Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells

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Abstract

Introduction: Human dental pulp stem/progenitor cells (DPSCs) show remarkable regenerative potential in vivo. During regeneration, DPSCs may interact with their inflammatory environment via toll-like receptors (TLRs). The present study aimed to depict for the first time the TLR expression profile of DPSCs. Methods: Cells were isolated from human dental pulp, STRO-1–immunomagnetically sorted, and seeded out to obtain single colony-forming units. DPSCs were characterized for CD14, CD34, CD45, CD73, CD90, CD105, and CD146 expression and for their multilineage differentiation potential. After incubation of DPSCs in basic or inflammatory medium (interleukin-1β, interferon-γ, interferon-α, tumor necrosis factor-α), TLR expression profiles were generated (DPSCs and DPSCs-i). Results: DPSCs showed all characteristics of stem/progenitor cells. In basic medium DPSCs expressed TLRs 1–10 in different quantities. The inflammatory medium upregulated the expression of TLRs 2, 3, 4, 5, and 8, downregulated TLRs 1, 7, 9, and 10, and abolished TLR6. Conclusions: The current study describes for the first time the distinctive TLR expression profile of DPSCs in uninflamed and inflamed conditions. (J Endod 2016;42:413–417)

Key Words

Flow cytometry, polymerase chain reaction, pulp, stem cells, TLR

The pulp-dentin complex stems embryonically from the multipotent neural crest—ectomesenchyme and constitutes a functional and physiological unit (1). As a highly vascularized and innervated connective tissue, it is composed of diverse cell populations, among which dental pulp stem/progenitor cells (DPSCs) are anticipated to continuously replenish odontoblasts to form secondary and tertiary reparative dentin throughout adult life as well as in response to various insults (2). DPSCs are considered an alternative and readily available source of multipotent stromal cells (MSCs) for tissue regeneration; they are characterized by their clonogenicity, their highly proliferative potential, their capability of self-renewal, and their multilineage differentiation aptitude (3) and are reported to hold promising attributes in the field of ameliorating ischemic, cardiac, and neurologic diseases (4, 5).

Toll-like receptors (TLRs), key molecules connecting innate and adaptive immunity, are germline encoded pattern recognition receptors sensing specific pathogen-associated molecular patterns (PAMPs), thereby promoting activation of immune cells, and are pivotal in pathogenesis of chronic inflammatory, autoimmune, and infectious diseases (6). To date, 10 functional human TLRs have been described. Depending on their cellular localization and PAMP ligands, TLRs are divided into extracellular ones, mostly identifying microbial membrane constituents including lipids and lipoproteins (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4), and flagellin (TLR5), and intracellular ones, which recognize double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8), and unmethylated CpG-DNA of viruses and bacteria (TLR9) (7).

MSCs of different origin express functional TLRs in characteristic patterns, making them particularly sensitive to certain microbial compounds. When activated by their ligands, TLRs modulate the migratory, proliferative, differentiation, and immunosuppressive potentials of MSCs (8). Varied expressions of TLRs 1, 2, 3, 4, 5, and 6 were reported on human and mural adipose MSCs and bone marrow–derived MSCs (BM-MSCs) as well as on human Wharton jelly MSCs (WJ-MSCs) (9). This distinctive TLR expression pattern could affect the therapeutic potential of MSCs during transplantation in vivo (10). To date, solely the expression of TLRs 2, 3, and 4 was described on DPSCs (11–14). The aim of the present investigation was to characterize for the first time a complete TLR expression profile of DPSCs under inflamed and uninflamed conditions.

Materials and Methods

Isolation and Culture of DPSCs

Human dental pulp tissue was obtained from patients (age, 15–20 years) undergoing extraction of non-curious third molars (n = 6) (Institutional Review Board approval number D-444/10). Teeth were disinfected and mechanically fractured, and the dental pulp was gently isolated with sterile forceps, rinsed several times in basic medium, and placed into 75-mL culture flasks (Sarstedt AG, Nümbrecht, Germany) for 30 minutes to adhere. Subsequently, the basic medium was carefully added, flasks were incubated in 5% carbon dioxide at 37°C, and cells were left to grow out.

After reaching 80%–85% confluence, cells were detached with 0.10% trypsin-EDTA (Biochrom Ltd, Cambridge, UK) and counted; their viability was tested by using trypan blue (Sigma-Aldrich, St Louis, MO) and finally seeded out at 30 cells/cm² density in basic medium. After the first passage cells reached 80%–85% confluence, they were immunomagnetically sorted by using anti-STRO-1 (BioLegend, San Diego, CA) and anti-
immunoglobulin M MicroBeads (Miltenyi-Biotec, Bergisch Gladbach, Germany) antibodies according to the manufacturer’s instructions (MACS; Miltenyi-Biotec). The STRO-1+–sorted cells (DPSCs) were seeded out to form colony-forming units (CFUs).

**CFUs**

DPSCs were cultured in basic medium at 1.63 cells/cm² density. Aggregates of 50 or more cells were scored as CFUs. On day 12, representative cultures were fixed with 4% formalin and stained with 0.1% crystal violet. The remainder of the CFUs were detached by using cell scrapers and seeded in new 75-mL flasks.

**Flow Cytometric Analysis**

After reaching confluence, DPSCs were characterized flow cytometrically for the MSCs’ surface markers: CD14, CD34, CD45, CD73, CD90, CD105, and CD146 (all from Becton Dickinson Co, Canaan, CT). Binding of primary antibodies and isotype controls was performed by using FcR Blocking Reagent (Miltenyi-Biotec), and their expression was evaluated with FACSCalibur E6370 and FACSGomp 5.1.1 software (Becton Dickinson Co).

**Multilineage Differentiation Potential**

For osteogenic differentiation, third passage 2 × 10⁴ DPSCs were cultured on 6-well plates in osteogenic medium (PromoCell, Heidelberg, Germany) and in basic medium (control). At day 14, cell cultures were stained with alizarin red (Sigma-Aldrich) to label calcified deposits. For adipogenic differentiation, third passage 3 × 10⁵ DPSCs were cultured on 6-well plates in adipogenic medium (PromoCell) and in basic medium (control). The presence of lipid droplets was evaluated after 21 days by oil red O (Sigma-Aldrich). For chondrogenic differentiation, micromasses of third passage 3 × 10⁴ DPSCs were incubated with chondrogenic medium (PromoCell) in 1.5-mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and in basic medium (control). Chondrogenic differentiation was evaluated at day 35 by staining of glycosaminoglycans with alcian blue and nuclear fast red counterstaining (Sigma-Aldrich).

**Inflammatory Medium**

To test the effect of the inflammatory environment on the TLR expression profile of DPSCs, standardized inflammatory medium, composed of 25 ng/mL interleukin (IL)-1β, 1 × 10⁻⁶ U/mL interferon (IFN)-α, 50 ng/mL tumor necrosis factor (TNF)-α, and 3 × 10⁶ U/mL IFN-δ (inflammatory medium; all from PeproTech, Hamburg, Germany) (15) added to the basic medium components, was used. DPSCs were incubated 18 hours in the inflammatory (DPSCs-i) or basic medium (DPSCs).

**mRNA Extraction and cDNA Synthesis**

mRNA extraction was performed for DPSCs and DPSCs-i by using RNeasy kit (Qiagen, Hilden, Germany). Obtained RNA was purified by using RNase-free DNase (Promega, Mannheim, Germany) and quantified photometrically. Complementary cDNA was synthesized from 1 to 13 μL RNA (1 ng/μL) by reverse transcription by using QuantiTect reverse transcription kit (Qiagen) in a volume of 20 μL reaction mixture containing 4 pmol of each primer, 10 μL of the LightCycler Probes Master mixture (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and 5 μL specimen cDNA. Real-time polymerase chain reaction (rt-PCR) (LightCycler-96 Real-Time-PCR System; Roche Molecular Biochemicals, Indianapolis, IN) was performed according to the manufacturer’s instructions. Relative quantities of each transcript were normalized according to the expression of phosphoglycerate kinase 1 (PGK1).

**Primers for TLRs 1–10 and PGK1 reference gene were supplied by Roche and tested on DPSCs and DPSCs-i (Table 1).**

**Flow Cytometric Determination of TLR Expression**

DPSCs and DPSCs-i were characterized flow cytometrically for the expression of TLRs 1–10 at protein level. For intracellular TLR staining, cells were fixed and permeabilized with Fix&Perm Kit (Imtac, Antwerpen, Belgium) before incubation. Antibodies used were anti-TLR1, anti-TLR3, and anti-TLR9 (eBioscience, San Diego), anti-TLR2, and anti-TLR4, and anti-TLR8 (Enzo Life Sciences, Lorrach, Germany), anti-TLR5 (R&D Systems, Hessen, Germany), anti-TLR6 (BioLegend), anti-TLR7 (Perbio Science, Bonn, Germany), and anti-TLR10 (Acris Antibodies, Herford, Germany). Binding of primary antibodies and corresponding isotype controls was performed by using FcR Blocking Reagent and evaluated with FACScalibur E6370 and FACSGomp 5.1.1 software (Becton Dickinson).

**Statistical Analysis**

Shapiro-Wilk test tested the normal distribution of data. Differences in TLR expression on mRNA and protein levels in DPSCs and DPSCs-i were evaluated by using the Wilcoxon signed rank test (SPSS software version 11.5; SPSS, Chicago, IL). The level of significance was P = .05.

**Results**

**Microscopy, CFUs, and Flow Cytometric Analysis**

After the initial adherence phase, fibroblast-like cells grew out of the pulp tissue masses (Fig. 1A). Twelve days after seeding, STRO-1+–sorted cells (DPSCs) showed CFUs (Fig. 1B) and were CD14+, CD34−, and CD45+ and CD73+, CD90+, CD105+, and CD146+ (Fig. 1C).

**Multilineage Differentiation**

The osteogenic differentiation of DPSCs was demonstrated by the formation of alizarin red–positive calcified deposits in contrast to their control (Fig. 1D and E). Adipogenic differentiation of DPSCs resulted in the formation of oil red O–positive lipid droplets in contrast to their control (Fig. 1F and G). The chondrogenic differentiation of DPSCs resulted in the formation of alcian blue–positive glycosaminoglycans in contrast to their control (Fig. 1H and I).

**TLR mRNA Expression**

On the mRNA level, DPSCs incubated in basic medium expressed (median gene copies/PGK1 copies, Q25/Q75) TLR1 (0.0013, 0.0008/0.0058), TLR2 (0.0002, 0.0000/0.0018), TLR3 (0.0015, 0.0009/0.0022), TLR4 (0.0067, 0.0024/0.0105), TLR5 (0.0000, 0.0000/0.0005), TLR6 (0.0017, 0.0007/0.0026), and TLR10 (0.0005, 0.0002/0.0006). Table 1 shows the TLR mRNA expression of DPSCs and DPSCs-i.

**Table 1. Primer Names and ID Used for rt-PCR (as supplied by Roche)**

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<th>Assay ID</th>
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<th>Roche accession ID</th>
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Inflammation (DPSCs-i) significantly upregulated the expression of TLR1 (0.0027, 0.0015/0.0114; \( P = .019 \)), TLR2 (0.0041, 0.0009/0.0075; \( P = .006 \)), and TLR3 (0.0160, 0.0049/0.0318; \( P = .002 \)) in addition to significantly lower expression of TLR6 (0.0006, 0.0004/0.0012; \( P = .012 \), Wilcoxon signed rank test) and downregulation of TLR10 (0.0003, 0.0001/0.0007). No expression for TLRs 7, 8, and 9 was recorded in both media on mRNA level (Fig. 2B).

**Flow Cytometric TLR Expression**

DPSCs incubated in basic medium expressed (% protein expression, Q25/Q75) TLR1 (30.67, 12.07/42.71), TLR2 (33.74, 22.78/52.94), TLR3 (0.60, −0.29/2.08), TLR4 (18.90, 14.92/43.13), TLR5 (23.42, 17.40/44.70), TLR6 (3.15, −0.23/10.60), TLR7 (9.14, 2.09/12.23), TLR8 (0.31, −0.62/59.59), TLR9 (11.18, 2.78/24.22), and TLR10 (37.43, 19.28/75.72) (Fig. 2C). Inflammatory medium upregulated TLR2 (45.45, 29.15/63.95), TLR3 (9.05, 0.12/2.34), TLR4 (19.84, 14.85/42.05), TLR5 (24.22, 7.78/42.07), and TLR8 (5.08, 2.39/34.06), downregulated TLR1 (24.40, 20.55/37.81), TLR7 (2.21, 0.60/12.27), TLR9 (3.55, 0.52/14.84), and TLR10 (23.58, 6.48/62.44), and diminished TLR6 expression (−0.91, −1.85/0.12; \( P = .008 \), Wilcoxon signed rank test) on DPSCs-i (Fig. 2D).

**Discussion**

The innate immune system, of which TLRs are fundamental components, is the host’s first defense line interacting with invading pathologic components. Inflammation follows most tissue injuries; it is an integral part of early healing processes, affecting in multiple ways the succeeding tissue reparative/regenerative phase (11). DPSCs may be exposed to such stimuli in many clinical and therapeutic conditions. Clinically active deep caries may result in localized odontoblastic damage or death through bacteria and their by-products, ultimately resulting in pulpitis (16). Through their sensing receptors (17), some of the surviving odontoblasts may participate in the pulpal response, whereas under the infected dentin, undifferentiated DPSCs are activated to extend the inflammation via their immunomodulatory properties and initiate tertiary/reparative dentin formation (16). In the field of tissue engineering, therapeutic approaches may further use a direct DPSC transplantation into inflamed environment, resulting ultimately in an interaction between DPSCs and PAMPs through their TLRs. The cell-specific TLR expression profile and its alteration in inflammatory environment play a decisive role in their aptitude for such interactions. Therefore, the aim of the present study was to characterize the distinctive TLR expression profile of DPSCs in inflamed and uninflamed conditions as a first stage of exploring this possible communication.

The putative STRO-1 stem cell marker has been exploited in the present study to isolate the DPSCs immunomagnetically. The sorted DPSCs showed all classic features for MSCs; they were CD73+, CD90+, CD105+, and CD146+, and CD14+, CD45+, and CD45−, and demonstrated remarkable CFU ability, plastic adherence, and multilineage differentiation potential.

PAMPs of gram-positive/gram-negative bacteria involved in pulpal pathologic conditions can induce an inflammatory reaction to which most MSCs are sensitive (9, 18), with an upregulation of multiple proinflammatory cytokines in the surrounding environment. To mimic such inflammatory condition, DPSCs were cultured in a standardized medium supplemented with IL-1β, IFN-γ, TNF-α, and IFN-α, the cytokines mostly present at inflammatory sites (15). Earlier investigations demonstrated multiple profound effects of these inflammatory cytokines on TLR signaling pathways. The same intracellular
signaling proteins activated through binding of IL-1β to IL-1 receptor (IL-1R) participate in signaling through other receptors with toll-IL-1 receptor domains (19). IL-1R, TNF-receptor (TNF-R), and TLR4 signaling pathways produce intracellular nuclear factor kappa B through converging on a common IκB kinase complex phosphorylating the nuclear factor kappa B inhibitory protein (IκBa), the inhibitor of nuclear factor kappa B kinase (20). TNF-R–associated factor 6, a pivotal signaling molecule regulating a diverse array of physiological processes including adaptive and innate immunity, is essential for the signaling downstream of the IL-1-R/TLR superfamily (21). Finally, a positive feedback loop exists between IFN levels and the expression of multiple TLRs (22).

On the protein level, the DPSCs in uninflamed environment expressed TLRs 1–10 in different quantities. According to their median expression values, TLR10 was the highest expressed, followed by TLRs 2, 1, 5, 4, 9, 8, 7, and 3 in a descending order. The mRNA level of most DPSC TLRs in inflamed and uninflamed conditions correlated mostly with their protein expression, with a statistically significant upregulation reached for TLRs 1, 2, and 3 and a down-regulation in TLR6. On mRNA level no expression of TLRs 7, 8, and 9 was noted.

Diverse TLR expression profiles have been described in human MSCs originating from different tissues. TLRs 1, 2, 3, 4, 5, 6, and 9 were expressed in umbilical cord MSCs (23). BM-MSCs demonstrated a wider expression profile with added TLRs 8 and 10 expression (9, 15). WJ-MSCs showed a comparable pattern with marginal/deficient TLR4 expression (9). Dental tissue–derived MSCs demonstrated the expression of TLRs 2, 3, and 4 in dental follicle MSCs (11) and DPSCs (11, 24), whereas TLRs 1, 2, 3, 4, 5, 6, 8, 9, and 10 were expressed in periodontal ligament MSCs (25). Similar to the current study’s results, inflammation upregulated the expression of TLR2 and TLR4 and down-regulated the expression of TLR6 in BM-MSCs (15).

Earlier studies proved direct relationships between levels of cellular TLR expression and responsiveness to their corresponding

Figure 2. mRNA and protein expression of TLRs in DPSCs and DPSCs-i. (A) mRNA expression of TLRs 1–10 in uninflamed condition. (B) mRNA expression of TLRs 1–10 in inflamed condition (n = 6, box and whisker plots with medians and quartiles). The green boxes show increased mRNA expression after stimulation by the inflammatory medium (Wilcoxon signed rank test, statistical significance marked with asterisk: ***P < .01, ****P < .001). (C) Protein expression of TLRs 1–10 in uninflamed condition. (D) Protein expression of TLRs 1–10 in inflamed condition (n = 6, box and whisker plots with medians and quartiles) (Wilcoxon signed rank test, statistical significance marked with asterisk: **P < .01).
ligands (26). The outlined TLR expression profile, especially under inflammation, may influence the DPSCs’ response to the corresponding ligands in vitro. An upregulation of the lipooxigenase acid–sensing TLR2 (27) and LPS-sensing TLR4 (28) could increase the DPSCs’ ability to recognize both gram-positive and gram-negative pathogens, respectively, under inflammation, whereas the inflammatory upregulation of TLR5 expression could favor the recognition of bacterial flagellin (28). A downregulation of TLR1 and TLR6 reduces the ability for recognition of lipoprotein (29), and a downregulation of TLR7 and TLR9 lessens the aptitude for recognition of viral pathogens (30).

A coactivation of multiple TLRs makes interpretation of this direct ligand-receptor relationship more complex. Studies described that co-transfection of different TLRs could either augment or inhibit the recognition of specific PAMPs (31, 32), suggesting that cellular responses to PAMPs are dependent on the entire repertoire of TLRs expressed on a cell, necessary cofactors, and levels of each PAMP present (26). Further complexity arises from the fact that some TLRs may in combined stimulation act as co-receptors (eg, TLR1, TLR6) for other TLRs (eg, TLR2) and can thereby stimulate or inhibit cellular responsiveness to their stimulating ligands (31, 33). To date, only effects of TLR 2, 3, and 4 activation have been characterized on DPSCs. Earlier studies reported on production of distinctive inflammatory cytokines by DPSCs, including an increase in IL-8 (12–14), IL-1 and IL-6 (13, 14) production in response to TLR4 activation. Activation of the expressed/upregulated TLRs during inflammatory conditions could further affect the migration, proliferation, and differentiation potentials of DPSCs. TLR4 activation could boost cell migration, downregulate their proliferation (13, 14), and enhance osteogenesis and suppress adipigenic potential of DPSCs through increased Wnt-5a expression (13). TLR4 activation further lowers the endogenous TGF-β and the immunosuppressive factor indolamine-2,3-dioxygenase production, affecting combined the immunosuppressive phenotype of DPSCs (11).

The current study describes for the first time the distinctive TLR expression profiles of DPSCs in inflamed and uninflamed conditions, which could impact their immunomodulatory and therapeutic potentials in vitro (10). Studies are further needed to test the effect of each of the outlined TLRs’ activation, solely or in combination, on the various cellular attributes of DPSCs.

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The authors deny any conflicts of interest related to this study.

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