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Original Article

A biological study establishing the endotoxin limit for osteoblast and adipocyte differentiation of human mesenchymal stem cells

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ABSTRACT

Introduction: Multipotent mesenchymal stem cells (MSCs) are widespread in adult organisms and are implicated in tissue maintenance and repair, regulation of hematopoiesis, and immunologic responses. Human (h)MSCs have applications in tissue engineering, cell-based therapy, and medical devices but it is unclear how they respond to unfavorable conditions, such as hypoxia or inflammation after transplantation *in vivo*. Although endotoxin testing is required for evaluating the quality and safety of transplanted MSCs, no reports on their dose response to endotoxins are available to establish the limits for *in vitro* MSC culture systems. In the present study, we aimed to accurately quantify the risk of endotoxin contamination in cell culture systems to establish an acceptable endotoxin limit for the differentiation of hMSC osteoblasts and adipocytes.

Methods: Three types of bone marrow-derived hMSCs (hMSC-1: 21-year-old, M/B; hMSC-2: 36-year-old, M/B; hMSC-3: 43-year-old, M/C) and adipose-derived stem cells (ADSCs; StemPro Human) were cultured in osteogenic or adipogenic differentiation media, respectively, from commercial kits, containing various concentrations of endotoxin (0.01–100 ng/ml). The degree of adipocyte and osteoblast differentiation was estimated by fluorescent staining of lipid droplets and hydroxyapatite, respectively. To clarify the molecular mechanism underlying the effect of endotoxin on hMSC differentiation, cellular proteins were extracted from cultured cells and subjected to liquid chromatograph-tandem mass spectrometry shotgun proteomics analysis.

Results: Although endotoxin did not effect the adipocyte differentiation of hMSCs, osteoblast differentiation was enhanced by various endotoxin concentrations: over 1 ng/ml, for hMSC-1; 10 ng/ml, for hMSC-2; and 100 ng/ml, for hMSC-3. Proteomic analysis of hMSC-1 cells revealed up-regulation of many proteins related to bone formation. These results suggested that endotoxin enhances the osteoblast differentiation of MSCs depending on the cell type.

Conclusions: Since endotoxins can affect various cellular functions, an endotoxin limit should be established for *in vitro* MSC cultures. Its no-observed-adverse-effect level was 0.1 ng/ml based on the effect on the hMSC osteoblast differentiation, but it may not necessarily be the limit for ADSCs.

1. Introduction

Regenerative medicine and tissue engineering are being revolutionized by the developments in the field of stem cell science. Naturally-derived biomaterials, such as collagen, gelatin, chitin, chitosan, hyaluronate, and alginate, are commonly used in cell culture scaffolds because of their biocompatibility. Recent advances in tissue engineering have enabled the use of naturally-derived biomaterials beyond the regulation of tissue response at the material interface, e.g., in the fabrication of three-dimensional culture matrices [1–7]. However, a major limitation of these materials is

Abbreviations: LPS, Lipopolysaccharide; FGF, Fibroblast growth factor; BMP, Bone morphogenetic protein; CD, cluster of differentiation; LC-MS/MS, liquid chromatograph-tandem mass spectrometry; NOAEL, no-observed-adverse-effect level; (h)ADSC, (human) adipose-derived stem cell; (h)MSC, (human) mesenchymal stem cell; TLR, Toll-like receptor.

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quality control; in particular, their microbial safety has not been well characterized and is difficult to control.

Multipotent mesenchymal stem cells (MSCs) are emerging as a desirable tool in regenerative medicine and cell therapy because of their wide-ranging differentiation potential, large expansion capacity, and lack of immune rejection following transplantation. Furthermore, MSCs are widespread in adult organisms, and have been implicated in tissue maintenance and repair, regulation of hematopoiesis, and immunological responses [8]. Human (h)MSCs have applications in tissue engineering, cell-based therapy, and medical devices, but it is unclear how they respond to unfavorable conditions, such as hypoxia or inflammation, after *in vivo* transplantation [9].

Toll-like receptors (TLRs) play an important role in the immune system by participating in the initial recognition of microbial pathogens and pathogen-associated components. TLR agonists can affect the proliferation and differentiation of hMSCs, which express TLRs, such as TLR-4 and the endotoxin receptor [8,10–12]. Most TLR agonists are microbial components, e.g., lipoprotein, glycoprotein, double-stranded RNA, non-methylated CpG DNA, flagellin, mycetoma-polysaccharide, and endotoxin, which exerts the greatest biological effect at the lowest dose [13,14]. Endotoxins are surface lipopolysaccharides (LPS) of gram-negative bacteria and typical pyrogens that elicit host immune responses even when present in trace amounts [13], and have various other biological activities *in vitro* and/or *in vivo* [11,14].

MSCs differentiate along several lineages via tightly regulated pathways. The human adipose tissue contains cell populations with characteristics similar to the bone marrow stromal cells. Wnt proteins are induced by stimulation by TLR agonists and have been linked to the proliferation and differentiation of various cell types, including MSCs [15]. E.g., endotoxin derived from *Porphyromonas gingivalis* inhibits osteoblast differentiation at doses over 100 ng/ml [16], whereas *Escherichia coli* endotoxin stimulates fibroblast proliferation after 6 d of exposure at concentrations of 50–500 ng/ml [17]. With the exception of CpG DNA, no TLR agonists that affect the proliferation of the human adipose-derived stem cell (hADSCs) are currently known. Endotoxin and peptidoglycans stimulate osteogenic differentiation, whereas CpG DNA inhibits it [9]. In addition, double-stranded RNA analogs do not affect adipogenic or osteogenic differentiation, but act synergistically with endotoxin or peptidoglycan to induce osteogenic differentiation. Pam3Cys, a TLR-2 ligand, inhibits the differentiation of MSCs into osteogenic, adipogenic, and chondrogenic lineages, while preserving their immunosuppressive function [8]. It was also reported that TLR ligands might antagonize MSC differentiation triggered by exogenous mediators and, consequently, support cells in an undifferentiated and proliferative state *in vitro*. Moreover, MSCs derived from a myeloid factor 88-deficient mouse lack the capacity to differentiate into osteogenic and chondrogenic cells [8].

The above reports suggest that TLRs and their ligands are regulators of cell proliferation and differentiation, and contribute to the maintenance of MSC multipotency. Furthermore, these effects differ according to the type of TLR agonist and source of cells. However, it remains unclear why endotoxin would exert different effects on the proliferative and differentiative capacities of each MSC, since the cells recognize it via TLR-4 and activate the same downstream signal transduction pathway. Furthermore, published studies used high concentrations of TLR ligands; this is especially true of endotoxin, which can induce biological responses in the concentration range of pg/ml or ng/ml, depending on the cell type.

Although endotoxin testing is required for the evaluation of the quality and safety of regenerative medicine products derived from the processing of autologous human somatic stem cells [18], as well as pharmaceuticals and medical devices, no reports on the dose response to endotoxin have been published to establish the endotoxin limits for *in vitro* MSC culture systems. Recently, we reported that the *in vitro* proliferation capacity of MSCs is enhanced by endotoxin at concentrations above 0.1 ng/ml, and that up-regulation of Fe/Mn-type superoxide dismutase may improve cell survival during endotoxin exposure [19]. In the current study, we investigated the no-observed-adverse-effect level (NOAEL) of endotoxin for several types of MSCs cultured in media containing various concentrations of endotoxin. We examined the effect of endotoxin on the cellular differentiation capacity and the underlying mechanisms to empirically establish the *in vitro* endotoxin limit for MSC differentiation.

2. Materials and methods

2.1. Reagents and materials

Three types of bone marrow-derived hMSCs (hMSC-1: 21-year-old, M/B; hMSC-2: 36-year-old, M/B; hMSC-3: 43-year-old, M/C) and the MSCGM BulletKit, hMSC Osteogenic Differentiation Medium BulletKit, hMSC Adipogenic Differentiation Medium BulletKit, and OsteoImage mineralization assay were purchased from Lonza (Walkersville, MD, USA). Hoechst 33258, BODPY lipid probes, hADSCs (StemPro Human), MesenPRO RS medium kit, StemPro osteogenesis differentiation kit, and StemPro adipogenesis differentiation kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). All tools made of glass, metal, or Teflon were autoclaved at 250 °C for more than 16 h prior to use.

2.2. Preparation of bacterial endotoxin

E. coli strain O3:K2a, K2b:H3 (ATCC no. 23501; American Type Culture Collection, Manassas, VA, USA) was cultured in a fermenter (50 l) at 37 °C for 16 h with gentle stirring, with an air flow of 1 l/min, in a minimum nutrient broth containing 0.2% (w/v) beef extract, 1% (w/v) peptone, and 0.5% (w/v) NaCl (pH 7.4). After neutralization of the culture medium pH and heat inactivation at 121 °C for 15 min, bacterial cells were collected by continuous centrifugation (7000×*g*) and washed three times with distilled water. This was followed by sequential extraction with ethanol, acetone, and diethyl ether to dehydrate the cells. Endotoxin was extracted from dried cells using the phenol-water method [20], and purified by repeated ultracentrifugation after deoxyribonuclease and ribonuclease treatments [21]. The activity of purified endotoxin was 27.5 EU/ng.

2.3. Cell culture, and analysis of cell proliferation and differentiation

Three types of bone marrow-derived hMSCs and hADSCs were cultured using the MSCGM BulletKit and MesenPRO RS medium kits, respectively, at 37 °C in a humidified atmosphere containing 5% CO_2, with a medium exchanged every 3 d. Once the cells reached an 80–90% confluence, they were trypsinized, counted, and passaged. Passage 3 or 4 cells, free of contamination, were used in subsequent experiments. To evaluate the effect of endotoxin on cell differentiation capacity, hMSC-1, hMSC-2, hMSC-3, and hADSC cells were cultured in each differentiation medium in the presence of various concentrations of endotoxin (0.01–100 ng/ml). Cells cultured without endotoxin served as a negative control. Adipogenic differentiation was performed using hMSC Adipogenic Differentiation Medium BulletKit. hMSCs (4.0×10^4 cells/cm^2) were plated in 96-well plates and cultured in the growth medium. At 100% confluence, the medium was replaced with adipogenic