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Research paper

TLR-induced immunomodulatory cytokine expression by human gingival stem/progenitor cells

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ABSTRACT

During therapeutic application, mesenchymal stem cells (MSCs) may interact with their environment via their expressed toll-like-receptors (TLRs) leading to pro- or anti-inflammatory immune responses. The present study aimed to describe the gingival margin-derived stem/progenitor cells' (G-MSCs) TLR-induced immune regulatory response to specific TLR agonists.

Gingival cells were obtained, immunomagnetically sorted via anti-STRO-1 antibodies and seeded out to achieve colony forming units (CFUs). G-MSCs were investigated for stem cell characteristics and TLR expression. Specific TLR agonists were applied and m-RNA expression of pro- and anti-inflammatory factors was analyzed via real-time polymerase chain reaction.

G-MSCs showed all characteristics of stem/progenitor cells. All TLR agonists induced pro-inflammatory cytokines, except for the TLR3 agonist, which significantly promoted the anti-inflammatory response. ($p \leqslant 0.05$, Wilcoxon-Signed-Ranks-Test).

TLR-induced immunomodulation by G-MSCs could impact their therapeutic potential in vivo. Two distinctive pro-inflammatory and an anti-inflammatory TLR-induced phenotypes of G-MSCs become noticeable in this study.

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1. Introduction

The human gingiva is a unique periodontal tissue, investing and surrounding the tooth-bearing alveolar bone. In the oral environment, the gingiva is usually subjected to high microbial challenges with resultant inflammatory changes [1,2]. Mesenchymal stem/progenitor cells (MSCs) are multipotent cells existing in various niches of the body, including the gingival connective tissue, demonstrating the capability to differentiate into various cell types [3]. In case of inflammation and tissue damage, MSCs migrate to the affected tissue sites, participating in regeneration and repair [4]. Their interaction with the surrounding inflammatory milieu is a vital part of this healing mechanism. In recent years, much knowledge has been gained about cellular and molecular processes implicated in the communication between MSCs and their inflammatory surrounding, thereby affecting and modulating their biological properties at sites of injury or repair [5–7].

Toll-like receptors (TLRs) are key cellular receptors, linking innate and adaptive immune responses. Their main function is the detection of invading pathogens through specific pathogen-associated molecular patterns (PAMPs), thereby promoting the activation of constituents of the immune response. TLR ligation activates both the innate and adaptive arms of the immune defensive mechanism [8]. In former studies, 10 functional TLRs have been identified in humans [9]. According to their localization, TLRs comprise of extracellular and intracellular types. Extracellular TLRs mostly recognize membrane constituents of invading microbes including lipoproteins and lipids (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4) and flagellin (TLR5). The intracellular group exists within the cells, to identify double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8) and

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unmethylated CpG DNA of viruses and bacteria (TLR9) [10–12]. Results indicated that explicit patterns of TLRs' expression differ according to the origin of the MSCs and the level of surrounding inflammation [13,14]. Differential expression patterns of TLRs 1–10 were described on human bone marrow (BM-MSCs), adipose derived MSCs (AD-MSCs), human Wharton Jelly MSCs (WJ-MSCs), human umbilical cord blood MSCs (UCB-MSCs) and on human MSCs from the oral tissues, including the dental pulp, the dental follicle and the free gingiva (G-MSCs) [14–17]. In a recent study, G-MSCs displayed a characteristic expression profile of TLRs 1, 2, 3, 4, 5, 6, 7 and 10 in basic culture condition and with inflammation up-regulated the expression of TLRs 1, 2, 4, 5, 7, and 10, while diminished TLR6 expression [17]. To date, no data exists regarding the TLR-induced immunomodulation by G-MSCs. The aim of the present study is to characterize such TLR-induced immunomodulation for the first time in G-MSCs and its possible pro- or anti-inflammatory effects on m-RNA level.

2. Material and methods

2.1. Isolation and culture of G-MSCs

Isolation of G-MSCs was performed as described in previous studies [3]. After acquiring patients' informed consents (Ethical Committee IRB – Approval number D 444/10), free gingival samples were collected surgically from five patients (n = 5) at the dental clinic of the Christian-Albrecht's-University-Kiel, Germany. The attained gingival tissue collars were detached, de-epithelized, rinsed with Minimum Essential Medium Eagle Alpha Modification (α-MEM; Sigma-Aldrich GmbH, Hamburg, Germany); supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; Biochrom AG, Berlin, Germany) and 1% amphotericin (Biochrom) and left inside dry 75 ml culture flasks (Sarstedt AG, Nümbrecht, Germany) for 30 min to allow their adherence. Consequently, basic medium comprised of 15% fetal calf serum (FCS, HyClone, Logan, UT, USA) added to α-MEM, 100 U/ml penicillin, 400 mmol/ml l-glutamine (Biochrom), 1% amphotericin and 100 μg/ml streptomycin was carefully added. The flasks were placed in an incubator and cells were left to grow out in a setting of 5% carbon dioxide and 37 °C. Throughout the cellular growth period, the culture flasks were checked regularly using a phase contrast inverted microscope and replaced the basic medium three times weekly. After attaining a confluence of 80–85%, cells were separated from the flasks with 0.10% trypsin-EDTA (Biochrom) and counted. Viability test was performed using Trypan Blue (Sigma-Aldrich GmbH, Hamburg, Germany) and cells were seeded at a density of 30 cells/cm^2 in 75 ml culture flasks in basic medium and incubated in the previously mentioned setting. Consequently, first passage cells were subjected to magnetic activated cell sorting (MACS), utilizing anti-STRO-1 (BioLegend, San Diego, CA, USA) and anti-IgM Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) antibodies according to the manufacturers' instructions. The positively sorted cell fraction (G-MSCs) were seeded out to grow colony-forming units (CFUs).

2.2. Colony-forming units (CFUs)

To evaluate the colony-forming efficiency, G-MSCs were left to grow under basic culture conditions at a density of 1.63 cells/cm^2. Clusters containing 50 or more cells were recorded as colonies. After 12 days, representative samples were fixed with 4% formalin and stained with 0.1% crystal violet. Single colonies from the remaining G-MSCs forming CFUs were subsequently detached by cell scrapers [18] to be seeded out in new 75 ml flasks and incubated in basic medium.

2.3. Flow cytometric analysis of surface MSC markers

Once G-MSCs reached confluence, a sample group of cells was characterized by flow cytometry, examining the predefined surface marker constellation of MSCs [19]; CD14, CD34, CD45, CD73, CD90 and CD105. Standard protocols were followed to bind the primary antibodies and the corresponding isotype controls (all from Becton Dickinson, Heidelberg, Germany), employing FcR Blocking Reagent (Miltenyi Biotec) and results were assessed with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson, Heidelberg, Germany).

2.4. Multilineage differentiation potential

To evaluate the osteogenic differentiation potential, 2×10^4 G-MSCs of the third passage were cultured on 6-well culture plates in an osteogenic inductive medium (PromoCell, Heidelberg, Germany). As a control group, G-MSCs were grown in basic medium. After 14 days, staining of the cell cultures was completed with Alizarin-Red (Sigma-Aldrich), to mark calcified sediments [19], while the expression of runt-related-transcription-factor-2 (Cbfa1/Runx2) and alkaline phosphatase (ALP) was assessed using real-time polymerase chain reaction (PCR; LightCycler; Roche Molecular Biochemicals, Indianapolis, IN, USA) [3]. To examine the potential of adipogenic differentiation, 3×10^5 third passage G-MSCs were left to grow on 6-well culture plates in an adipogenic inductive medium (PromoCell). As a control group G-MSCs were cultured in basic medium. Lipid droplet formation was assessed by Oil-Red-O staining (Sigma-Aldrich) [19] and the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ; an early adipogenic marker) and lipoproteinlipase (LPL) was evaluated by PCR after 21 days [3]. The chondrogenic differentiation potential was tested by incubating micro-masses of third passage 3×10^4 G-MSCs with chondrogenic inductive medium (PromoCell) in 6-well culture plates (Sarstedt). G-MSCs were cultured in basic medium as a control group. The evaluation occurred at day 35 by staining of the samples via Alcian-Blue (Sigma-Aldrich), to label the produced glycosaminoglycans [19] and by analysis of the m-RNA expression of aggrecan (ACAN), also known as cartilage-specific proteoglycan core protein [3]. Media change took place three times weekly.

2.5. Flow cytometric determination of TLR expression

G-MSCs were characterized by flow cytometry for the presence of the different TLRs 1–10 at protein level as a second stage confirmatory assessment of a previous G-MSCs' TLR profiling [17]. For staining of intracellular TLRs, cells were first fixed and permeabilized with Fix & Perm cell permeabilization kit (Imtec, Antwerpen, Belgium) before incubation. The subsequent antibodies were utilized: anti-TLR1, anti-TLR3 and anti-TLR9 (all from eBioscience), anti-TLR2, anti-TLR4 and anti-TLR8 (all from Enzo Life Sciences, Lörrach 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 the primary antibodies and the corresponding isotype controls was performed according to standard protocols using FcR Blocking Reagent (Miltenyi Biotec) and evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson). The investigation of G-MSCs' TLR expression was performed as confirmation.

2.6. TLR stimulation

To test the effect of TLR stimulation on G-MSCs' expression of immune regulatory genes, 3×10^5 G-MSCs of the third passage were cultured on 6 well plates under basic culture conditions. After

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