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ABSTRACT

Discoveries of immunomodulatory functions in mesenchymal stem cells (MSCs) have suggested that they might have therapeutic utility in treating immune diseases. Recently, a novel MSC population was identified from dental pulp of human supernumerary teeth, and its multipotency characterized. Herein, we first examined the *in vitro* and *in vivo* immunomodulatory functions of human supernumerary tooth-derived stem cells (SNTSCs). SNTSCs suppressed not only the viability of T-cells, but also the differentiation of interleukin 17 (IL-17)-secreting helper T (Th17) -cells in *in vitro* co-culture experiments. In addition, systemic SNTSC transplantation ameliorated the shortened lifespan and elevated serum autoantibodies and nephritis-like renal dysfunction in systemic lupus erythematosus (SLE) model MRL/lpr mice. SNTSC transplantation also suppressed *in vivo* increased levels of peripheral Th17 cells and IL-17, as well as *ex vivo* differentiation of Th17 cells in MRL/lpr mice. Adoptive transfer experiments demonstrated that SNTSC-transplanted MRL/lpr mouse-derived T-cell-adopted immunocompromised mice showed a longer lifespan in comparison with non-transplanted MRL/lpr mouse-derived T-cell-adopted immunocompromised mice, indicating that SNTSC transplantation suppresses the hyper-immune condition of MRL/lpr mice through suppressing T-cells. Analysis of these data suggests that SNTSCs are a promising MSC source for cell-based therapy for immune diseases such as SLE.

KEY WORDS: supernumerary teeth, dental pulp, mesenchymal stem cells, T-cells, immunotherapy, systemic lupus erythematosus.

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Immune Therapeutic Potential of Stem Cells from Human Supernumerary Teeth

INTRODUCTION

Immunomodulatory functions of human mesenchymal stem cells (MSCs), which are capable of differentiating into multi-lineage cells (Bianco *et al.*, 2001), have been discovered against a variety of immune cells such as T- and B-lymphocytes and dendritic cells (Corcione *et al.*, 2006; Spaggiari *et al.*, 2006; Ramasamy *et al.*, 2007) and have offered MSC-based cell therapy to several immune diseases such as acute graft-vs.-host-disease (GVHD; Le Blanc *et al.*, 2004) and systemic lupus erythematosus (SLE; Sun *et al.*, 2009). Human oral tissues serve a variety of unique MSC populations such as dental pulp stem cells, stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells, stem cells from root apical papilla, and gingival stem cells (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2004; Sonoyama *et al.*, 2006; Zhang *et al.*, 2009). These oral MSCs also express an immunosuppressive capacity to T-cells (Wada *et al.*, 2009; Zhang *et al.*, 2009; Yamaza *et al.*, 2010; Zhao *et al.*, 2012) and provide a possible treatment for immune diseases (Zhang *et al.*, 2009; Yamaza *et al.*, 2010; Zhao *et al.*, 2012).

Recently, a new MSC population has been identified from the dental pulp of human supernumerary teeth, and its capability for colony formation and multi-differentiation has been characterized (Huang *et al.*, 2008). However, no one has evaluated whether the supernumerary tooth-derived stem cells (SNTSCs) possess an immunomodulatory capacity and offer therapeutic efficacy for immune diseases. In this study, we examined an *in vitro* immunoregulatory property of SNTSCs for T-cells and show an *in vivo* immune effect of SNTSCs in human SLE model MRL/lpr mice.

MATERIALS & METHODS

Source of Supernumerary Teeth

Human maxillary supernumerary teeth, mesiodens, were obtained as clinically discarded biological samples from five patients (from 5 to 7 yrs old) with their parents' informed consent at the Department of Pediatric Dentistry of Kyushu University Hospital, according to approved Institutional Review Board guidelines (Kyushu University, Protocol number: 393-01).

Antibodies and Reagents

All antibodies and reagents used in this study are described in the Appendix.

Mice

Immunocompromised NOD SCID mice (female, 8-week-old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). C57BL/6 and C57BL/6J-*lpr/lpr* (MRL/*lpr*) mice (female, 8-week-old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were performed under an institutionally approved protocol for the care and use of laboratory animals (Kyushu University, Protocol number: A21-044-1).

Culture of SNTSCs and hBMSCs

SNTSCs isolated from the dental pulp tissues of supernumerary teeth were cultured as described previously (Yamaza *et al.*, 2010) and as in the Appendix. hBMSC were isolated as described previously (Yamaza *et al.*, 2009) and cultured as in the Appendix.

In vitro Immunomodulatory Assay

T-cell Survival Assay

SNTSCs or hBMSCs were co-cultured with phytohemagglutinin (PHA)- or anti-human CD3 antibody-activated human peripheral blood mononuclear cells (PBMNCs) as described in the Appendix. The cell viability and apoptosis of T-cells were analyzed as described in the Appendix.

Induction of Interleukin 17 (IL-17)-secreting Helper T (Th17) -cells and Regulatory T-cells (Tregs)

Induction and analysis of Th17 cells and Tregs co-cultured with SNTSCs or hBMSCs are described in the Appendix.

Assay of SNTSC-treated MRL/lpr Mice

Cultured SNTSCs or hBMSCs ($0.1 \times 10^6/10$ g body weight/100 μ L PBS) were intravenously transplanted into MRL/*lpr* mice at the age of 16 wks as described previously (Sun *et al.*, 2009; Yamaza *et al.*, 2010). The therapeutic efficacy on the mice was assessed as described in the Appendix.

In vivo Tracing of SNTSCs

The distribution of transplanted SNTSCs into MRL/*lpr* mice was assayed as described previously (Ma *et al.*, 2012) and as in the Appendix.

Culture and Cell Viability Assay of Mouse PanT-cells

PanT-cells from mouse spleen were cultured and assayed as in the Appendix.

Adoptive Transfer of Mouse PanT-cells into SCID Mice

Mouse PanT-cells ($10 \times 10^6/\text{mouse}/100$ μ L PBS) were intravenously infused into SCID mice and their survival assayed as described in the Appendix.

Statistical Analysis

Data were assayed by a one-way ANOVA F test. *P* values less than .05 were considered significant.

RESULTS

SNTSCs Display MSC Properties

Cells isolated from the dental pulp of supernumerary teeth were able to develop attached colonies consisting of fibroblastic cells on plastic dishes (Appendix Fig. 1A). The colonies expressed different sizes and various densities. The colony-forming efficiency was 88.0 ± 2.0 (means $\pm$ SD, $n = 5$) *per* 1×10^6 . The frequency of colony formation was significantly increased depending on the number of plating cell densities (Appendix Fig. 1B). SNTSCs exhibited *in vitro* prolonged, but limited, cell proliferation (total population-doubling score: 65.4 ± 3.2 , $n = 5$) by population-doubling assay. Bromodeoxyuridine (BrdU) was largely incorporated into the nuclei of SNTSCs ($74.1 \pm 4.0\%$, $n = 5$). Flow cytometry demonstrated that SNTSCs were negative to hematopoietic cell markers CD34, CD45, and CD14 and positive to MSC markers CD73 ($99.7 \pm 0.3\%$), CD105 (97.5 ± 1.7), and CD90 ($99.8 \pm 0.1\%$) and an embryonic stem cell marker stage-specific embryonic antigen 4 ($27.3 \pm 1.6\%$) ($n = 5$) (Appendix Fig. 1C). SNTSCs also expressed genes for both ES cell markers, *NANOG* and *octamer 4*, and neural crest cell markers, *NOTCH1*, *NESTIN*, and *p75* (Appendix Fig. 1D).

In dentinogenic/osteogenic conditions, the SNTSCs were capable of forming mineralized tissues and expressed odontoblast-/osteoblast-specific genes (*runt-related transcription factor 2*, *alkaline phosphatase*, *osteocalcin*, and *dentin sialophosphoprotein*) by Alizarin red staining and reverse-transcription/polymerase chain-reaction (RT-PCR) assay (Appendix Fig. 2A). Immunofluorescence showed that SNTSCs expressed neural cell markers glial fibrillary acidic protein, neurofilament M, and tubulin β III (Appendix Fig. 2B) and endothelial cell markers CD31 and CD34 (Appendix Fig. 2C) under neural and endothelial cell differentiation conditions, respectively.

Ten clonogenic CFU-F were obtained from the dental pulp tissue of a supernumerary tooth by a limited dilution method. The single-colony-derived SNTSCs displayed different population-doubling scores, various BrdU incorporation capacities, and various Alizarin-red-positive area formations (Appendix Fig. 3).

Immunomodulatory Capacity of SNTSCs in vitro

To examine the *in vitro* immunomodulatory effects of SNTSCs, we co-cultured SNTSCs with human PBMNCs or T-cells. SNTSCs inhibited the cell viability of PHA-stimulated human PBMNCs in an increased SNTSC ratio-dependent manner (Fig. 1A) and induced Annexin-V⁺7AAD⁺ apoptotic cells of anti-CD3 antibody-activated human PBMNCs (Fig. 1B). In a Th17-cell differentiation condition, SNTSCs inhibited the differentiation of CD4⁺IL-17⁺interferon-gamma (IFN γ)⁺ Th17 cells (Fig. 1C) and the secretion of IL-17 (Fig. 1C). Conversely, SNTSCs enhanced the differentiation of CD4⁺CD25⁺Foxp3⁺ cells (Fig. 1D) and IL-10 secretion (Fig. 1D) in a Treg differentiation condition. SNTSCs expressed higher immunomodulatory functions than hBMSCs (Fig. 1). Further studies will be necessary to examine in more detail the immunomodulatory capacities of SNTSCs, including T-cell proliferation and immune cell differentiation.

Systemic SNTSC Transplantation Improves SLE-like Disorders in MRL/lpr Mice

SLE patients have received allogenic 1×10^6 MSCs/kg body weight and experienced subsequent therapeutic efficacy and safety (Sun *et al.*, 2009, 2010; Wang *et al.*, 2013). To examine the *in vivo* immunomodulatory effects of SNTSCs, we preliminarily challenged 16-week-old MRL/lpr mice with 4 SNTSC doses (0.001 , 0.01 , 0.1 , and $1.0 \times 10^6/10$ g body weight) *via* systemic transplantation. The dose of $0.1 \times 10^6/10$ g ($10 \times 10^6/\text{kg}$) showed the most effective therapeutic efficacy on serum anti-nuclear antigen (ANA) and urine protein in MRL/lpr mice 4 wks after transplantation (unpublished observations) and was used as the optimal cell dose in this study. The discrepancy between optimal cell doses for human and mouse recipients may relate to the differences of factors such as graft type (allogenic or xenogenic), recipient species (human or mouse), and recipient background (drug pre-treatment patients or CD95 mutation mice).

SNTSCs ($0.1 \times 10^6/10$ g) were systemically transplanted into 16-week-old MRL/lpr mice (Fig. 2A). SNTSC transplantation showed a significant prolongation of the shortened lifespan of MRL/lpr mice compared with that of non-transplanted MRL/lpr mice ($p < .005$) (Fig. 2B). By the Mantel-Haenszel test, the median survival times were found to be 216, 210, and 162 days in the SNTSC-, hBMMSC-, and non-transplanted groups, respectively.

MRL/lpr mice at the age of 20 wks expressed over-production of ANA and anti-dsDNA IgG and IgM antibodies compared with age-matched C57BL/6 mice (Fig. 2C). Non-transplanted MRL/lpr mice at 20 wks showed typical histological glomerulonephritis, including compression of the glomerular capillary, thickness of the capillary basal membrane, and deposition of collagen and IgG in the mesangium (Fig. 2D), and biochemical glomerulonephritis, including increased urine protein and serum creatinine and reduced serum albumin (Fig. 2E) when compared with those of control wild-type C57BL/6 mice. In contrast, systemic transplantation of SNTSC significantly reduced the elevated serum levels of the autoantibodies in MRL/lpr mice at 20 wks. The SNTSC transplantation unpacked the capillary compression, reduced the hyperthickness of the basement membrane, and diminished the deposition of collagen and IgG in the glomerulus (Fig. 2D). The levels of urine protein, serum albumin, and serum creatinine were recovered after the SNTC transplantation into MRL/lpr mice (Fig. 2E). The therapeutic effects

Figure 1. Immunosuppressive effects of SNTSCs on human T-cells. (A) Inhibition of cell viability of PHA-activated human PBMNCs (PHA-PBMNC). (B) AnnexinV+7AAD+ apoptotic cells of CD3 and anti-CD28 antibody-activated T-cells by flow cytometry. (C) Suppression of CD4+IL-17+IFN γ (Th17) cell differentiation by flow cytometry and IL-17 secretion in the culture supernatants (Sup IL-17) by ELISA. T: anti-CD3 and anti-CD28 antibody-activated CD4+CD25+ T-cells. (D) Induction of CD4+CD25+Foxp3+ cell (Treg) differentiation by flow cytometry and IL-10 secretion in the culture supernatants (Sup IL-10) by ELISA. T: anti-CD3 and anti-CD28 antibody-activated CD4+CD25+ T-cells. (A-D) $n = 5$ for all groups. * $p < .05$, *** $p < .005$. The bar graph represents mean $\pm$ SD.

in the SNTSC group were similar to those in the hBMMSC-transplant group (Figs. 2C-2E).

SNTSC transplantation Suppresses Hyper-immunoreactivity of T-cells, Especially Th17 Cells, in MRL/lpr Mice

In 20-week-old MRL/lpr mice, CD4+IL-17+IFN γ Th17 cells and IL-17 were significantly increased, and CD4+CD25+Foxp3+ Tregs and IL-10 were reduced in the peripheral blood (Figs. 3A, 3B), and the ratio of Tregs/Th17 cells was markedly decreased (Fig. 3A) in comparison with that in the age-matched control C57BL/6 mice. Systemic transplantation of SNTSCs affected the recovery of the Treg/Th17 cell ratio, with the down-regulation of Th17 cell function and the up-regulation of Treg function. The immune efficacy of SNTSC transplantation was higher than that in the hBMMSC-transplanted group (Fig. 3).

Figure 2. Systemic SNTSC transplantation improves the lifespan and SLE-like disorders in MRL/lpr mice. **(A)** A scheme of systemic MSC transplantation into MRL/lpr mice. **(B)** Kaplan-Meier survival curve of MRL/lpr mice. **(C)** ELISA of ANA (serum ANA), anti-dsDNA IgG (serum dsDNA IgG), and anti-dsDNA IgM (serum dsDNA IgM) antibodies in the peripheral blood serum. **(D)** Representative images of kidneys. HE, hematoxylin and eosin staining; TC, Gomori trichrome staining; PAS, Periodic acid-Schiff staining. IgG/DAPI: Immunofluorescence of mouse IgG. Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI). Dot-circled area, G: glomerular. Bars = 50 μ m. **(E)** Levels of urine protein, serum albumin, and creatinine. **(B-E)** BL/6, C57BL/6 group; MRL/lpr, non-transplanted MRL/lpr group; hBMMSC, hBMMSC-transplanted MRL/lpr group; and SNTSC, SNTSC-transplanted MRL/lpr group. (B) $n = 7$, (C-E) $n = 5$ for all groups. (C, E) * $p < .05$, ** $p < .01$, *** $p < .005$. The bar graph represents mean $\pm$ SD.

Systemically Infused SNTSCs Localize to Spleen and Kidney in MRL/lpr Mice and Regulate the Local Immune Environment

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled SNTSCs were intravenously injected into 16-week-old MRL/lpr mice. The high frequency of CFSE-labeled SNTSCs was observed in the spleen and kidneys, particularly in the glomeruli, one day after the transplantation (Fig. 4A). The frequency of CFSE-labeled SNTSCs decreased gradually in both tissues from the 7th day after transplantation (Fig. 4A). The distribution of SNTSCs was similar to that of hBMMSCs (Fig. 4A). The levels of inflammatory cytokine IL-17 in the spleen and kidneys were significantly reduced in SNTSC- and hBMMSC-transfused MRL/lpr mice when compared with those in control MRL/lpr mice (Fig. 4B).

To analyze T-cell toxicity in MRL/lpr mice, we isolated splenic T-cells from non-, SNTSC-, and hBMMSC-transplanted MRL/lpr mice, termed T-MRL/lpr, T-SNTSC, and T-hBMMSC, respectively, and stimulated them for 3 days with anti-CD3 and anti-CD28 antibodies. Supernatant levels of IL-17 of T-SNTSC and T-hBMMSC cultures were markedly lower than those of T-MRL/lpr (Figure 4C). We transplanted these T-cells into SCID mice (Fraziano *et al.*, 1994) and assessed *in vivo* survival damage. The transplantation of T-SNTSC- and T-hBMMSC extended the shortened lifespan of T-MRL/lpr-transplanted mice (Fig. 4D). The median survival time was significantly prolonged in T-SNTSC- (37 days, $p < .005$) and T-hBMMSC- (36 days, $p < .005$) transplanted groups when compared with the T-MRL/lpr-transplanted group (10 days).

DISCUSSION

To date, hBMMSC therapy has enjoyed substantial success in regenerative medicine (Le Blanc *et al.*, 2004; Sun *et al.*, 2009, 2010; Wang *et al.*, 2013). In contrast, human bone marrow has been shown to have not only invasive harvesting problems but also donor age-related problems, associated with harvestable stem cell numbers, *ex vivo* expansion, and potential (Stolzinger *et al.*, 2008). These problems represent significant difficulties for the utilization of hBMMSCs in regenerative medicine. Various MSC candidates have been identified from umbilical cord blood (Erices *et al.*, 2000) and adipose tissues (Zuk *et al.*, 2001). Present and current studies (Huang *et al.*, 2008) demonstrated

Figure 3. SNTSC transplantation modulates peripheral Treg/Th17 cell balance in MRL/lpr mice. **(A)** Flow cytometry of peripheral CD4⁺IL-17⁺IFN γ (Th17) cells and CD4⁺CD25⁺Foxp3⁺ cells (Tregs) and the ratio of Tregs/Th17 cells (Tregs/Th17) in MRL/lpr mice. **(B)** Serum levels of IL-17 and IL-10 in MRL/lpr mice by ELISA.

the existence of multipotential MSCs in the dental pulp of supernumerary teeth. Since supernumerary teeth cause esthetic, oral hygiene, and masticatory problems, they are usually extracted and discarded clinically. Therefore, supernumerary teeth might be a promising stem cell source with minimal ethical issues in regenerative medicine.

SLE has been known to be a typically fatal autoimmune disease with autoantibody-caused multiple organ disorders including lupus nephritis. Allogenic transplantation of hBMMSCs and human umbilical-cord-derived MSCs (UCMSCs) has shown successful therapeutic efficacy in refractory SLE patients (Sun *et al.*, 2009, 2010; Wang *et al.*, 2013). MRL/lpr mice show SLE-like disorders, including shortened lifespan, abundant autoantibodies, glomerulonephritis, and a breakdown of self-tolerance similar to that of human SLE patients (Theofilopoulos and Dixon, 1985). Currently, systemic transplantation of human MSCs, including BMMSCs, UCMSCs, and SHED, improves SLE-like disorders in MRL/lpr mice (Zhou *et al.*, 2008; Gu *et al.*, 2010; Yamaza *et al.*, 2010), indicating that the xenogenic transplantation of human MSCs into MRL/lpr mice might be a novel discovery of a new cell-based

Figure 4. SNTSC transplantation suppresses hyper-activity of Th17 cells in MRL/lpr mice. **(A)** Homing of systemically infused CFSE-labeled SNTSCs (CFSE-SNTSC) and hBMMSCs (CFSE-hBMMSC) to spleen and kidney of MRL/lpr Mice after 1- (Day 1) or 7- (Day 7) day transplantation. Dot-circled area, G: glomerular. CFSE, CFSE staining images; DAPI, DAPI staining images; Merged, Merged images. Bar = 50 μ m. **(B)** IL-17 levels in spleen (Spleen IL-17) and kidney (Kidney IL-17). **(C)** IL-17 level in the culture supernatants of splenic T-cells (Spleen-T IL-17) isolated from MRL/lpr mice. **(D)** Adoptive transfer of T-cells from MRL/lpr mice. A scheme of adoptive transfer of Spleen-T into SCID mice. Kaplan-Meier survival curve of SCID mice. (A-C) $n = 5$. (D) $n = 7$ for all groups. (B) BL/6, C57BL/6 group; MRL/lpr, non-transplanted group; hBMMSC, hBMMSC-transplanted group; and SNTSC, SNTSC-transplanted group. (C) T-MRL/lpr, T-hBMMSC, T-SNTSC: Spleen-T from non-transplanted, hBMMSC-transplanted, and SNTSC-transplanted groups, respectively. (D) Control, T-MRL/lpr-T, T-hBMMSC-T, T-SNTSC-T: non-, T-MRL/lpr-, T-hBMMSC-, and T-SNTSC-adoptive transferred groups, respectively. (B, C) $*p < .05$, $**p < .01$, $***p < .005$. The bar graph represents mean $\pm$ SD.

therapeutic approach for human SLE. In this study, systemically transplanted SNTSCs reduced hyperproduction of autoantibodies and recovered renal dysfunction in MRL/lpr mice. Therefore, analysis of these data suggests that SNTSCs might have a therapeutic benefit for SLE disorders.

The immunoregulatory mechanisms of MSCs are associated with both a paracrine effect of MSC-producing factors [indoleamine 2,3-dioxygenase, IL-10, transforming growth factor- β 1, and tumor necrosis factor- α (TNF α)-stimulated gene/protein 6, etc.] and a cell-contact-dependent mechanism (Jagged1/Notch1, Fas/Fas ligand and programmed death-1/programmed death-ligand 1 pathways) (DelaRosa *et al.*, 2012; English, 2013). MSCs also induce Tregs and inhibit Th17 cells to display immune tolerance by shifting the balance of Tregs/Th17 cells (Sun *et al.*, 2009; Yamaza *et al.*, 2010). The immunoregulatory properties of MSCs are primed by IFN γ , IL-1 β , and TNF α released from activated immune cells (Krampera *et al.*, 2006; Prasanna *et al.*, 2010). The present systemic transplantation experiment, as well as these co-culture experiments, demonstrated that transplanted SNTSCs regulate the disturbed immune system in MRL/lpr mice. In addition, the present adoptive transfer experiment supports *in vivo* immunosuppressive effects of systemically infused SNTSCs on hyper-activated T-cells in MRL/lpr mice. Infused SNTSCs homed in on local lesions, including the kidney and spleen of MRL/lpr mice, might control systemic and regional immune conditions through local inflammatory environments. However, the *in vivo* detailed mechanism of SNTSC-mediated immunomodulation is not fully understood. Therefore, further studies will be necessary to elucidate the detailed immunoregulatory mechanisms of SNTSC-based immunotherapy.

In conclusion, the present study evaluates, at least partially, the *in vitro* immunomodulatory functions of SNTSCs on T-cells. Moreover, systemic SNTSC transplantation ameliorates SLE disorders *via* suppressing hyper-immune reaction in human SLE-like model MRL/lpr mice. Analysis of these data suggests that SNTSCs offer an accessible and executable MSC source for cell-based immune therapies for human autoimmune diseases.

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