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

Yusuke Nomura, Chie Fukui, Yuki Morishita, Yuji Haishima*

Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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.

© 2018, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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...
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 pathogens and pathogen-associated components. TLR agonists can induce the activation of 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 immunogens 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]. For example, 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. Pams3Cys, 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 non-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, BODIPY lipid probes, hADSCs (StemPro Human), MesenPRO RS medium kit, StemPro osteogenesis differentiation kit, and StemPro adipogenesis differentiation kit were purchased from Thermo Fisher Scientific (Walther, 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% CO2, 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 a hMSC Adipogenic Differentiation Medium BulletKit. hMSCs (4.0 × 10^4 cells/cm²) were plated in 96-well plates and cultured in the growth medium. At 100% confluence, the medium was replaced with adipogenic
induction medium. Following this, the cells were cultured for 3 days, after which the medium was supplemented with adipogenic maintenance medium for a further 4 days of culturing. After three cycles of induction/maintenance, the cells were cultured for 7–14 days in adipogenic maintenance medium, with the medium being replaced every 2–3 days. Osteogenic differentiation was performed using Osteogenic Differentiation Medium BulletKit. hMSCs (5.7 × 10⁵ cells/cm²) were plated in 96-well plates and grown in a growth medium. After 24 h, the medium was replaced by induction medium. The cells were then cultured for 2–4 weeks, and the medium was changed every 3–4 days. Cell nuclei, hydroxyapatite (HAp), and lipid droplets were stained by using Hoechst 33342, OsteoImage mineralization assay, and BODIPY lipid probes, respectively. The number of stained cells and the stained area (μm²) were analyzed using BZ-9000 (KEYENCE, Osaka, Japan). The differentiation capacities of osteoblasts and adipocytes were evaluated using the following equations: osteoblast differentiation score (OD score) = (HAp area)/(number of cells), and adipocyte differentiation score (AD score) = (lipid droplet area)/(number of cells), respectively. Data were analyzed by one-way analysis of variance (ANOVA). A post-hoc Tukey’s test was performed on all datasets that were shown to be significantly different by ANOVA (p < 0.05).

3. Results

3.1. Effect of endotoxin on adipocyte differentiation of hMSCs and hADSCs

hMSCs and hADSCs were cultured in the presence or absence of endotoxin, and the effect on adipocyte differentiation was evaluated based on cell number and lipid droplet area (Fig. 1). The AD scores of hMSC-1 cells cultured for 14 d with different concentrations of endotoxin (0–100 ng/ml) varied only slightly (24.53–33.69) (Fig. 2a). Similarly, the AD scores of hADSC cells cultured for 7 and 14 d in the presence of various concentrations of endotoxin, did not vary significantly (Fig. 2). On the other hand, the AD score of hMSC-1 cells was significantly higher (p < 0.05) in the high dose group (100 ng/ml) than in the control group (0 ng/ml) after 7 d of exposure; the scores of hMSC-2 and 3 cells were significantly lower (p < 0.05) in the high dose groups (1, 10, or 100 ng/ml for hMSC-2; 100 ng/ml for hMSC-3) than in the control groups after 14 d (Fig. 2). Although the differences were statistically significant, the actual change of the numerical values was not pronounced. These observations suggested that the adipocyte differentiation capacity of hMSCs and hADSCs slightly varied depending on the cell types and culture conditions, but it might not be affected by endotoxin because of the slight change of the AD scores observed.

3.2. Effect of endotoxin on osteoblast differentiation of hMSCs and hADSCs

In vitro osteoblast differentiation of hMSCs and hADSCs was performed in an osteoblast induction medium in the presence of various concentrations of endotoxin. The effect of endotoxin on osteogenic differentiation was evaluated based on the HAp area and cell number (Fig. 1). The OD scores of hMSC-1 cells cultured for 21 d with different concentrations of endotoxin (0–100 ng/ml) varied considerably (0.56–4.12) (Fig. 3a). The OD scores of hMSC-2 cells cultured for 21 d in the presence of 0–0.1 ng/ml endotoxin were zero, and then increased up to 8.94 in the presence of increasing amounts of endotoxin (Fig. 3b). The OD scores of hMSC-3 cells cultured in the presence of 0–10 ng/ml endotoxin were also zero, and increased to 4.57 after 21 d in the presence of 100 ng/ml endotoxin (Fig. 3c). Thus, the osteoblast differentiation capacity of hMSCs was significantly enhanced by endotoxin at concentrations exceeding 1.0 ng/ml, for hMSC-1 cells; 10 ng/ml, for hMSC-2 cells; and 100 ng/ml, for hMSC-3 cells. On the other hand, the osteoblast differentiation capacity of hADSCs was not affected by endotoxin even at the highest concentration tested (100 ng/ml) (Fig. 3d).

3.3. Proteomic analysis of intracellular proteins in endotoxin-stimulated hMSC

To identify the molecular mechanism by which endotoxin enhances the osteoblast differentiation capacity of MSCs, proteins of hMSC-1 cells cultured in the presence or absence of 1000 ng/ml endotoxin for up to 4 d were extracted and analyzed by LC-MS/MS shotgun proteomics. In total, 6130 proteins (p < 0.05) were identified, including ones associated with the immune system and...
osteogenesis, as well as the Wnt signaling pathway (Tables 1–3). The list of identified protein functions is shown in Fig. 4. The relative abundance of each protein induced by endotoxin stimulation varied, but in general, the immune system and osteogenesis-related proteins were up-regulated. E.g., 1 d after the beginning of the hMSC-1 cell culture, the levels of the following proteins were increased: TLR-4; glutamate receptors GRIK3 and GRM3; cell-surface co-receptor of Wnt/β-catenin signaling (LRP6; plays a pivotal role in bone formation); osteoblast differentiation-regulated proteins CHD9 and NO66; regulator of the insulin-like growth factor signaling pathways PHF7; type I procollagen enhance factor proliferating cell nuclear antigen (PCNA); homeobox proteins HXA6 and ZHX3; cytokines LKHA4, IL1R, and NLF1; steroid receptors FKBP4; ion homeostasis proteins SCN1A, SCN2A, and SCN3A; and NF-κB–related proteins FGD2 and NLRX1. On days 2 and/or 3, the levels of bone morphogenetic-related proteins (BMPR1A and BMP3B), vitamin D-coupled transcription regulation-related factor (AR11A), estrogen-responsive protein (GREB1), Fe/Mn-type superoxide dismutase (SODM), and nuclear factor NF-κB p100 subunit (NFKB2) were increased. Furthermore, on day 4, the levels of the growth factor and related proteins (ESRP1, MINT, and TISB) were increased.

4. Discussion

In the current study, we investigated the effect of endotoxin on MSC differentiation in an in vitro culture system and the underlying mechanism of that effect, to accurately evaluate the risks associated with endotoxin contamination in culture systems used for tissue engineering, and to establish endotoxin limits based on empirical evidence. In a culture system, endotoxin contamination always indicates the presence of live or dead gram-negative bacteria, but it may also indicate the presence of other microbes, such as gram-positive bacteria and fungi. Although the effect of endotoxins on
cell culture should be estimated by bacterial cell counts, the detection of endotoxin activity in a system should be interpreted as an indication of contamination level. It is therefore important to determine admissible limits of endotoxin levels to assure the safety and quality of MSC-based products.

The osteoblast differentiation capacity of hMSCs used in the current study decreased with cellular aging, and, in particular, hMSC-3 cells only produced small amounts of HAp during the culture period. However, the capacity was enhanced by endotoxin in a dose-dependent manner not only in hMSC-1 and hMSC-2 cells but also in hMSC-3 cells. It has been reported that various stressors, including endotoxin, induce the expression of growth factors in hMSC [22]. Fibroblast growth factor (FGF) and related proteins were indeed detected by the proteomics approach in the current study (Table 1).

The lowest observed adverse effect level for stimulating osteoblast differentiation of hMSCs was 1.0 ng/ml, which was considerably lower than the dose previously reported [9]. The endotoxin preparation method used in the current study resulted in a lower-heterogeneity preparation, with most molecules fully acylated [19]. Further, endotoxin is unstable in aqueous solution. Perhaps these explain why a relatively large amount of endotoxin was required to alter the behavior of MSCs, as compared with previous studies that employed a commercially available E. coli endotoxin.

The expression of cluster of differentiation (CD)80, CD86, major histocompatibility complex-II, TLR-4, and tumor necrosis factor-α in MSCs was found to be most effectively induced by endotoxin at a concentration of 10 μg/ml [10]. However, the dose seems to be too high for increasing the expression at a molecular level because, in the current study, the differentiation ability of MSCs was perceptibly increased by endotoxin at concentrations exceeding 1 ng/ml, and alteration of the related gene and protein expression levels in MSCs would be expected to be induced by a lower dosage. The ability of endotoxin to enhance MSC differentiation might be beneficial in regenerative medicine; however, since endotoxin may also affect other cellular functions, a concentration limit should be set for MSC cultures to assure their safety and quality. Although the precise amount of endotoxin that affects MSCs at the molecular level remains unclear, an NOAEL of 0.1 ng/ml was established in the current study based on the effect on MSC osteoblast differentiation. The NOAEL for hMSC-1 cells was 0.01 ng/ml when the cells were pre-cultured with endotoxin (100 ng/ml) prior to the culture in osteoblast differentiation medium (data not shown).

Little is known about the effect of endotoxin on MSCs in vivo. Several studies on the host response to biomaterials with spiked-in bacterial components, such as endotoxin, have been published [23-27], but none have focused on their effect on MSCs or the dose response to establish endotoxin limits at specific sites of the body. In the only quantitative analysis published to date, we reported that a collagen sheet containing dried E. coli cells implanted into a cranial or femoral defect in rats led to a dose-dependent delay of the osteoanagenesis with a NOAEL of 9.6 EU/mg [28]. This was not observed when an untreated collagen sheet or one containing Staphylococcus aureus cells were used. These observations suggested that endotoxin affected the process of osteoanagenesis and that the delayed formation of new bone was caused by the dried cells that suppressed the development of the connective tissue covering the defective areas, as well as the proliferation and differentiation of MSCs (intra-membranous ossification), since the pathology analysis did not reveal any osteoclasts or inflammation [28]. Thus, endotoxin exhibits different effects in vivo and in vitro.

Fig. 3. Effect of endotoxin on hMSC osteoblast differentiation. Osteoblast differentiation scores (OD scores) of hMSC-1 (a), hMSC-2 (b), hMSC-3 (c), and hADSC (d) cells cultured for 14, 21, or 28 d in media containing various endotoxin concentrations. OD score = (HAp area)/(number of cells). Data are presented as the mean ± SD (n = 4); *p < 0.05 vs. the control (0 ng/mL) group.
Table 1
Abbreviated list of osteogenesis proteins induced in hMSCs by endotoxin.

| Protein ID | Peptide count | Expression ratio [LPS(+) / control] |
|------------|---------------|-------------------------------------|
| Code       | Score         | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| FGFR2      | 32.0          | 1     | 11.3  | –     | 1.0   | 0.2   |
| PHF7       | 32.0          | 1     | 1.0   | –     | –     | –     |
| SH3C1      | 54.5          | 2     | 3.0   | 0.6   | 1.4   | 0.0   |
| ST5A       | 29.4          | 1     | 55.7  | 19.9  | 0.7   | –     |
| TSC1       | 35.6          | 2     | 2.1   | 8.7   | 0.5   | 11.0  |
| AKT3       | 23.8          | 2     | 1.3   | 2.1   | 1.6   | 1.7   |
| BMP1A      | 25.7          | 1     | –     | –     | –     | –     |
| CORL2      | 22.3          | 1     | –     | 7.5   | –     | –     |
| IRS4       | 24.9          | 1     | –     | 30.1  | –     | –     |
| UCHL3      | 43.0          | 2     | –     | 2.1   | –     | 116.1 |
| AKT1       | 30.2          | 3     | 1.2   | 1.4   | 4.3   | 4.0   |
| CD109      | 24.5          | 2     | 0.9   | –     | 2.4   | –     |
| FGF12      | 34.7          | 1     | 1.2   | –     | 8.1   | –     |
| FGFR2      | 26.5          | 1     | –     | –     | –     | –     |
| NED4L      | 25.8          | 1     | –     | –     | –     | –     |
| PHF7       | 19.7          | 1     | –     | –     | 14.5  | –     |
| ST5B       | 28.5          | 2     | –     | –     | 17.6  | –     |
| STA5B      | 28.5          | 2     | –     | –     | 17.6  | 0.4   |
| SH3C1      | 31.2          | 2     | 0.8   | 1.1   | 3.2   | –     |
| AKT3       | 49.0          | 3     | –     | 1.5   | –     | –     |
| TWST1      | 20.9          | 1     | –     | –     | 3.0   | –     |
| BMP1B      | 19.6          | 1     | –     | –     | –     | 2.6   |
| ESRP1      | 34.8          | 1     | 1.4   | 0.9   | 0.2   | –     |
| IF2B1      | 66.7          | 4     | –     | 1.2   | 1.1   | 2.2   |
| IF2B3      | 31.2          | 2     | –     | 0.8   | 1.1   | 3.2   |
| MINT       | 42.6          | 4     | 1.1   | 0.6   | 1.3   | 2.0   |
| TISB       | 27.2          | 1     | –     | –     | –     | 26.1  |
| WWF1       | 22.9          | 1     | –     | –     | –     | 18.0  |
| AKT1C3     | 88.9          | 4     | 2.7   | 1.1   | 41.8  | 3.2   |
| AK1C4      | 50.6          | 2     | 22.9  | 1.1   | 177.9 | 1.3   |
| ARIP4      | 23.2          | 1     | 0.0   | 0.3   | –     | –     |
| CSN6       | 42.1          | 2     | 3.5   | 0.7   | –     | –     |
| FKBP4      | 61.1          | 2     | 3.1   | 0.3   | –     | –     |
| MCR        | 27.4          | 1     | –     | –     | –     | –     |
| PCM5       | 52.9          | 1     | –     | 0.7   | 2.8   | 1.3   |
| PRP6       | 27.1          | 2     | 11.3  | 0.7   | –     | –     |
| SRCAP      | 32.0          | 1     | –     | 0.7   | –     | –     |
| GREB1      | 39.2          | 2     | 1.3   | 172.9 | 0.7   | –     |
| MAGAB      | 24.8          | 1     | –     | 4.9   | –     | –     |
| PGCR1      | 60.8          | 3     | 1.1   | 2.0   | 3.6   | 0.4   |
| AK1C1      | 122.3         | 7     | 1.6   | 1.1   | 3.1   | 1.9   |
| AK1C2      | 130.2         | 5     | 2.0   | 1.1   | 2.2   | 1.2   |
| ANM1       | 113.3         | 11    | 0.6   | 1.0   | 2.4   | 1.5   |
| DHB11      | 18.1          | 1     | –     | –     | 6.7   | –     |
| NCOA2      | 27.1          | 1     | –     | –     | –     | –     |
| STAM2      | 36.0          | 1     | 0.8   | 1.3   | –     | 3.5   |
| CMC1       | 29.1          | 2     | 3.4   | 1.1   | 71.4  | 0.3   |
| GRIK3      | 21.2          | 1     | –     | –     | –     | –     |
| CRN3       | 29.5          | 1     | –     | –     | –     | –     |
| RIC8A      | 82.2          | 6     | 2.0   | 1.7   | 1.2   | 1.2   |
| NARG2      | 86.3          | 4     | 1.6   | 3.0   | 1.8   | 1.1   |
| SH3C1      | 65.2          | 2     | 1.2   | 2.5   | 0.9   | 0.9   |
| NMDE3      | 26.5          | 1     | –     | –     | 51.6  | –     |
| GRIK5      | 23.1          | 1     | –     | –     | –     | 37.3  |
| AR11A      | 26.7          | 2     | –     | 74.4  | –     | –     |
| NR2C2      | 29.8          | 1     | –     | –     | –     | 6.9   |
| SMRC1      | 115.1         | 3     | 1.0   | 1.1   | 0.9   | 3.9   |
| SPI100     | 40.4          | 2     | 0.1   | 1.1   | 1.1   | 56.8  |
| ACBP       | 66.6          | 3     | 2.5   | 0.5   | 1.2   | 0.7   |
| ARMET      | 90.5          | 7     | 2.6   | 0.5   | 0.5   | 0.7   |

(continued on next page)
induce bone formation [29]. Further, they act as osteoinductive growth factors that may as well as to regulate cell growth, apoptosis, and differentiation of several tissues and organs during vertebrate development, and have been shown to participate in the patterning and specification of skeletal morphogenesis [30,31]. BMP3B and BMP3 are regarded as a protein that in human is encoded by the BMP3B gene [30]. It plays a role in head formation and may have multiple roles in cartilage development. BMP receptors are a family of transmembrane serine/threonine kinases that include the type I receptors BMPR1A and BMPR1B, and the type II receptor BMPR2.

In the current study, we observed that endotoxin affects the expression of many osteogenesis-related proteins, such as TRL-4, BMP3B, BMPR1A, FGF2, FGF2R, GREB1, GRIK3, GRM3, and LRP6. BMP3B, also known as growth differentiation factor 10 (GDF10), is a protein that in human is encoded by the BMP3B gene [30]. It plays a role in head formation and may have multiple roles in skeletal morphogenesis [30,31]. BMP3B and BMP3 are regarded as a separate subgroup within the TGF-beta superfamily [30]. Similar to other BMPs, BMP3B is known for its ability to induce bone and cartilage development. BMP receptors are a family of transmembrane serine/threonine kinases that include the type I receptors BMPR1A and BMPR1B, and the type II receptor BMPR2. Overexpression of a constitutively active form of BMPR1A in

**Table 1 (continued)**

| Protein ID      | Name                                                | Peptide count | Expression ratio [LPS+/control] |
|-----------------|------------------------------------------------------|---------------|--------------------------------|
|                 |                                                      |               | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| RCTD8           | BTD/POZ domain-containing protein RCTD8              | 47.6          | 3     | 48.1  | 1.7   | 0.9   | 1.3   |
| [Ephrin]        | Ephrin type-A receptor 7                             | 23.2          | 1     | –     | –     | –     | 7.9   |
| [Hedgehog]      | Uncharacterized protein C6orf170                     | 24.0          | 1     | 5.4   | –     | –     | 0.0   |
| DISP1           | Protein dispatched homolog 1                         | 35.4          | 1     | –     | 54.3  | –     | –     |
| PTBP2           | Polypyrimidine tract-binding protein 2               | 60.3          | 3     | 1.3   | 2.4   | 1.0   | 1.3   |
| [Homeobox]      | Homeobox protein Hox-A6                              | 31.6          | 2     | 20.4  | –     | –     | –     |
| ZHX3            | Zinc fingers and homeoboxes protein 3                | 35.7          | 1     | 25.5  | –     | –     | –     |
| [ECM]           | A disintegrin and metalloproteinase with thrombospondin motifs 5 | 33.5          | 1     | 20.8  | –     | –     | –     |
| ITH2            | Inter-alpha-trypsin inhibitor heavy chain H2        | 33.0          | 1     | 22.8  | 0.0   | 0.7   | 1.8   |
| CO1A1           | Collagen alpha-1(I) chain                            | 177.3         | 14    | 2.0   | 0.7   | 0.7   | 1.4   |
| CO7A1           | Collagen alpha-1(VII) chain                          | 42.2          | 1     | 58.6  | 0.8   | –     | –     |
| PDLI1           | PDZ and LIM domain protein 1                         | 133.9         | 9     | 2.2   | 0.8   | 0.7   | 2.5   |
| C0K1A           | Collagen alpha-1(XX) chain                           | 30.4          | 2     | 1.3   | 8.8   | 0.0   | –     |
| COOA1           | Collagen alpha-1(XXIV) chain                         | 27.7          | 1     | –     | 0.0   | 1.2   | –     |
| LAMB2           | Laminin subunit beta-2                               | 34.0          | 1     | 1.3   | 0.8   | 0.8   | 1.4   |
| TENR            | Tenasin-R                                            | 32.2          | 1     | –     | 0.5   | 3.9   | –     |
| CHST9           | Carbohydrate sulfotransferase 9                     | 24.4          | 1     | –     | –     | 2.3   | –     |
| CO4A4           | Collagen alpha-4(IV) chain                           | 37.9          | 2     | 1.3   | 1.2   | 2.4   | 1.2   |
| CO6A2           | Collagen alpha-2(III) chain                          | 149.2         | 8     | 0.5   | 1.3   | 2.1   | 0.9   |
| COFA3           | Collagen alpha-1(III) chain                          | 21.3          | 1     | –     | –     | 2.1   | –     |
| COFA1           | Collagen alpha-1(XXXV) chain                         | 28.6          | 2     | 1.3   | 0.6   | 2.5   | 1.7   |
| ITAV            | Integrin alpha-V                                     | 118.0         | 10    | 1.5   | 0.8   | 2.2   | 1.4   |
| TSP2            | Thrombospondin-2                                     | 46.1          | 1     | 1.0   | 0.8   | 3.0   | 1.2   |
| PCOC2           | Procollagen C-endopeptidase enhancer 2              | 30.6          | 2     | 0.9   | 0.9   | 1.3   | 9.1   |
| HPS2            | Heparanase-2                                         | 28.4          | 1     | 0.0   | 0.7   | 104.5 | –     |
| LAMMA5          | Laminin subunit alpha-5                              | 27.0          | 1     | –     | –     | –     | 3.6   |
| LRP6            | Low-density lipoprotein receptor-related protein 6   | 33.5          | 2     | –     | 0.9   | 0.0   | 0.0   |
| SEM7A           | Semaphorin-7A                                        | 44.4          | 2     | 2.4   | –     | –     | –     |
| APC             | Adenomatous polyposis coli protein                   | 30.5          | 1     | 1.2   | 3.5   | –     | 151.9 |
| CYBP            | Calcylcin-binding protein                            | 67.0          | 3     | 1.0   | 4.2   | 1.2   | 1.3   |
| MESDD2          | Mesoderm development candidate 2                    | 23.1          | 1     | 0.8   | 67.9  | –     | 1.4   |
| PIITK1          | Serine/threonine-protein kinase PIITK1-1             | 28.3          | 1     | 1.0   | 3.1   | –     | –     |
| MACF1           | Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 | 35.9          | 2     | 0.0   | 1.0   | 2.1   | 1.0   |
| PYG02           | Pygopus homolog 2                                    | 20.4          | 1     | –     | 4.3   | –     | –     |
| WNT7A           | Protein Wnt-7a                                       | 29.3          | 1     | –     | 0.0   | 12.1  | –     |
| GLIS2           | Zinc finger protein GLIS2                            | 21.4          | 1     | 0.0   | –     | –     | 3.9   |
| TFR1            | Transferrin receptor protein 1                       | 77.9          | 7     | 0.7   | 1.4   | 1.1   | 2.0   |
| [Other]         | A disintegrin and metalloproteinase with thrombospondin motifs 4 | 18.2          | 1     | –     | –     | –     | –     |
| CHD9            | Chromodomain-helicase-DNA-binding protein 9          | 32.5          | 1     | 0.0   | 0.0   | –     | –     |
| NLP             | Ninine-like protein                                  | 30.3          | 1     | 168.8 | 0.0   | 50.1  | –     |
| NO66            | Nucleolar protein 66                                 | 26.8          | 1     | –     | –     | –     | –     |
| PCNA            | Proliferating cell nuclear antigen                   | 229.7         | 10    | 2.5   | 1.8   | 1.0   | 0.9   |
| LBN             | Limbin                                              | 49.1          | 1     | –     | 10.1  | 0.5   | 1.2   |
| OMD             | Osteomodulin                                        | 31.6          | 1     | –     | 6.6   | –     | –     |
| TENC1           | Tensin-like C1 domain-containing phosphatase         | 27.4          | 1     | 0.5   | 10.6  | 0.7   | –     |
| ARSE            | Arylsulfatase E                                     | 26.0          | 1     | –     | –     | –     | –     |
| ZNRF2           | E3 ubiquitin-protein ligase ZNRF2                    | 26.3          | 2     | 0.0   | –     | 1.2   | 9.2   |

Over 2-fold changes in expression levels are displayed in shadowed boxes.

hMSCs give rise to osteoblasts to form bone. The process beings with the differentiation of osteoprogenitor cells into pre-osteoblasts, which eventually develop into mature osteoblasts. Subsequently, the mature osteoblasts will become entombed in an osteoid to become osteocytes. Osteoblast differentiation requires the expression of proteins associated with osteogenesis, immune system, and Wnt signaling. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and have been shown to participate in the patterning and specification of several tissues and organs during vertebrate development, as well as to regulate cell growth, apoptosis, and differentiation [29]. Further, they act as osteoinductive growth factors that may induce bone formation in vivo and in vitro.
| Protein ID | Name | Cytokine | Score | 100 | 1000 | 1000 | 1000 |
|-----------|------|----------|-------|-----|------|------|------|
| HNRPQ     | Heterogeneous nuclear ribonucleoprotein Q | 147.6 | 12 | 2.4 | 0.9 | 1.5 | 1.0 |
| I17R8     | Interleukin-1 receptor B | 38.9 | 1 | 2.7 | 1.0 | 0.9 | 1.6 |
| IL16      | Pro-interleukin-16 | 21.3 | 1 | 7.5 | 0.9 | – | – |
| IL1R1     | Interleukin-1 receptor type 1 | 27.0 | 1 | 0.8 | 0.2 | – | – |
| LK1H4     | Leukotriene A-4 hydrolase | 10.9 | 5 | 79.4 | 0.6 | 1.1 | 0.6 |
| NALP4     | NACHT, LRR and PYD domains-containing protein 4 | 22.7 | 1 | 6.5 | 0.0 | – | – |
| NIF1      | Nuclear-localized factor 1 | 24.5 | 1 | 2.8 | 0.4 | – | – |
| CCR9      | C-C chemokine receptor type 9 | 24.6 | 1 | – | - | – | 0.0 |
| GBP2      | Interferon-induced guanylate-binding protein 2 | 33.8 | 2 | – | 1.5 | – | – |
| K56AS     | Ribosomal protein S6 kinase alpha-5 | 28.8 | 1 | 1.5 | 6.5 | – | – |
| MAST2     | Microtubule-associated serine/threonine-protein kinase 2 | 28.4 | 1 | – | 12.7 | – | – |
| SENP5     | Senrin-specific protease 5 | 29.4 | 1 | – | 3.0 | 0.4 | – |
| UCRP      | Interferon-induced 17 kDa protein | 171.1 | 5 | – | 69.9 | 14.9 | 25.6 |
| CEBPG     | CCAAT/enhancer-binding protein gamma | 28.0 | 1 | – | – | 44.6 | – |
| IL5RB     | Interleukin-6 receptor subunit beta | 32.0 | 1 | – | – | 91.2 | 1.3 |
| NALP7     | NACHT, LRR and PYD domains-containing protein 7 | 15.0 | 1 | – | – | 12.9 | – |
| PAI1      | Plasminogen activator inhibitor 1 | 69.6 | 5 | 1.0 | 0.5 | 2.7 | 3.1 |
| TYK2      | Non-receptor tyrosine-protein kinase TYK2 | 24.9 | 1 | 1.0 | – | 29.1 | – |
| CSDE1     | Cold shock domain-containing protein E1 | 119.5 | 5 | 0.8 | 1.0 | 0.9 | 2.5 |
| ELAV1     | ELAV-like protein 1 | 84.0 | 4 | 0.8 | 1.0 | 0.4 | 27.2 |
| NALP4     | NACHT, LRR and PYD domains-containing protein 11 | 32.5 | 1 | – | – | – | 8.0 |
| SRCA      | Sarcalumin | 28.0 | 1 | 0.9 | 0.1 | 0.0 | 16.5 |
| ZCH11     | Zinc finger CCHC domain-containing protein 11 | 29.1 | 2 | – | 0.8 | – | 43.6 |

**Table 2**

Abbreviated list of immune system proteins induced in hMSCs by endotoxin.

| Protein ID | Name | Score | Peptide count | Expression ratio [LPS (+)/control] |
|-----------|------|-------|---------------|----------------------------------|
| FG2D      | FYVE, RhoGEF and PH domain-containing protein 2 | 22.9 | 101.4 | – | – | 1.1 |
| NLRX1     | NLR family member X1 | 28.5 | 3 | 24.8 | 0.0 | 0.7 | 1.0 |
| COMD5     | COMM domain-containing protein 5 | 38.5 | 1 | – | – | 3.4 | – |
| HBZ       | HTLV-1 basic zipper factor | 33.3 | 1 | – | – | 4.9 | 1.6 |
| JIP3      | C-jun-amino-terminal kinase-interacting protein 3 | 31.2 | 2 | 0.0 | 2.0 | 0.9 | 0.9 |
| JIP4      | C-jun-amino-terminal kinase-interacting protein 4 | 39.1 | 2 | 1.1 | 0.0 | 2.0 | – |
| UBR2K     | Ubiquitin-conjugating enzyme E2 R2 | 31.3 | 1 | – | – | 0.0 | – |
| IKLB      | NF-kappa-B inhibitor-like protein 2 | 27.5 | 1 | – | – | 8.1 | – |
| FER       | Proto-oncogene tyrosine-protein kinase FER | 23.8 | 1 | – | – | 3.4 | – |
| LYRIC     | Protein LYRIC | 32.0 | 1 | – | – | 1.2 | 3.3 |
| NPKB2     | Nuclear factor NF-kappa-B p100 subunit | 16.6 | 1 | – | – | 130.9 |
| SP5Y      | Spemmine synthase | 95.5 | 4 | 1.0 | 0.5 | 2.9 | 0.6 |
| TNIP1     | TNF receptor-interacting protein 1 | 37.7 | 2 | – | – | – | – |
| TRA2B     | Transform-2 protein homolog beta | 127.8 | 3 | 1.2 | 0.8 | 2.1 | 1.6 |
| PAIRB     | Plasminogen activator inhibitor 1 RNA-binding protein | 142.7 | 5 | 0.9 | 0.4 | 1.3 | 2.1 |
| NCOA3     | Nuclear receptor coactivator 3 | 31.0 | 2 | – | 1.3 | 1.4 | – |
| NLR5C     | Protein NLR5C | 29.8 | 2 | – | 1.0 | – | 19.5 |
| NLR5C     | Protein NLR5C | 38.8 | 3 | 0.8 | 0.7 | 0.6 | 2.1 |

**[Other]**

| Protein ID | Name | Score | Peptide count | Expression ratio [LPS (+)/control] |
|-----------|------|-------|---------------|----------------------------------|
| ITIT3     | Interferon-induced protein with tetratricopeptide repeats 3 | 49.5 | 4 | 42.4 | 2.6 | 21.2 | – |
| M4K2      | Mitogen-activated protein kinase kinase kinase kinase 2 | 19.1 | 1 | 56.5 | – | – | 0.0 |
| NF2P2     | NFATC2-interacting protein | 24.0 | 1 | 5.4 | – | – | 0.0 |
| PO210     | Nuclear pore membrane glycoprotein 210 | 25.3 | 1 | – | – | 0.1 | – |
| PMSE1     | Proteasome activator complex subunit 1 | 119.6 | 7 | 2.8 | 1.2 | 21.2 | 2.7 |
| SIN1A     | Paired amphilactic helix protein Sin3a | 30.8 | 1 | – | – | – | – |
| SODM      | Superoxide dismutase [Mn], mitochondrial | 301.4 | 15 | 2.7 | 14.1 | 3.7 | 9.6 |
| TRAP      | Transformation/transcription domain-associated protein | 25.3 | 1 | – | – | 0.0 | – |
| ZBT2      | Zinc finger and BTB domain-containing protein 32 | 25.6 | 1 | 44.6 | – | – | – |
| IFT1      | Interferon-induced protein with tetratricopeptide repeats 1 | 43.7 | 1 | – | 20.0 | 57.4 | – |
| IK2F3     | Zinc finger protein Aiolos | 26.9 | 1 | – | 21.8 | – | – |
| ITLN2     | Interleukin-2 | 29.4 | 1 | – | – | 3.0 | 0.4 |
| M3K7      | Mitogen-activated protein kinase kinase 7 | 27.3 | 1 | – | 9.1 | – | – |
| NFAC1     | Nuclear factor of activated T-cells, cytoplasmic 1 | 25.4 | 1 | – | 25.6 | – | – |
| PAVR      | PRKC apoptosis WT1 regulator protein | 61.3 | 2 | – | 2.3 | 1.6 | 1.2 |
| SEM3B     | Semaphorin-3B | 26.3 | 1 | – | 3.1 | – | – |
| TACT      | T-cell surface protein tactile | 38.0 | 1 | – | – | 98.4 |
| TNAF5     | Tumor necrosis factor, alpha-induced protein 2 | 21.1 | 1.9 | 3.5 | – | – | – |
| TRIS6     | Tripartite motif-containing protein 56 | 33.1 | 1 | – | 3.7 | – | – |

(continued on next page)
chicken limb buds suggests that signaling through this receptor also can regulate chondrocyte differentiation [32]. FGF2 exerts both positive and negative effect on the growth and differentiation of MSCs. E.g., it was shown to promote osteoblast differentiation by inducing the osteocalcin gene expression in MSCs and enhances calcium deposition [33,34]. GREB1 is up-regulated in both positive and negative effect on the growth and differentiation of MSCs. E.g., it was shown to promote osteoblast differentiation by inducing the osteocalcin gene expression in MSCs and enhances calcium deposition [33,34]. GREB1 is up-regulated in

Table 2
Abbreviated list of ion homeostasis proteins induced in hMSCs by endotoxin.

| Protein ID | Name | Peptide count | Expression ratio [LPS(+) /control] |
|------------|------|---------------|----------------------------------|
| Code       | Score| Day 1 | Day 2 | Day 3 | Day 4 |
| AT1B3      | 40.2 | 2    | 4.7   | 2.6   | 0.8  | 1.3  |
| DIP6       | 30.9 | 2    | 2.2   | 0.0   | –    | –    |
| GP143      | 34.4 | 1    | 4.5   | 1.7   | –    | –    |
| KCAB3      | 25.3 | 1    | –     | 1.4   | –    | –    |
| KN29A      | 25.3 | 1    | –     | 1.2   | –    | –    |
| SCN4A      | 25.3 | 1    | –     | 0.0   | 0.5  | –    |
| SCN4B      | 33.4 | 2    | 50.6  | 0.1   | 2.3  | 1.2  |
| SCN5B      | 25.7 | 2    | 416.5 | 0.0   | 1.8  | –    |
| SL9A4      | 27.6 | 1    | 67.8  | –     | –    | –    |
| UNC79      | 26.4 | 1    | –     | –     | –    | –    |
| WEE2       | 35.8 | 1    | 2.9   | –     | –    | –    |
| AT2C1      | 32.5 | 2    | –     | –     | 20.5 | –    |
| MX1        | 90.9 | 7    | 1.0   | 8.6   | –    | –    |
| RYR3       | 25.1 | 1    | 1.2   | 96.3  | –    | –    |
| S39AD      | 33.6 | 1    | –     | 33.1  | –    | –    |
| WNK3       | 44.3 | 12   | –     | 32.4  | 4.3  | –    |
| ACDC       | 241.2| 13   | 1.0   | 1.7   | 4.0  | 2.4  |
| AT2B1      | 114.1| 2    | 1.1   | 1.0   | 73.6 | 0.5  |
| AT2B3      | 92.8 | 1    | 1.1   | 1.0   | 73.6 | 0.9  |
| AT2B4      | 118.8| 5    | 0.8   | 1.0   | 3.2  | 1.0  |
| CAC3B      | 26.0 | 2    | –     | 0.1   | 247.1| –    |
| DYSF       | 40.2 | 2    | 1.8   | 1.0   | 2.9  | 1.4  |
| TRPC1      | 29.2 | 1    | –     | –     | 103.7| –    |
| TRPM5      | 31.0 | 1    | –     | 0.7   | –    | 1.3  |
| CA2D2      | 31.3 | 1    | –     | –     | –    | 2.6  |
| CA1D      | 35.2 | 1    | 1.4   | 0.0   | –    | 79.7 |
| SCN2B      | 28.0 | 1    | 0.9   | 0.1   | 0.0  | 16.3 |

Table 3
Abbreviated list of ion homeostasis proteins induced in hMSCs by endotoxin.

| Protein ID | Name | Peptide count | Expression ratio [LPS(+) /control] |
|------------|------|---------------|----------------------------------|
| Code       | Score| Day 1 | Day 2 | Day 3 | Day 4 |
| AT1B3      | 40.2 | 2    | 4.7   | 2.6   | 0.8  | 1.3  |
| DIP6       | 30.9 | 2    | 2.2   | 0.0   | –    | –    |
| GP143      | 34.4 | 1    | 4.5   | 1.7   | –    | –    |
| KCAB3      | 25.3 | 1    | –     | 1.4   | –    | –    |
| KN29A      | 25.3 | 1    | –     | 1.2   | –    | –    |
| SCN4A      | 25.3 | 1    | –     | 0.0   | 0.5  | –    |
| SCN4B      | 33.4 | 2    | 50.6  | 0.1   | 2.3  | 1.2  |
| SCN5B      | 25.7 | 2    | 416.5 | 0.0   | 1.8  | –    |
| SL9A4      | 27.6 | 1    | 67.8  | –     | –    | –    |
| UNC79      | 26.4 | 1    | –     | –     | –    | –    |
| WEE2       | 35.8 | 1    | 2.9   | –     | –    | –    |
| AT2C1      | 32.5 | 2    | –     | –     | 20.5 | –    |
| MX1        | 90.9 | 7    | 1.0   | 8.6   | –    | –    |
| RYR3       | 25.1 | 1    | 1.2   | 96.3  | –    | –    |
| S39AD      | 33.6 | 1    | –     | 33.1  | –    | –    |
| WNK3       | 44.3 | 12   | –     | 32.4  | 4.3  | –    |
| ACDC       | 241.2| 13   | 1.0   | 1.7   | 4.0  | 2.4  |
| AT2B1      | 114.1| 2    | 1.1   | 1.0   | 73.6 | 0.5  |
| AT2B3      | 92.8 | 1    | 1.1   | 1.0   | 73.6 | 0.9  |
| AT2B4      | 118.8| 5    | 0.8   | 1.0   | 3.2  | 1.0  |
| CAC3B      | 26.0 | 2    | –     | 0.1   | 247.1| –    |
| DYSF       | 40.2 | 2    | 1.8   | 1.0   | 2.9  | 1.4  |
| TRPC1      | 29.2 | 1    | –     | –     | 103.7| –    |
| TRPM5      | 31.0 | 1    | –     | 0.7   | –    | 1.3  |
| CA2D2      | 31.3 | 1    | –     | –     | –    | 2.6  |
| CA1D      | 35.2 | 1    | 1.4   | 0.0   | –    | 79.7 |
| SCN2B      | 28.0 | 1    | 0.9   | 0.1   | 0.0  | 16.3 |

Over 2-fold changes in expression levels are displayed in shadowed boxes.

preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis. Mutations in the gene encoding a Wnt co-receptor, the low-density lipoprotein receptor-related protein 5 (LRP5), are causally linked to the alterations in human bone mass [36]. LRP6 is another cell-surface co-receptor for Wnt signaling, and plays a pivotal role in bone formation. Msx2-interacting nuclear target protein (MINT) synergizes with RUNX2 to enhance FGF2-mediated activation of the osteocalcin FGF-responsive element in osteoblasts. TLRs play an important role in the immune system by participating in the initial recognition of microbial pathogens and pathogen-associated components. Further, TLR agonists can affect the proliferation and differentiation of hMSCs [8,10–12].
Fig. 4. List of protein functions. Molecular function (a), cellular components (b), and biological processes (c).
In the current study, although the levels of TLR-1 and 3 were not affected by endotoxin exposure, TLR-4 levels increased on day 1, suggesting that all changes in the osteoblast differentiation ability and protein expression in hMSCs originated from signal transduction via TLR-4, an endotoxin receptor. The supersoxide dismutase and some cytokine levels were elevated on day 1. Subsequently, the levels of NF-kB-related proteins increased. Furthermore, the presence of endotoxin resulted in the increase in levels of osteogenesis- and vitamin-related proteins, growth factors, etc., and induced Wnt/b-catenin signaling, which promotes bone morphogenesis. These data indicated that endotoxin enhanced the osteoblast differentiation capacity of hMSCs. On the other hand, preliminary DNA array analysis revealed that the expression of genes encoding an LPS-binding protein and alkaline phosphatase was induced upon endotoxin stimulation (data not shown). This indicated poor correlation between proteomics and DNA microarray data for these proteins. However, changes in the levels of TLR, BMP, cytokine, Wnt/b-catenin signaling, and NF-kB-related proteins, etc., showed good correlation with the microarray data (data not shown).

The differentiation of MSCs towards adipogenic or osteogenic cells depend on a variety of signaling and transcription factors. The other hand, several lines of experimental evidence suggest that an inverse correlation exists between adipogenesis and osteogenesis [37]. Indeed, in the current study, endotoxin was shown to affect the differentiation of hMSCs into osteoblasts but not adipocytes, although the detailed mechanism whereby endotoxin promotes the osteoblast differentiation of hMSCs remains unknown. Collectively, the presented data may be used for the specification of endotoxin limit for biomaterials used for osteogenesis. Quantitative analyses to establish the endotoxin limit for the in vitro proliferation and differentiation ability of induced pluripotent stem cells, another cell source for regenerative medicine, are currently in progress in our laboratory.

5. Conclusions

The current study constitutes follow-up research for a previous report [19], describing endotoxin specifications for MSC proliferation, with applications in tissue engineering. The NOAEL for the enhancement of osteoblast differentiation capacity, observed in an in vitro culture system, was 0.1 ng/ml (2.75 EU/ml). Future studies should focus on determining the limits for the proliferation or differentiation capacity of induced pluripotent stem cells.

Conflicts of interest

The authors declare no conflicts of interest.

References

[1] Arahia T, Todo M. Effects of proliferation and differentiation of mesenchymal stem cells on compressive mechanical behavior of collagen/beta-TCP composite scaffold. J Mech Behav Biomed Mater 2014;39:218–30.
[2] Chen G, Lu Y, Dong C, Yang L. Effect of internal structure of collagen/hydroxyapatite scaffold on the osteogenic differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther 2015;10:99–108.
[3] Sun K, Li H, Li R, Nian Z, Li D, Xu C. Silk fibroin/collagen and silk fibroin/chitosan blended three-dimensional scaffolds for tissue engineering. Eur J Orthop Surg Traumatol 2015;25:243–9.
[4] Snyder TN, Madhavan K, Intrator M, Dregalla RC, Park D. A fibrin/hyaluronic acid hydrogel for the delivery of mesenchymal stem cells and potential for articular cartilage repair. J Biol Eng 2014;8:10.
[5] Hiwatashi N, Hirano S, Mizuta M, Tatemya I, Kanemaru S, Nakamura T, et al. Biocompatibility and efficacy of collagen/gelatin sponge scaffold with sustained release of basic fibroblast growth factor on vocal fold fibroblasts in 3-dimensional culture. Ann Otol Rhinol Laryngol 2015;124:116–25.
[6] Sapir Y, Ruvinov E, Polyak B, Cohen S. Magnetically actuated alginate scaffold: a novel platform for promoting tissue organization and vascularization. Methods Mol Biol 2014;1113:49–55.
[7] Curtin CM, Tierney EG, McSorley K, Cryan SA, Duffy GP, O’Brien FJ. Combinatorial gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. Adv Healthc Mater 2015;4:223–7.
[8] Pevsner-Fischer M, Morad V, Cohen-Sfady M, Rousso-Noori L, Zanin-Zhorov A, Cohen S, et al. Toll-like receptors and their ligands control mesenchymal stem cell functions. Blood 2007;109:1422–32.
[9] Faya Cho H, Bae YC, Jung JS. Role of toll-like receptors on human adipose-derived stem cells. Stem Cells 2006;24:7442–52.
[10] Shi J, Liu XM, Hu YY, Wang JS, Fang Q. The effect of lipopolysaccharide on the expression and activity of Toll-like receptor 4 in mesenchymal stem cells. Zhonghua Xue Ye Xue Za Zhi 2006;28:828–31.
[11] Wang ZJ, Zhang FM, Wang LS, Yao YW, Zhao Q, Gao X. Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor (TLR)-4 and PI3K/Akt. Cell Biol Int 2009;33:665–74.
[12] He X, Wang H, Jin T, Xu Y, Mei L, Yang J. TLR4 activation promotes bone marrow MSC proliferation and osteogenic differentiation via Wnt3a and Wnt5a signaling. PLoS One 2016;11:e0149876.
[13] Retschel ETBL, Schade U, Seydel U, Zähner U, Lindner B, Morgan AP, et al. Chemical structure and biological activity of lipopolysaccharides. In: Baumgartner JD, Calandra T, Carlet J, editors. Endotoxin from pathophysiology to therapeutic approaches. Paris: Flammarion Medicine-Sciences; 1990. p. 11–18.
[14] Retschel E, Wollenweber HW, Zähner U, Lindner B, Morgan AP, et al. Chemical and biological activity of lipopolysaccharides. In: Baumgartner JD, Calandra T, Carlet J, editors. Endotoxin from pathophysiology to therapeutic approaches. Paris: Flammarion Medicine-Sciences; 1990. p. 11–18.
[15] Yang H, Kaneko M, He C, Hughes MA, Cherry GW. Effect of a lipopolysaccharide from E. coli on the proliferation of fibroblasts and keratinocytes in vitro. Photother Res 2002;16:43–7.
[16] Hayakawa T, Aoi T, Umezu M, Otsawa K, Sato Y, Sawa Y, et al. A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from the processing of autologous human somatic stem cells. Regen Ther 2015;2:57–69.
[17] Nomura Y, Fukui C, Morishita Y, Hashima Y. A biological study establishing the endotoxin limit for in vitro proliferation of human mesenchymal stem cells. Regen Ther 2017;7:45–51.
[18] Westphal G, Jann K. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr Chem 1965;5:83–91.
[19] Hashima Y, Murai T, Nakagawa Y, Hirata M, Yagami T, Nakamura A, Chemical and biological evaluation of endotoxin contamination on natural rubber latex products. J Biomed Mater Res 2001;55:424–32.
[20] Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. Am J Physiol Cell Physiol 2008;294:C675–83.
[21] Daly KA, Liu S, Agrawal Y, Brown BN, Huber A, Johnson SA, et al. The host response to endotoxin-contaminated dental matrix. Tissue Eng Part A 2012;18:1293–303.
[22] Ho TY, Chen YS, Hisang CY. Noninvasive nuclear factor–kappaB bioluminescence imaging for the assessment of host-biomaterial interaction in transgenic mice. Biomaterials 2007;28:4370–7.
[23] Martinez Avila H, Schwarz S, Feldmann EM, Mantas A, von Bombard A, Catenholm P, et al. Biocompatibility evaluation of densified bacterial nanocelluloselike hydrogel as an implant material for auricular cartilage regeneration. Appl Microbiol Biotechnol 2014;98:7423–35.
[24] van Putten SM, Wubben M, Wubben M, Plantinga JA, Hennek WE, van Luijn JM, et al. Homma JY, Kanegasaki S, Lüderitz O, Shiba T, Westphal O, editors. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr Chem 1965;5:83–91.
[25] Hashima Y, Hasegawa C, Todoki K, Lüderitz O, Westphal O, editors. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr Chem 1965;5:83–91.
[26] Curtin CM, Tierney EG, McSorley K, Cryan SA, Duffy GP, O’Brien FJ. Combinatorial gene therapy accelerates bone regeneration: non-viral dual delivery of VEGF and BMP2 in a collagen-nanohydroxyapatite scaffold. Adv Healthc Mater 2015;4:223–7.
[27] Schulte RJ, Xie L, Klitzmann B, Reichert WM. In vivo cytokine-associated responses to biomaterials. Biomaterials 2009;30:160–8.
[28] Hashima Y, Hasegawa C, Todoki K, Sasaki K, Niimi S, Ozono S. A biological study establishing the endotoxin limit of biomaterials for bone regeneration in cranial and femoral implantation of rats. J Biomed Mater Res A 2011;98:527–34.
[29] Oszczka AM, Leboy PS. Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase signaling. Endo
terocrinology 2005;146:3428–37.
[30] Cunningham NS, Jenkins NA, Gilbert DJ, Copeland NG, Reddi AH, Lee SJ. Growth/differentiation factor-10: a new member of the transforming growth factor-beta superfamily related to bone morphogenetic protein-3. Growth Factors 1995;12:99–109.
[31] Hino J, Kangawa K, Matsuo H, Nohno T, Nishimatsu S. Bone morphogenetic protein-3 family members and their biological functions. Front Biosci 2004;9:1520–9.

[32] Zou H, Wieser R, Massague J, Niswander L. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev 1997;11:2191–203.

[33] Hanada K, Dennis JE, Caplan AI. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. J Bone Miner Res 1997;12:1606–14.

[34] Scutt A, Bertram P. Basic fibroblast growth factor in the presence of dexamethasone stimulates colony formation, expansion, and osteoblastic differentiation by rat bone marrow stromal cells. Calcif Tissue Int 1999;64:69–77.

[35] Liu T, Gao Y, Sakamoto K, Minamizato T, Furukawa K, Tsukazaki T, et al. BMP-2 promotes differentiation of osteoblasts and chondroblasts in Runx2-deficient cell lines. J Cell Physiol 2007;211:728–35.

[36] Krishnan V, Bryant HH, Macdougald OA. Regulation of bone mass by Wnt signaling. J Clin Invest 2006;116:1202–9.

[37] James AW. Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. Scientifica (Cairo) 2013;2013, 684736.