new stem cell population which recently has been studied for MSC markers and it has been shown that SHED have the properties of MSCs.[14-16] Hence it is expected that SHED can suppress the immune responses, but this immune-inhibitory has not been investigated adequately, only a few studies explored it.[17] In this research, the immunosuppression of SHED, focusing on T lymphocytes as the main cells of immune system was explored.

MATERIALS AND METHODS

The expansion of SHED

A T25 cell culture flask of SHED (passage 2) was generously provided by Torabi negad Research Center (Dental School, Isfahan University of Medical Sciences) for this research. These cells had been isolated from extracted pulp tissues of normal exfoliated deciduous teeth of 6 to 9-year-old children. SHED were subcultured for further passages to be expanded. When SHED got to passage 4, a sufficient amount of them was achieved for the study.

Isolation of T lymphocytes

T lymphocytes were isolated from buffy-coats of a healthy volunteer, using RosetteSep® Human T Cell Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada) according to manufacture protocol. The enriched T cells were washed two times with phosphate buffer saline (PBS) +2% fetal bovine serum. The lymphocytes were suspended in completed Roswell Park Memorial Institute (RPMI1640) (RPMI1640 with L-glutamine (Sigma) supplemented with 10%

fetal calf serum (FCS) (Gibco) and 1% penicillin/streptomycin (Roche)) and T cell suspension was adjusted at 2×10^6 Cells/ml.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by density gradient centrifugation using ficoll lymphopreb solution from heparinized vein blood samples, obtained from healthy volunteer (that was allogenic to one who donated T lymphocytes). The isolated cells then were washed two times with PBS and suspended in RPMI1640 with L-glutamine (Sigma) supplemented with 10% FCS (Gibco) and 1% penicillin/streptomycin (Roche). To stop their proliferation, a final concentration of mitomycin C (Roche) in PBMCs suspension was adjusted at 20 μg/ml. After 30 min incubation at 37°C, the cells were washed with PBS, three times. Finally, a cell suspension of 2×10^6 viable cells/ml was prepared.

Co-cultures

Direct co-cultures

Mixed lymphocyte culture

In these cultures T cells were specifically activated by alloantigens (here allogenic PBMCs) and were co-cultured with SHED. First SHED were trypsinized and with 7.5 μg/ml of mitomycin C (Roche) were incubated for 2 h to be inactivated and prevent proliferation. Then, cells counted and the suspension of 2×10^6 viable cells/ml in Dulbecco's modified Eagle's medium (completed medium) (Sigma) was prepared.

To two four-well sets of a 96 well cell culture plate respectively, 2×10^4, 8×10^3, 4×10^3 and 2×10^3 cells of SHED in final volume of 100 μl were added. Each culture was done in triplicate (collectively, 12 cultures). Another 12-culture set of cultures were done identically as standard control. Moreover, in a well 2×10^4 cells of SHED in 100 μl (for modified mixed lymphocyte culture [mMLC]) were added. To three extra wells, only 100 μl of RPMI (completed medium) were added for controls. These cultures also were done in triplicate. At first, SHED were incubated at 37°C in a 90% humidified atmosphere of 5% CO_2 for 18-20 h to allow cell adhesion and stability. Then, 2×10^5 T cell in final volume 50 μl plus 50 μl of completed medium involved the same number inactivated PBMCs (stimulator cells) were added to primary 12 cultures. As a result, the co-cultures of SHED and MLC with different SHED: T lymphocytes ratios (1:10, 1:25, 1:50, 1:100) were obtained. Only 100 μl of completed medium were added to 12-culture set of standard controls (only SHED). In the well of mMLC, 2×10^5 T cells in 50 μl of completed medium plus 50 μl of completed medium (without stimulator cells) were added. In mMLC, unstimulated T cells were cultured with SHED, exploring whether the stem cells alone can stimulate T lymphocytes or not. To a well of controls, 2×10^5 T lymphocytes plus the same number of inactivated PBMCs in final volume 100 μl were added (positive control; MLC without SHED). To another well of controls, 2×10^5 T lymphocytes in final volume of 100 μl were added (negative control; unstimulated T cell culture). To the last well of controls, only 100 μl of completed medium were added. Finally, the plate was incubated for 96 h in the incubator.

Lymphocyte transformation test

In these cultures T lymphocytes were nonspecifically activated by mitogens (here phytohemagglutinin [PHA]) and were co-cultured with SHED. First SHED were cultured as mentioned about MLCs, except for mMLC in a 96 well cell culture plate that were incubated at 37°C in a 90% humidified atmosphere of 5% CO_2 for 18-20 h for cell adhesion and stability. After this time, 2×10^5 T cell involved PHA (Roche) (at the concentration of 8 μg/ml) in final volume of 100 μl were added to primary 12 cultures. As a result, the co-cultures of SHED and LTTs with different SHED: T lymphocytes ratios (1:10, 1:25, 1:50 and 1:100) were obtained. Only 100 μl of completed medium were added to 12-culture set of standard controls. To a well of controls, 2×10^5 T lymphocytes involved PHA in final volume 100 μl were added (positive control; LTT without SHED). To another well of controls, 2×10^5 T lymphocytes in final volume of 100 μl were added (negative control; unstimulated T cell culture). To the last well of controls, only 100 μl of completed medium were added. Finally, the plate was incubated for 48 h in the incubator.

Indirect co-cultures

For these cultures, ThinCert flat-bottom 24 well cell culture plates were used. In these plates, every well is divided into two chambers by a permeable membrane. The SHED were cultured in lower chambers and stimulated T cells were cultured in upper chambers. The details of doing co-cultures were the same as direct co-cultures, expect all volumes were multiplied 2.5 times. As before mentioned all cultures were in triplicate.

The proliferation of lymphocytes was assayed with a Cell Proliferation ELISA, Bromodeoxyuridine (BrdU) (colorimetric) kit. 20 h before the end of incubation time, BrdU (a pyrimidine analog which is incorporated instead of thymidine into the replicating deoxyribonucleic acid [DNA]) was added to the all cultures at the concentration of 5 μg/ml. After the incubation time, first the cultures were centrifuged; their supernatants were collected and

stored in −80°C. Then, the amount of incorporated BrdU in new-synthesized DNA (that represents the amount of lymphocyte proliferation) as manufacturer's protocol was evaluated through the assessment of the absorbance values of the cultures (ELISA).

Statistical analysis

The data was analyzed using the statistical SPSS (Statistical Package for the Social Sciences) software for Windows, version 16 (SPSS Inc., USA). Statistical significance was calculated using *t*-test analyses and univariate analyze of variance and the $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Stem cells

SHED obtained in Torabi negad Research Center Research, were adherent cells with fibroblast appearance which presented the immunophenotype of MSCs.[18] Their potential of differentiation into osteoblast and chondrocyte were assayed *in vitro* (the unpublished data).

Calculation of proliferation index (stimulation index) and inhibition

To obtain the pure absorbance of proliferated T lymphocytes, the absorbance of each only SHED cultures (with different number of cells) were subtracted from corresponding co-cultures (with the same number of SHED).

For proliferation assay in various cultures, the proliferation (or stimulation) index was calculated as follow:

Proliferation (stimulation) index = The absorbance of the sample/the absorbance of T cells.

SHED suppress the proliferation of T cells

The proliferation indexes for three different culture types were calculated:

- Cultures of (mitogen/alloantigen) stimulated T cells with different number of SHED
- Cultures of unstimulated T cells with SHED (mMLC)
- Cultures of (mitogen/alloantigen) stimulated T cells without SHED (positive controls).

The results are summarized in Table 1. In general, the presence of SHED resulted in a statistically significant decrease in PHA/alloantigen-induced proliferation of T lymphocytes. Although in mMLC, which contained SHED and unstimulated T cells, SHED themselves did not induce T cells and no proliferation of T lymphocytes were observed.

As shown in Figure 1, in these cultures, T lymphocytes were activated specifically by alloantigens (here allogenic PBMNC) or non-specifically by mitogens (here PHA) and cultured with SHED. The same numbers of activated T cells (2×10^5) were cultured with different number of SHED and the T cell proliferation was assayed. As it is seen when SHED: T lymphocytes ratio increased, the mean of T cell proliferation is decreased. "None" refer to the culture of activated T lymphocytes alone, without SHED (positive control) and "mMLC" refer to the culture of unstimulated T lymphocytes with SHED. (*) represent the statically significant different ($P < 0.05$) between each group by its previous and next groups.

SHED significantly impaired stimulated T cell proliferation in a dose-dependent manner. This means that the more number of SHED in co-cultures caused the more decrease in T cell proliferation. These results are seen well in both LTT and MLC cultures [Table 1]. Table 1 represents the means of proliferation indexes of (mitogen/alloantigen) stimulated T cells in the presence of different

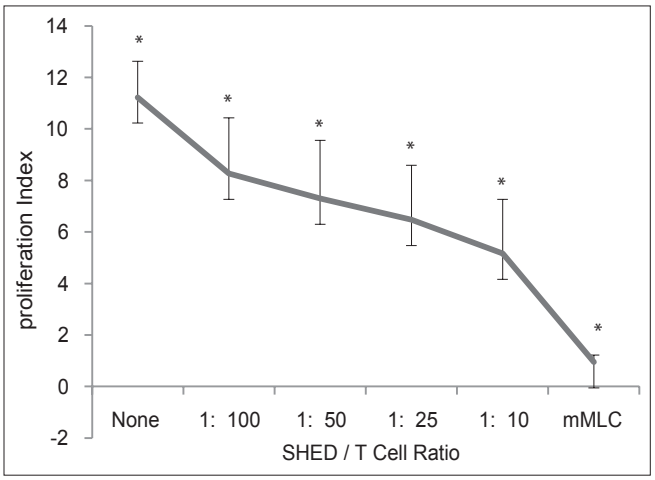

Figure 1: Stem cell from human exfoliated deciduous teeth inhibit the proliferation of activated T lymphocytes

Table 1: The proliferation of activated T lymphocytes in MLC and LTT cocultures

SHED/ T cell ratio	Proliferation index		
	LTT	MLC	Total
1:100	9.83±2.95	7.29±0.48	8.27±2.16
1:50	8.37±3.17	6.63±1.27	7.30±2.25
1:25	7.73±2.12	5.69±1.79	6.47±2.11
1:10	6.65±1.94	4.24±1.69	5.16±2.1
mMLC	-	0.95±0.2712	0.95±0.2712
None	12.32±1.12	10.54±1.11	11.22±1.4
P value	0.00*	0.00*	0.00*

*Stand for the statically significant different ($P<0.05$) between each group by its previous and next groups. MLC: Mixed lymphocyte culture, LTT: Lymphocyte transformation test, mMLC: Modified mixed lymphocyte culture, SHED: Stem cells from human exfoliated deciduous teeth

numbers of SHED (three repeats), with their standard deviation (SEM).

Table 1 represents the mean of proliferation indexes of triplicate cultures of T lymphocytes which were activated specifically by alloantigens (here allogenic PBMNC; MLCs) and nonspecifically by mitogens (here Phytohaemagglutinin; LTTs) with different number of SHED, separately. In both types of co-cultures, the same numbers of activated T cells (2×10^5) were cultured with different number of SHED and the T cell proliferation was assayed. As it is seen when SHED: T lymphocytes ratio increased, the mean of T cell proliferation is decreased. "None" refer to the culture of activated T lymphocytes alone, without SHED (positive control) and "mMLC" refer to the culture of unstimulated T lymphocytes with SHED.

SHED suppress the proliferation of T cells in direct and indirect cultures

The proliferation indexes of activated T cell cultures in usual cultures (direct contact to SHED) and transwell cultures (that T cells are isolated from SHED by a permeable membrane; indirect contact to SHED) are shown in Table 2. Table shows the means of stimulation indexes of (mitogen/alloantigen) stimulated T cells in the presence of different numbers of SHED (three repeats), with their standard deviation (SEM).

Statistical analysis revealed no significant difference between the mean proliferation of T cells in direct and indirect contact to SHED in general.

As shown in Table 2, the mean of calculated proliferation indexes of triplicate co-cultures of PHA-activated T lymphocytes which were in direct physical contact or indirect contact to SHED in the first column; and the same parameters for alloantigen (allogenic PBMNC)-activated T lymphocytes are shown in second column. The last column represents calculated proliferation indexes of activated T lymphocytes which were in direct or indirect contact to SHED, in general (without regard to the type of activation). As it is seen the difference between T cell proliferation in direct or indirect contact to SHED, whether stimulated by alloantigens or mitogen (PHA) – also in converse manner - were significant. However in general the difference is not significant.

However, the statistically significant difference between the proliferation indexes of each individual culture type (LTT or MLC) in direct and indirect contact to SHED was detected and the most inhibition was seen for MLCs in direct contact but, for LTTs in indirect contact to SHED.

Table 2: SHED inhibit the proliferation of activated T cells in both direct and indirect contact

Contact	Proliferation index		
	LTT	MLC	Total
Direct	9.99±1.58	5.41±1.66	6.94±2.72
Indirect	5.37±0.86	7.61±0.91	6.49±1.43
P value	0.00*	0.00*	0.57

*Stand for the statically significant different ($P<0.05$) between each group by its previous and next groups. MLC: Mixed lymphocyte culture, LTT: Lymphocyte transformation test, SHED: Stem cells from human exfoliated deciduous teeth

DISCUSSION

Abundant reports have ever demonstrated that MSCs are able to suppress an ongoing immune response by inhibiting the stimulated T-cell proliferation. SHED are a relative new cell population of MSCs that their potential immunosuppressive effect and their interaction with immune cells has not been studied yet, as much as necessary. Therefore, we examined the MSC-mediated immunosuppressive effect of the SHED.

In this study, SHED were co-cultured with *in vitro* stimulated T lymphocytes isolated from a volunteer. The results showed that SHED, similar to MSCs from other sources such as bone marrow derived Mesenchymal stem cells (BMMSC),[19,20] Adipose derived Stem Cells (ADSC),[21,22] umbilical cord blood (UCB)-derived MSC,[23] placenta-derived MSC,[24] periodontal ligament stem cells and dental pulp stem cells[25] could suppress the proliferation of activated T cells. Though potentially with different mechanism, MSC has been showed that suppress the proliferation of mitogen activated T cells and either T lymphocytes stimulated by alloantigens.[19,24] According to our results, SHED inhibited the proliferation of both PHA-stimulated T lymphocytes (in LTT cultures) and T cells activated by allogeneic PBMCs (in MLC cultures).

Additionally, in this experiment, SHED co-cultured with equal number of unstimulated T lymphocytes (in mMLCs) could not elicit the proliferation of allogeneic T cells. This result is in agreement with several other reports about MSC derived from different tissues.[19,22] However, Le Blanc,[26] Potian[27] and Prasanna[28] displayed MSC as immunostimulatory cells that induce lymphoproliferation when co-cultured with allogeneic unstimulated lymphocytes. It is probably that the different reports about the immunogenicity of MSCs are due to different culture conditions.[29,30]

Many scientists have reported that the immunosuppression of MSC is dose-dependent.[25,31,32] This means that with the reduction of the number of MSC present in contact to lymphocytes, the inhibition of activated T cells diminishes. However, the ratio of MSC/lymphocytes in which the suppression disappears

totally and the amount of the suppression in various ratios differs noticeably in diverse studies; as reported by Yanez *et al*.[21] at MSC/lymphocyte ratio of 1/100, the inhibitory effect on lymphocyte proliferation is undetectable anymore. Other researchers often considered higher ratios.[23,33] The reports implying the immunosuppressive effects of MSC at 1/100 ratio or lower are restricted to studies on murine MSC.[34] However, Beyth *et al*.[32] demonstrated a large repression of human BMMSC at 1/100 ratio. In the present work, we detect a significant proliferation inhibition at SHED to T cell ratio of 1/100.

It is remarkable that even in the studies demonstrating the immunostimulatory effect of MSC,[26,27,35] these cells inhibited the activated lymphocytes at a define ratio ranges, but in lower ratios (1/1000 and 1/10000) increased the proliferation of stimulated lymphocytes.

Another parameter that has been considered in immunosuppressive of MSC is the cell contact. Numerous researchers have demonstrated the inhibitory function of MSC do not necessarily require the cell-to-cell contact (direct contact) of MSC and lymphocytes, although the suppression is reduced somewhat by the physical separation of MSCs and immune cells (indirect contact), this have been shown about MSC from different sources.[19,32,33]

In our study, the results obtained from MLCs co-cultured with SHED showed a more inhibition of T cell proliferation in usual co-cultures (direct contact) than transwell co-cultures that is consistent with the stated reports.[19,21,36]

It has been ever revealed that the immunosuppressive effect of MSC may exert by the cell-to-cell interactions[4,5] and either by soluble factors secreted by MSC or lymphocytes-affected MSC,[5,21,25,37] Therefore, it is conceivable that the physical separation of lymphocytes and MSC, eliminating the cell-to-cell dependent mechanisms, reduce the suppression. The impairment of inhibitory effect by a transwell membrane could also be explained by the dilution of the inhibitory factor because of the distance between stem cells and lymphocytes. Moreover, if the factors is unstable,[30,38] it could be destroyed before reaching its target.

Otherwise, some investigators reported that lymphocyte proliferation was not affected by the physical separation of the two populations; Yang *et al*.[39] worked on murine MSC and showed that MSC culture supernatant had the same suppressive activity as the MSCs themselves, Dela Rosa *et al*.[22] have reported that ADSC mediated suppression of CD8 + T cells (but not CD4 + T cells) in transwell conditions was the same to contact conditions, Krampera *et al*.[7] observed no variation in the inhibitory effect of BMMSC on CD4 + T cells in direct and indirect contact to stem cells. Our results in the whole (MLCs and LTTs together) indicate that the suppression of T cell proliferation by SHED is independent of physical contact. Interestingly, in this study the maximum inhibition of T cell proliferation in LTT co-cultures was observed when SHED physically were separated from T lymphocytes (in transwell contact).

We could not find any report including mitogen-stimulated T cells and MSC, resemble to our results and also no other study that explored the immunosuppression of SHED on mitogen stimulated lymphocytes. However, this is not the first report suggests that the removal of physical contact results in stronger MSC-mediated suppression; in 2005 Puissant *et al*.[40] compared the immunomodulation of ADSC and BMMSC and observed that the inhibitory effect of ADSC on proliferation in transwell MLCs (but not LTTs) was less than usual MLC cultures. In addition, several studies[28] exist to demonstrate that differently stimulated T lymphocytes are variously influenced by MSC. Moreover, investigations has established that MSC from different tissue origins share in many fractures but are not identical and have individual characteristics.[27]

In the other hand, although the stimulation of lymphocytes with mitogen or alloantigen results in similar process (proliferation and cytokine production), but they are essentially different; alloantigens activate T cells in a specific manner and need the costimulatory molecules. Whereas mitogens induce proliferation whiteout need to costimulatory molecules and in a polyclonal manner.[41] Thus, SHED as a MSC population, may affected differently activated lymphocytes by their own various mechanisms.

In the general this study showed that SHED likewise other MSC populations can suppress the activation of T lymphocytes, which can be used instead of BM derived MSCs in many investigational and clinical applications. Needless to say, further investigations should be done to confirm these results and to be explained the mechanisms.

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