EXPERIMENTAL CELL RESEARCH 316 (2010) 2676–2682

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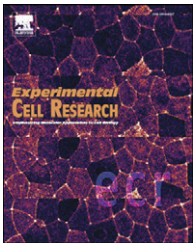

Research Article

Toll-like receptor 9 ligands enhance mesenchymal stem cell invasion and expression of matrix metalloprotease-13

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ARTICLE INFORMATION

Article Chronology:
Received 29 March 2010
Revised version received 20 May 2010
Accepted 21 May 2010
Available online 27 May 2010

Keywords:
Mesenchymal stem cells
Toll-like receptor-9
Matrix metalloprotease-13
Invasion
Organotypic culture

ABSTRACT

Human mesenchymal stem cells (hMSCs) are multipotent cells that are found in the bone marrow. Inflammation and tissue damage mobilize MSCs and induce their migration towards the damaged site through mechanisms that are not well defined. Toll-like receptor-9 (TLR9) is a cellular receptor for microbial and vertebrate DNA. Stimulation of TLR9 induces inflammatory and invasive responses in TLR9-expressing cells. We studied here the expression of TLR9 in human MSCs and the effects of synthetic TLR9-agonists on their invasion. Constitutive expression of TLR9 was detected in human MSCs but the expression was suppressed when MSCs were induced to differentiate into osteoblasts. Using standard invasion assays and a novel organotypic culture model based on human myoma tissue, we discovered that stimulation with the TLR9 agonistic, CpG oligonucleotides increased the invasion capacity of undifferentiated MSCs. Simultaneously, an increase in MMP-13 synthesis and activity was detected in the CpG-activated MSCs. Addition of anti-MMP-13 antibody significantly diminished the CpG-induced hMSC invasion. We conclude that treatment with TLR9-ligands increases MSC invasiveness, and this process is at least partially MMP-13-mediated.

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Abbreviations: CAF, cancer-associated fibroblast; ECM, extracellular matrix; IL, interleukin; MMP, matrix metalloprotease; MSC, mesenchymal stem cell; NF-κB, nuclear factor κB; TGF-β, transforming growth factor β; TLR, toll-like receptor

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doi:10.1016/j.yexcr.2010.05.024

Introduction

Human mesenchymal stem cells (MSCs) are multipotent cells that can be found in many tissues, and most commonly they are isolated and cultured from bone marrow. Depending on the surrounding conditions, MSCs can further differentiate into osteoblasts, chondroblasts or adipocytes, and also into other cell lineages [1]. Physiological stress factors, such as inflammatory processes or tissue injuries, initiate cytokine cascades that can mobilize and attract mesenchymal stem cells to begin the tissue regeneration process [2]. During this process, MSCs are mobilized from the bone marrow and they migrate to the wounded or damaged sites. The mechanisms that control MSC homing into regenerating tissues are not clear but several different cytokines might be involved [3,4]. Also malignancies cause secretion of inflammatory cytokines, and several reports from the recent years have demonstrated that MSCs home towards tumor tissues as well [5–8]. The exact mechanisms how the mesenchymal stem cells are recruited, and the molecules that can enhance their capacities to cross tissue barriers and migrate towards the injured site or tumors, are, however, poorly understood.

It has been suggested that human MSC migration is increased through toll-like receptor (TLR) activation [9]. Toll-like receptors are a family of transmembrane proteins that were initially discovered to recognize pathogen-associated molecular patterns. Their activation triggers the innate immune system that leads to expression of various proinflammatory cytokines (reviewed in [10]). TLR9 is a cellular receptor for microbial and vertebrate DNA [10]. In addition to the inflammatory effects, stimulation of TLR9 with bacterial DNA or its synthetic analog (CpG-DNA sequence containing synthetic oligonucleotides) can induce cell migration and invasion, which are mediated via TLR9. For example TLR9 activation has been shown to increase invasion of the breast, brain and prostate cancer cells [11–13]. Furthermore, this invasion in cancer cells was shown to be mediated via matrix metalloprotease-13 (MMP-13) activation [11]. MMP activity, on the other hand, has been associated with tissue destruction during cancer invasion [14,15].

The aim of this study was to investigate TLR9 expression in human MSCs, to evaluate the effects of TLR9-ligands on MSC invasion capacity, and to elucidate the mechanisms involved, in particular whether MMPs are involved in this process.

Materials and methods

Cell culture

Human bone marrow-derived mesenchymal stem cells were harvested and cultured as described previously [16]. The human bone marrow cells used in this particular study were obtained from patients who were operated for hip fracture or osteoarthritis. The ethical committee of Oulu University Hospital has approved the study protocol and the patients gave their written consent for participation in the study. MSCs were separated by virtue of their adherence to plastic, and after two days of initial culture, the nonattached cells were rinsed off from the cell culture flasks. MSCs were cultured in α-MEM (all reagents used in this study were from Sigma-Aldrich, unless otherwise indicated) with 10% fetal bovine serum (PromoCell), 20 mM Hepes buffer, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine at 37 °C in 5% CO_2 and 95% air. The differentiation potentials of the cell lines were assessed by inducing osteogenic differentiation of the cells as described earlier [16]. A total number of 16 separate human MSC cell lines, obtained from 16 patients were characterized and used for the experiments described below. All experiments were carried out before the fourth passage of the cells.

TLR9 and MMP-13 immunohistochemistry

For TLR9 immunocytochemistry the cells were cultured on glass slides and fixed with 3% paraformaldehyde. The samples were treated with 3% hydrogen peroxide in methanol and 0.1% Triton X-100 in PBS prior to the staining. The cells were then incubated with 2.5 μg/ml mouse anti-TLR9 (Biosite) at +4 °C overnight. The Histostain Plus Kit (Zymed laboratories) was used for detection of the primary antibody, according to the manufacturer's instructions. For MMP-13 immunohistochemistry, 6-μm cryosections were fixed with ice cold acetone for 10 min. Non-specific binding was blocked with normal goat serum in 2% BSA/PBS for 30 min at room temperature, and the specimens were then further incubated with 1 μg/ml primary mouse anti-human MMP-13 antibody (clone 181-15A12; Calbiochem) in a humidified chamber at + 37 °C for 30 min, and then at +4 °C overnight. FITC-conjugated Alexa Fluor 488 goat anti-mouse secondary antibody (0.2 μg/ml) (Molecular Probes) was applied for 2 h at room temperature. The sections were mounted with mounting media containing DAPI (Vector). In the negative controls, either PBS or normal serum was used instead of primary antibodies.

RNA isolation, quantitative real-time PCR and nested PCR

Total RNA was isolated from the samples according to RNeasy Mini Kit-protocol (Qiagen) and complementary DNA was synthesised from 1 μg of total RNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) or DyNAmo™ SYBR®Green 2-Step qRT-PCR Kit (Finnzymes); the latter was also used in quantitative RT-PCR according to the manufacturer's protocol. Specific primers for TLR9 were 5′-GGACCTCTGGTACTGCTTCCA-3′ (forward) and 5′-AAGCTCGTTGTACACCCAGTCT-3′ (reverse) and for GAPDH the primers were 5′-TGGAAGGACTCATGACCACA-3′ (forward) and 5′-TTCAGCTCAGGGATGACCTT-3′ (reverse). The PCR assay cycles were the following: 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s.

The cDNA synthesis and semi-quantitative MMP-13 nested PCR were performed according to Wahlgren *et al.* [17] with a few modifications; the amount of RNA used in the RT-reaction was 1 μg, the DNA polymerase was AmpliTaq Gold (Roche) and the number of PCR-cycles was 40. The size of the nested MMP-13 PCR product was 238 bp. The housekeeping gene β-actin was used as a control according to Hämäläinen *et al.* [18]. The intensities of the MMP-13 PCR-products were scanned and quantified with Scion-Image software to obtain densitometric values and they were normalised to the corresponding β-actin intensities.

Western blotting

For the detection of the TLR9 protein, the cells were grown in culture flasks until near confluence, after which the cells were