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Inflammatory stimuli differentially modulate the transcription of paracrine signaling molecules of equine bone marrow multipotent mesenchymal stromal cells

R. Vézina Audette, A. Lavoie-Lamoureux, J.-P. Lavoie, S. Laverty*

Comparative Orthopedic Research Laboratory, Département de sciences cliniques, Faculté de Médecine vétérinaire, Université de Montréal, St Hyacinthe, QC, Canada

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SUMMARY

Objective: Osteoarthritis (OA) is a degenerative disease of joint tissues that causes articular cartilage erosion, osteophytosis and loss of function due to pain. Inflammation and inflammatory cytokines in synovial fluid (SF) contribute to OA progression. Intra-articular (IA) injections of multipotent mesenchymal stromal cells (MSCs) are employed to treat OA in both humans and animals. MSCs secrete paracrine pro-inflammatory and anabolic signaling molecules that promote tissue repair. The objective of this study was to investigate the effects of OASF on the gene expression of paracrine signaling molecules by MSCs.

Methods: The effects of Lipopolysaccharide (LPS) and interleukin (IL)-1β as well as both normal (N) and osteoarthritis (OA) SF stimulations on the expression of paracrine pro-inflammatory (tumor necrosis factor (TNF)-α, IL-1β, IL-8), modulatory (IL-6) and anabolic (vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1 and insulin-like growth factor (IGF)-1) signaling molecules by equine bone marrow multipotent mesenchymal stromal cells (eBM-MSCs) was investigated employing reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: In contrast with NSF, OASF significantly up-regulated the expression of VEGF in eBM-MSCs. Both NSF and OASF significantly down-regulated the expression of IL-1β. LPS and IL-1β significantly increased the expression of pro-inflammatory cytokines (TNF-α, IL-8 and IL-6; and IL-1β and IL-8 respectively).

Discussion: We conclude that the transcription of paracrine signaling molecules in eBM-MSCs is modulated by SF. Furthermore, OA alters the properties of SF and the response of eBM-MSCs. Finally, the effects of LPS or IL-1β stimulation are distinct to that observed following stimulations with OASF.

Introduction

Multipotent mesenchymal stromal cells (MSCs) secrete trophic factors with anti-inflammatory properties

In recent years, there has been a paradigm shift in the understanding of multipotent MSCs potential therapeutic mechanisms: They may exert their effects by environment-dependent secretion of paracrine signaling molecules rather than differentiation into target tissue cells[1,2]. MSCs undergo genome-wide expression changes in an inflammatory microenvironment[3,4] and secrete paracrine signaling molecules that modulate the innate immune response and trophic factors that influence cell response to injury[5,6]. Evidence, from both *in vitro* and *in vivo* studies, suggests that MSCs may act as cellular sentinels of inflammation and tissue damage that respond by secreting a plethora of signaling molecules that may also assist tissue repair[7]. These molecules include growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and transforming growth factor β (TGF-β1)[8,9], cytokines such as TNF-α and interleukin (IL)-6 and chemokines such as IL-8[3,10]. These molecules influence a wide range of biological processes that include proliferation, differentiation, migration, and apoptosis.

MSC therapy for osteoarthritis (OA)

OA is a progressive, degenerative disease of joint tissues that results in a characteristic articular cartilage erosion, osteophytosis

* Address correspondence and reprint requests to: S. Laverty, Comparative Orthopaedic Research Laboratory, Département de sciences cliniques, Faculté de médecine vétérinaire, Université de Montréal, CP 5000, St Hyacinthe, Québec J2S 7C6, Canada. Tel: 1-514-343-6111x8267; Fax: 1-450-778-8158.
E-mail address: sheila.laverty@umontreal.ca (S. Laverty).

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and loss of function due to pain. Poor repair capacity and chondrocyte loss are characteristic features of this disease. Inflammation is also recognized as an important component of the pathobiology of OA and IL-1β, TNF-α and IL-6 were shown to be key cytokines involved in OA[11]. IL-1β itself drives the degradation of the articular cartilage matrix[12]. An optimal therapeutic approach to OA would ideally reduce joint inflammation and concurrently stimulate joint tissue repair. Intra-articular (IA) MSC therapy offers this potential.

IA injections of MSCs lead to a significant decrease in cartilage degeneration, subchondral sclerosis, osteophytosis and promote meniscal regeneration at the site of meniscal resection in surgical joint instability models[13]. In addition, MSCs appear to contribute to the repair of damaged articular tissue[13] *via* their engraftment and the production of extracellular matrix components locally[14]. IA BM-MSCs also produce a significant decrease in synovial prostaglandin e2 (PGE2) levels in OA joints, suggesting that IA eBM-MSCs have the capacity to modulate inflammation in OA joints[15].

As pre-existing joint inflammation may impact the secretory profile of MSCs and alter their therapeutic efficacy[16], it is crucial to determine the effect of inflammatory microenvironments on the expression of paracrine signaling molecules by BM-MSCs. There is still a considerable knowledge gap concerning the behavior, biology and effects of IA MSCs, particularly in the context of an injured joint. This knowledge is essential toward optimization of this form of therapy.

Synovial fluid (SF) molecules signal to MSCs

Few studies have been conducted on the influence of SF from diseased joints on MSC function. Most have focused on the pro-migratory and differentiation effects of SF on MSCs[2,17–20]. Healthy SF contains chemotactic signaling molecules that stimulate MSC migration, and are significantly decreased in OASF and associated with slower fracture repair[17]. *In vitro* stimulation of MSCs with SF from rheumatoid arthritis joints elicits potent migratory responses in MSCs, and, to a lesser degree, OASF[19]. OASF also stimulates the expansion of synovium-resident MSC populations[18]. Coleman and colleagues also reported that 5% SF from OA articulations significantly inhibited chondrogenesis *in vitro* by MSCs[2]. The changes in SF composition observed in naturally occurring OA may alter the gene expression profile of MSCs and their production of paracrine anabolic and pro-inflammatory molecules[5,10].

A complete understanding of the inducible changes in gene expression of MSCs in an IA environment is very important from a clinical perspective as stem cell therapy is currently being investigated for joint disease therapy with an accompanying inflammatory component that could potentially influence the secretory profile and subsequent efficacy of the administered cells. Although it has been previously demonstrated that MSCs react to the presence of inflammatory cytokines and growth factors, the specific aim of this study was to characterize the gene expression profile of equine bone marrow multipotent mesenchymal stromal cells (eBM-MSCs) to the inflammatory molecules present in OASF *in vitro*. Using RT-PCR, we studied the expression of paracrine pro-inflammatory molecules such as cytokines and chemokines (IL-1β, TNF-α, IL-6 and IL-8) as well as paracrine anabolic molecules (TGF-β1, VEGF, IGF-1) in eBM-MSCs in response to stimulation with lipopolysaccharide (LPS), IL-1β or SF from healthy and OA-affected articulations.

Materials and methods

Experimental procedures performed were approved by the Animal Care and use Committee of the Faculty of Veterinary Medicine, Université de Montréal.

Collection of SF

Equine limbs were obtained from a local abattoir. SF was aseptically harvested from normal (NSF: $N = 3$) or OA (OASF: $N = 2$) joints based on the macroscopic appearance (presence of articular cartilage erosions and osteophytes) of the articular surfaces.

SF samples were transferred to heparin-treated tubes, were filtered with 45 μm filters and centrifuged at 1500 rpm for 12 min at 12°C (Beckman Coulter, Brea, California, USA). Aliquots were stored at −20°C.

MSC isolation and culture

Bone marrow samples from healthy horses ($n = 3$) (6–9 years old) were harvested in heparinized tubes, and eBM-MSCs were isolated using gradient density separation techniques as described previously[21].

Induction of MSC into mesenchymal lineages

eBM-MSCs differentiation into adipocyte, osteoblast and chondrocyte lineages was confirmed as we described previously[21] (data not shown).

MSC stimulation

eBM-MSCs from each horse were seeded, separately, in six-well dishes. When the cells reached 80% confluence, undifferentiated cells were washed with Dulbecco's phosphate buffered saline (DPBS) and stimulated with LPS (100 ng/ml) (Sigma–Aldrich, Oakville, On; Clone number 0127:B8), IL-1β (10 ng/ml) (Sigma–Aldrich, catalogue# I9401) and SFs either normal (NSF) or OA (OASF) following filtration through 0.45 μm filters (Sarstedt). Unstimulated wells served as controls. Cells from all horses were subjected to each condition (including stimulations and control untreated) in duplicate wells. LPS, a bacterial endotoxin, is a recognized stimulus for *in vitro* models of inflammation and induces the expression of pro-inflammatory cytokines at this concentration in equine species[22]. The dose of IL-1β (10 ng/ml) was employed as it is frequently used to model OA *in vitro*[23]. 10% SF dilutions were employed as described by Song and colleagues[19].

Each treatment was carried out in duplicate for 1, 3, 6, 12 and 24 h in a total volume of 2.5 ml per well. At experimental endpoints, cells were washed with DPBS and incubated at 37°C for 7 min with 1.5 ml of trypsin 0.25% (Invitrogen) per well to detach adherent cells from the culture surface. The cells were collected, duplicates were pooled and centrifuged at 1500 rpm for 10 min at 18°C. The supernatant was discarded and the pellet was resuspended in 1 ml of media and transferred to a 2 ml Eppendorf tube and centrifuged for 2 min.

Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Each cell pellet from pooled duplicates was resuspended in 1 ml of Trizol® Reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. RNA concentration in diethylpyrocarbonate (DEPC)-treated H_2O was measured spectrophotometrically (Nanodrop1000, ThermoScientific, Wilmington, DE, USA). We then reverse transcribed 2 μg into cDNA using the Superscript III Reverse Transcription System (Invitrogen) in the presence of RNasin Ribonuclease Inhibitor (60 U, Promega, Fisher Scientific) using oligo$(dT)_{12-18}$ primers (500 ng, Invitrogen) in a

final 30 μl reaction volume. The resulting volume was then diluted to a total volume of 120 μl (4× dilution) with RNAse-free water.

Quantitative PCR (qPCR)

The cDNA from pooled duplicate cells of each horse was used to assess the levels of each gene of interest outlined above using qPCR. For each gene of interest and housekeeping genes, cDNA from each horse was studied in duplicates. Pipetting of the reaction mix was performed using the QiAgility (Qiagen, Mississauga, ON, Canada) and in duplicate. qPCR was performed using a RotorGene thermal cycler (Rotor-Gene Real-Time Centrifugal DNA Amplification System 3000, Corbett Research, Montreal Biotech, Montreal, QC, Canada) and the Quantitect SYBR Green PCR kit (Quantitect SYBR Green PCR kit, Qiagen, Mississauga, ON, Canada). Reactions were run in a volume of 20 μl using 4 μl of diluted cDNA. The concentration of oligonucleotide primers in each reaction was 5 μM. The amplification protocols are listed in Table I. Finally a melting curve using 0.5°C steps of 10 s duration starting from 57°C was determined.

Quantification was performed using optimized gene-specific standard curves made of serial dilutions (10×) of polymerase chain reaction (PCR) products (QIAGEN's Gel Extraction Kit) with reproducible efficiency coefficients between 95% and 105% and a calibrator sample in the target run. Absolute values were corrected relative to ubiquitin expression used as a reference gene. The equine specific primers for paracrine pro-inflammatory cytokines (TNF-α, IL-1β, IL-8), the modulatory cytokine (IL-6) and the anabolic signaling molecules (VEGF, IGF-1 and TGF-β1) employed are listed in Table I.

Statistical analysis

qPCR data is reported as mean ($N = 3$) fold increase representing the ratio of stimulated cell normalized gene expression over control unstimulated cell normalized gene expression (corresponding to a value of 1). The resulting fold increase values were transformed to a log scale, and a repeated-measures analysis of variance (ANOVA) analysis was performed to investigate the effects of stimulations (LPS, IL-1β, NSF and OASF) and timepoints (1, 3, 6, 12 and 24 h). 95% confidence intervals are provided for the fold increases or decreases. A priori contrasts were carried out between pairs of means, and alpha levels for each comparison were adjusted using the sequential Bonferroni procedure. Statistical analyses were carried out with SAS v. 9.3 (Cary, NC). The level of statistical significance was set at 0.05 throughout.

Results

The level of mRNA in the control wells at the 3 h timepoint for one of the three experiments was too low to analyze and the ratios between stimulated vs control could not be used. Thus, at the 3 h timepoint, only an $N = 2$ is used for analysis of our data. This was acceptable for the statistical analyses we performed.

eBM-MSC response to LPS stimulation

LPS stimulation provoked a rapid but transient increase in the transcription of TNF-α mRNA in eBM-MSCs after 1 h (23 fold (9.6–56.4), $P < 0.0001$, $n = 3$) and 3 h (seven fold (2.3–19.6), $P = 0.001$, $n = 2$) when compared to control non-stimulated cells [Fig. 1(A)]. Whereas no change in IL-1β gene expression occurred [Fig. 1(B)], a rapid increase in the transcription of IL-8 mRNA was observed following LPS stimulation relative to unstimulated controls [Fig. 1(C)] at the earliest timepoint of 1 h (nine fold (3.6–22.9), $P < 0.0001$, $n = 3$) and persisted at all timepoints to 24 h (3 h: nine fold (3.2–27.3), $P = 0.0003$, $n = 2$, 6 h: five fold (1.9–12.3), $P = 0.002$, $n = 3$, 12 h: 13 fold (5.0–32.2), $P < 0.0001$, $n = 3$, 24 h: six fold (2.2–14.5), $P = 0.0008$, $n = 3$). eBM-MSCs stimulated with LPS significantly up-regulated IL-6 at 3 h only (510 fold (19.1–13638.4), $P = 0.0007$, $n = 2$) relative to controls [Fig. 1(D)]. IL-1β, VEGF, TGF-β1 and IGF-1 gene expression did not change following exposure of eBM-MSC to LPS when compared with unstimulated controls at any timepoint [Fig. 2(A–C)].

eBM-MSC response to IL-1β stimulation

Stimulation with IL-1β induced an increase in IL-1β, which was significant at 1 h (10 fold (2.9–31.6), $P = 0.0007$, $n = 3$) and 12 h (11 fold (3.3–35.1), $P = 0.0005$, $n = 3$) in comparison to controls [Fig. 1(B)]. A significantly enhanced expression of IL-8 was also observed when eBM-MSCs were stimulated with IL-1β at 1 h (five fold (1.9–11.9), $P = 0.002$, $n = 3$), 3 h (seven fold (2.3–19.8), $P = 0.001$, $n = 2$), 12 h (nine fold (3.3–21.5), $P = 0.0001$, $n = 3$) and 24 h (10 fold (3.9–25.3), $P < 0.0001$, $n = 3$) [Fig. 1(C)]. TNF-α, IL-6, VEGF, TGF-β1 and IGF-1 gene expression did not change following exposure of eBM-MSCs to IL-1β when compared with unstimulated controls at any timepoint [Figs. 1(A and D) and 2(A–C)].

eBM-MSC response to SF stimulation

IL-1β expression was significantly down-regulated after 24 h exposure to NSF and OASF (NSF: 0.10 fold decrease (0.031–0.33),

Table I
List of primers and amplification protocols used for qPCR

	Target	Forward primer	Reverse primer	Amplification protocol
Pro-inflammatory	TNF-α	CTTGTGCCTCAGCCTCTTCTCCTTC	CAGCTGGTTGTCTGTCAGCTTC	95°C 10 min (polymerase activation), 55 cycles of 95°C 15 s, 55°C 25 s, 72°C 35 s
	IL-1β	GACTGACAAGATACCTGTGGCCT	AGACAACAGTGAAGTGCAGCCT	95°C 10 min (polymerase activation), 55 cycles of 95°C 15 s, 55°C 25 s, 72°C 25 s
	IL-8	CTTTCTGCAGCTCTGTGTGAAG	GCAGACCTCAGCTCCGTTGAC	95°C 10 min (polymerase activation), 50 cycles of 95°C 15 s, 63°C 25 s, 72°C 20 s
Modulatory	IL-6	CCTGGTCCAGATCCTGATGCAAAA	AAGGCTTCGAAGGATGAGGTGAGT	95°C 10 min (polymerase activation), 65 cycles of 95°C 15 s, 56°C 25 s, 72°C 20 s
Anabolic	VEGF	TGCAACGACGAGGGCCTA	ACTGTTCGGCTCCGCCA	95°C 10 min (polymerase activation), 65 cycles of 95°C 15 s, 59°C 25 s, 72°C 20 s
	TGF-β1	CAGCATGTGGAGCTGTACCAGAAA	TGACATCAAAGGACAGCCATTCCG	95°C 10 min (polymerase activation), 55 cycles of 95°C 15 s, 55°C 25 s, 72°C 25 s
	IGF-1	GTGTGTGGAGACAGGGGCTTTTAT	ACTTCCTTCTGAGCCTTGGGCATA	95°C 10 min (polymerase activation), 55 cycles of 95°C 15 s, 55°C 20 s, 72°C 20 s
Reference	UBQ	TAGCAGTTTCTTCGTGTCCGT	TGTAATCGGAAAGAGTGCGG	95°C 10 min (polymerase activation), 50 cycles of 95°C 15 s, 60°C 25 s, 72°C 20 s

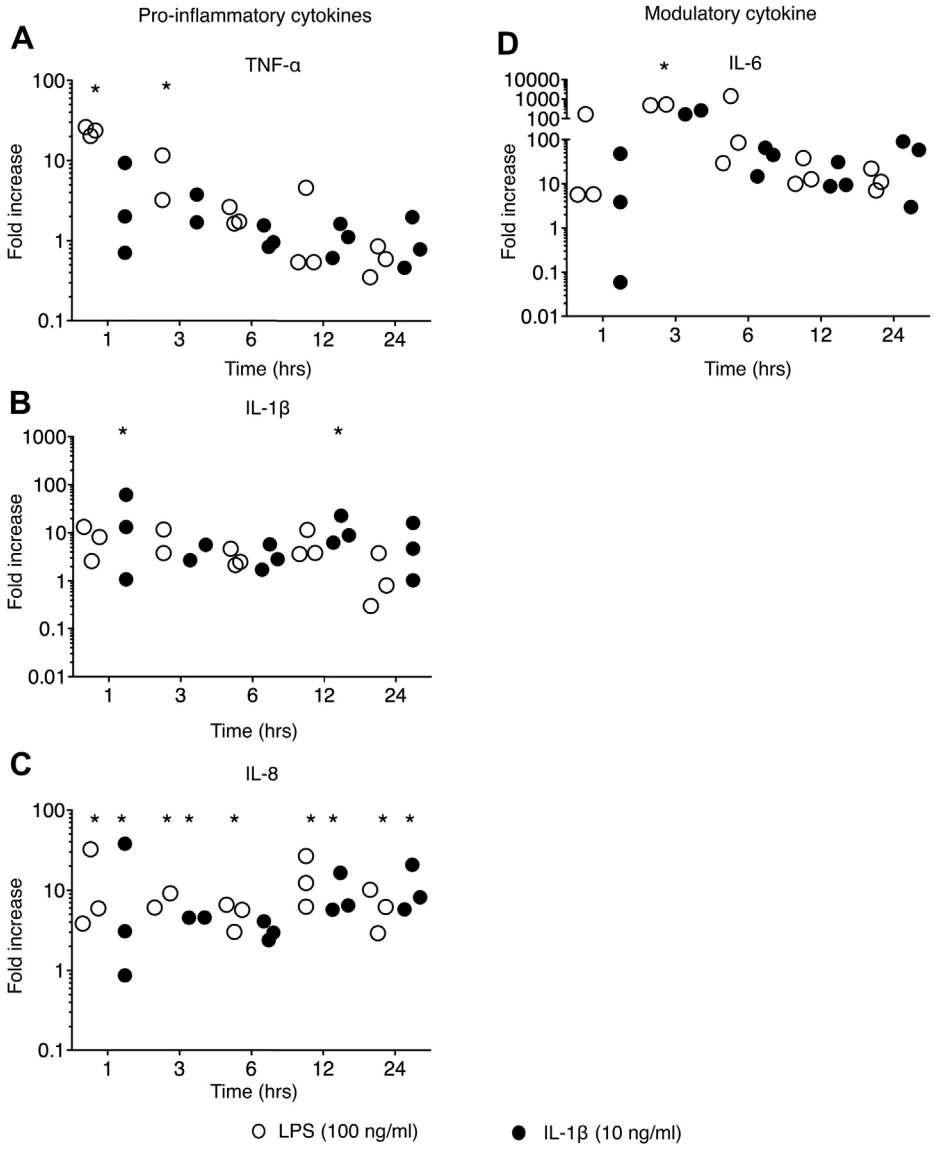

Fig. 1. eBM-MSCs' expression of paracrine pro-inflammatory and modulatory signaling molecules implicated in the pathology of OA is influenced by stimulation with LPS or IL-1β *in vitro*. LPS significantly increased the transcription of (A) TNF-α at 1 h (23 fold (9.6–56.4), $P < 0.0001$, $n = 3$) and 3 h (seven fold (2.3–19.6), $P = 0.001$, $n = 2$), (C) IL-8 at all timepoints (1 h: nine fold (3.6–22.9), $P < 0.0001$, $n = 3$, 3 h: nine fold (3.2–27.3), $P = 0.0003$, $n = 2$, 6 h: five fold (1.9–12.3), $P = 0.002$, $n = 3$, 12 h: 13 fold (5.0–32.2), $P < 0.0001$, $n = 3$, 24 h: six fold (2.2–14.5), $P = 0.0008$, $n = 3$) and (D) IL-6 at 3 h (510 fold (19.1–13638.4), $P = 0.0007$, $n = 2$) compared to unstimulated eBM-MSCs but (B) IL-1β expression was not affected by this stimulation. Conversely, IL-1β stimulation significantly increased the transcription of (B) IL-1β at 1 h (10 fold (2.9–31.6), $P = 0.0007$, $n = 3$) and 12 h (11 fold (3.3–35.1), $P = 0.0005$, $n = 3$) and (C) IL-8 at 1 h (five fold (1.9–11.9), $P = 0.002$, $n = 3$), 3 h (seven fold (2.3–19.8), $P = 0.001$, $n = 2$), 12 h (nine fold (3.3–21.5), $P = 0.0001$, $n = 3$) and 24 h (10 fold (3.9–25.3), $P < 0.0001$, $n = 3$) but (A) TNF-α and (D) IL-6 expression was unchanged by IL-1β stimulation compared to unstimulated eBM-MSCs. Open circles represent LPS-treated eBM-MSCs, solid circles represent IL-1B-treated eBM-MSCs and * represent statistically significant fold increases ($P \leq 0.05$).

$P = 0.0006$, $n = 3$; OASF; 0.07 fold decrease (0.023–0.24), $P = 0.0002$, $n = 3$) relative to controls [Fig. 3(B)]. Stimulation of eBM-MSCs with OASF, but not NSF, induced a rapid, but transient, significant increase (three fold (1.49–4.97), $P = 0.002$, $n = 3$) in the levels of VEGF mRNA after 1 h in culture [Fig. 4(A)]. TNF-α, IL-8, IL-6, TGF-β1 and IGF-1 gene expression did not change following exposure of eBM-MSCs to OASF when compared with NSF at any timepoint [Figs. 3(A, C and D) and 4(B and C)]. The anabolic cytokines TGF-β1 and IGF-1 gene expression was not significantly up-regulated by exposure to either NSF or OASF [Fig. 4(B and C)].

Discussion

Several important findings on eBM-MSC paracrine cytokine responses, relevant to their IA therapeutic applications, were identified in the current investigation modeling inflammatory joint conditions. First, the eBM-MSC pro-inflammatory cytokine gene expression was up-regulated when exposed to inflammatory stimuli and was agonist dependent: LPS stimulation enhanced eBM-MSC expression of the pro-inflammatory cytokine TNF-α and also the predominantly chemotactic cytokine IL-8, in addition to the modulatory cytokine IL-6, but not IL-1β. Stimulation with IL-1β also up-regulated IL-8 gene expression. However, the IL-1β stimulus enhanced eBM-MSCs' IL-1β gene expression, providing evidence for a positive feedback loop. Second, the inflammatory molecules did not increase nor decrease eBM-MSCs' expression of any of the anabolic genes studied, namely VEGF, IGF-1 and TGF-β1. Third, unlike the inflammatory molecules LPS and IL-1β, OASF did not up-regulate the eBM-MSC pro-inflammatory genes, but instead, induced a significant up-regulation in VEGF. Combined these

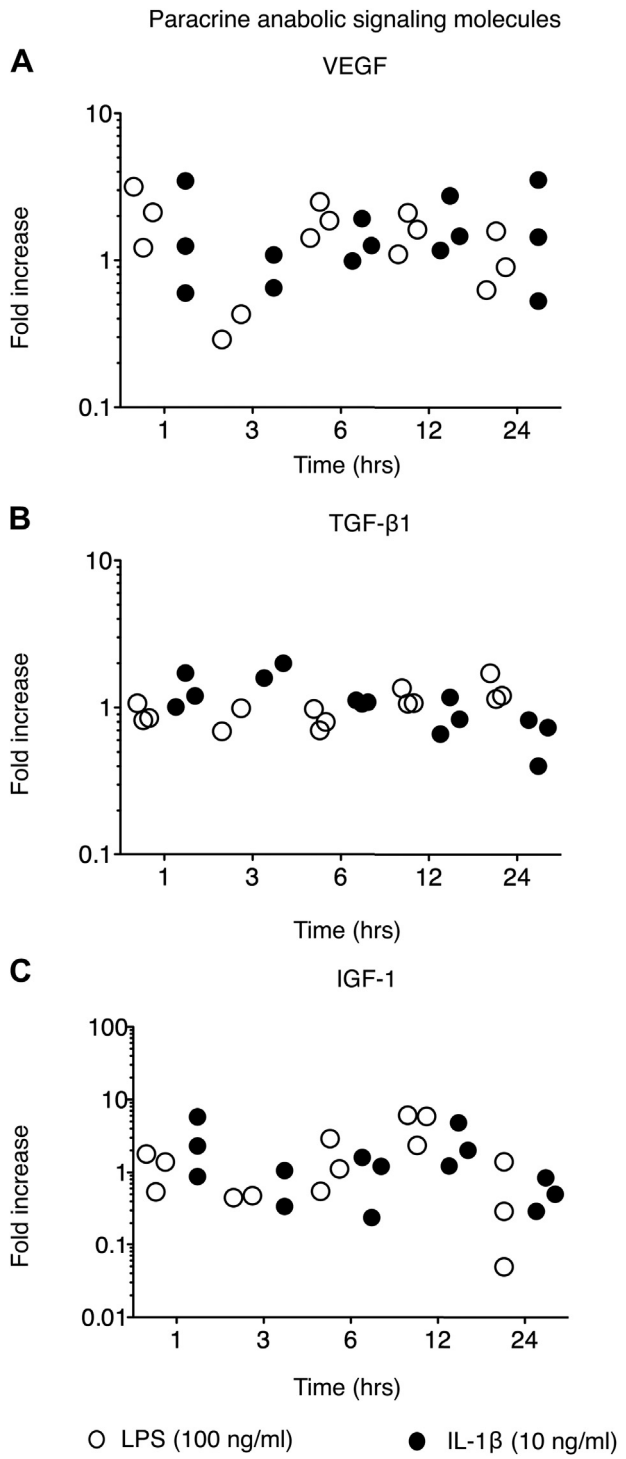

Fig. 2. eBM-MSCs' expression of the paracrine anabolic signaling molecules implicated in the pathology of OA is unaffected by stimulation with LPS or IL-1β. (A) VEGF, (B) TGF-β1 and (C) IGF-1 gene expression is not significantly different in cultured eBM-MSCs stimulated with LPS or IL-1β compared to unstimulated eBM-MSCs. Open circles represent LPS-treated eBM-MSCs, solid circles represent IL-1B-treated eBM-MSCs and * represent statistically significant fold increases ($P \leq 0.05$).

results further underpin the capacity of eBM-MSCs to respond and modify their secretory phenotype to different inflammatory cues in their environment. Of importance from a clinical perspective, no adverse pro-inflammatory effects were identified when eBM-MSCs were exposed to OASF but instead an anabolic effect was identified.

LPS and IL-1β stimulation underpins BM-MSCs capacity to contribute to inflammation

The results of the LPS and IL-1β stimulations of eBM-MSCs are in agreement with previous investigators that MSCs are sensitive to hostile microenvironments[10,16,24–26]. LPS signals through the toll-like receptor TLR4[25,26]. Toll-like receptors are important for the innate and adaptive immune system responses as they recognize both pathogen and damage-associated molecular patterns. The rapid and transient increase in eBM-MSC TNF-α transcription in response to LPS is comparable to the response observed in human monocytes[27], suggesting some analogous functions of MSCs and cellular effectors of the innate immune system as suggested by others[24,26]. TNF-α plays a major role in joint inflammation[11] and this finding reveals a capacity of resident MSCs to contribute to inflammation in the acute phase, at least in the presence of sepsis. IL-8 gene expression, on the other hand, was augmented throughout the 24 h stimulation period and to a similar degree on stimulation with the pro-inflammatory cytokine IL-1β, consistent with a previous study employing of hMSCs[25]. IL-8 is chemotactic for neutrophils, modulates their secretory response and is angiogenic[28]. This IL-8 response is also in line with MSCs pro-inflammatory role and confirms and extends the findings of others[24,26]. The sharp and sustained increase in IL-8 expression suggests that MSCs may amplify the communication between injured or inflamed tissues and the cells of the innate immune system, and therefore contribute to tissue neutrophilia.

The inability of LPS stimulation (100 ng/ml) to enhance IL-1β gene expression in eBM-MSCs contrasts markedly with data from hMSCs, where 4 h stimulation with 10 ng/ml of LPS increased IL-1β gene expression 135 fold[25]. This differential response may be explained by putative evolutionary divergence in the TLR4 genes, which may affect the nature of downstream intracellular signaling events in different species[29]. Moreover, the above-described discrepancies may be caused by the 10 fold increase in LPS concentration used in our experiments.

IL-1β stimulation induces a pro-inflammatory signature in BM-MSC

IL-1β is the principal instigator of articular cartilage degradation in OA through protease induction and has been identified in both osteoarthritic cartilage and SF[12,30–32]. Consequently MSCs employed for therapeutic purposes IA in OA would be exposed to this pro-inflammatory cytokine. The stimulation of MSCs by IL-1β caused an up-regulation of IL-8 similar to LPS, but, unlike LPS, also caused a biphasic up-regulation of IL-1β and no up-regulation of IL-6. The cellular receptors for LPS and IL-1β are closely interlinked and described as the TLR-IL-1 receptor (IL-1R) superfamily of receptors. When activated they induce a series of intercellular signaling pathways that converge on the transcription factor nuclear factor KappaB (NF-KappaB) resulting in pro-inflammatory cytokine expression[9,33]. These results suggest that the induction of IL-8 arises potentially from similar intracellular pathways but the differential IL-1β response and absence of IL-6 response to IL-1β may indicate alternative receptor signaling effects or either dose or time dependent effects. Nonetheless, both stimuli, LPS and IL-1β, induced the eBM-MSCs to adopt a pro-inflammatory phenotype. From a clinical standpoint this is particularly important in respect to IL-1β as it is present in OA joint tissues and indicates that eBM-MSCs could potentially contribute to the inflammatory processes in joint disease.

Together, the results reveal that eBM-MSCs have the capacity, depending on the joint environmental conditions, to contribute to joint inflammation by up-regulation of transcription of all the pro-inflammatory cytokines we investigated, i.e., TNF-α, IL-1β, IL-8 and IL-6. Furthermore, neither of the inflammatory stimuli, LPS or IL-1β,

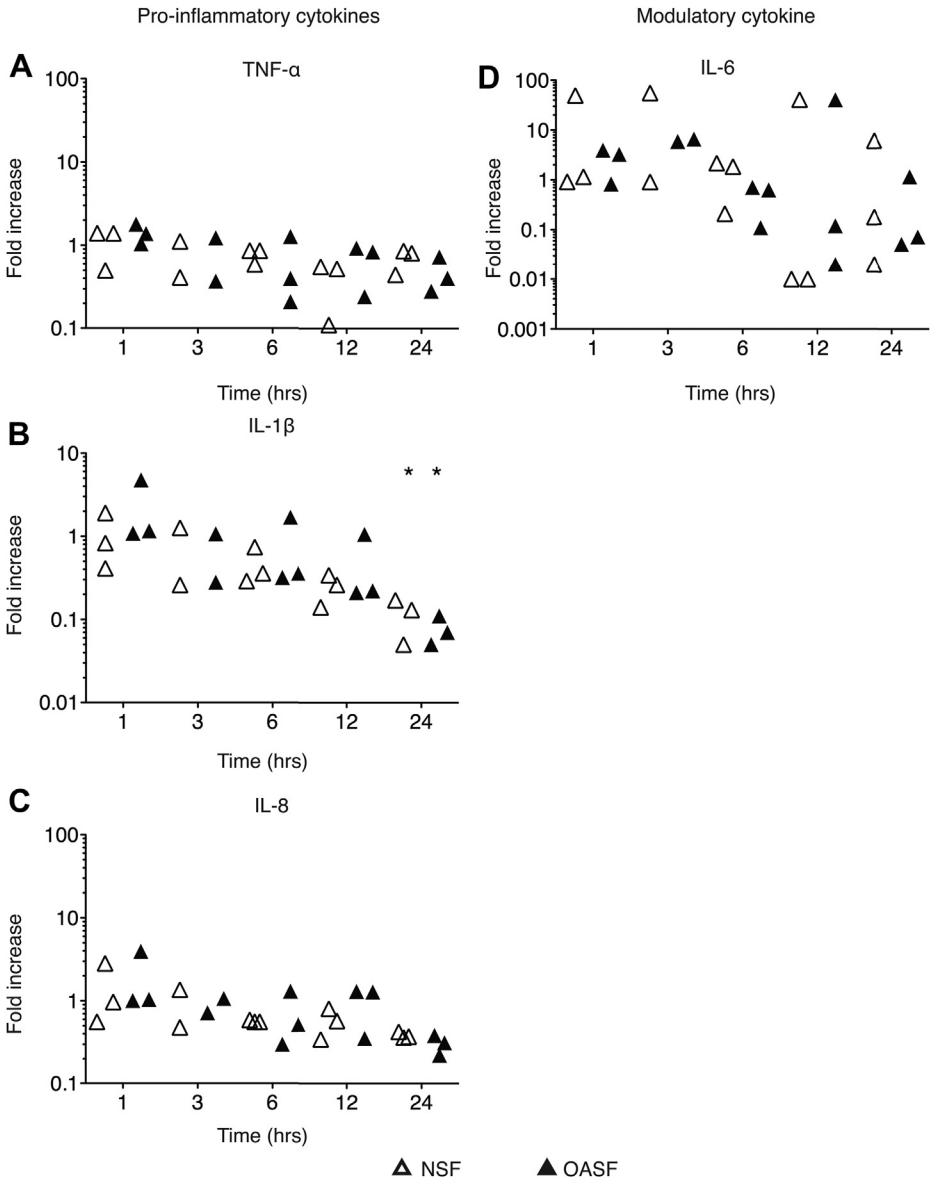

Fig. 3. eBM-MSCs' expression of paracrine pro-inflammatory and modulatory signaling molecules implicated in the pathology of OA is influenced by stimulation with NSF or OASF. (A) TNF-α, (C) IL-8 and (D) IL-6 gene expression at all timepoints was not significantly different in cultured eBM-MSCs stimulated with SFs from either normal or OA joints. However, the transcription of (B) IL-1β at 24 h was significantly decreased upon stimulation with both NSF and OASF (NSF: 0.10 fold decrease (0.031–0.33), $P = 0.0006$, $n = 3$; OASF; 0.07 fold decrease (0.023–0.24), $P = 0.0002$, $n = 3$) compared to unstimulated eBM-MSCs. Open triangles represent NSF-treated eBM-MSCs, solid triangles represent OASF-treated eBM-MSCs and * represent statistically significant fold decreases ($P \leq 0.05$).

caused changes in the eBM-MSCs' expression levels of the anabolic cytokines. The apparent discrepancy between these results and a recent study employing hMSC where VEGF was down-regulated by LPS stimulation, may be due to different concentrations of agonists or duration of stimulation[25].

OASF does not induce a BM-MSC pro-inflammatory cytokine profile

Importantly, unlike LPS and IL-1β stimulation, none of the pro-inflammatory cytokines were up-regulated by *in vitro* stimulation of eBM-MSCs with either NSF or OASF. This suggests that a component of SF acts on BM-MSCs to down-regulate the expression of IL-1β, one of the most important pro-inflammatory cytokines produced in OA joints, thereby evading an MSC pro-inflammatory phenotype, but the mechanism by which it acts requires elucidation. Inherent anti-inflammatory properties of SF may also account for the finding that eBM-MSCs' expression of pro-inflammatory cytokines were not up-regulated following stimulation with OASF. However, it is important to point out that the fold changes were very modest in the study herein, as expected, when compared to the effects observed with LPS or IL-1β stimulation alone. Overall, this lack of induction of an MSC pro-inflammatory phenotype on exposure to OASF is encouraging news from a clinical perspective. However caution should be exercised in interpreting the data. OASF composition will be variable depending on the disease status at aspiration, joint and age and these combined variables mean that a different response may have been elicited if other SF samples had been employed e.g., early vs late stage of disease. Additional characterization of the OA SF inflammatory status by routine (total protein and leukocyte counts) and inflammatory cytokine analysis could have provided important information about its composition. Furthermore, the

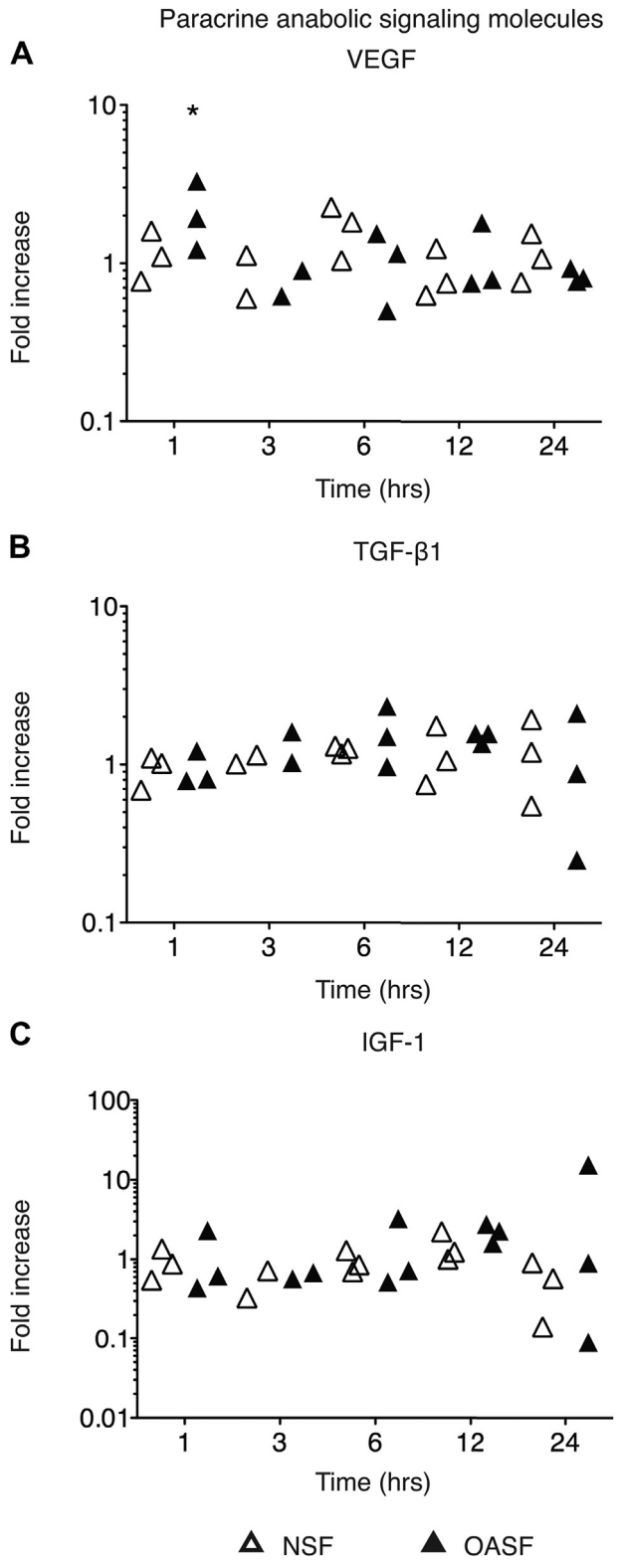

Fig. 4. eBM-MSCs' expression of the paracrine anabolic signaling molecule VEGF is significantly up-regulated on exposure to OASF for 1 h. (A) VEGF levels are significantly increased upon stimulation with OASF at 1 h (three fold (1.49–4.97), $P = 0.002$, $n = 3$) but not longer stimulations whereas (B) TGF-β1 and (C) IGF-1 levels are not significantly different in cultured eBM-MSCs stimulated with either NSF or OASF compared to unstimulated controls at any timepoint. Open triangles represent NSF-treated eBM-MSCs, solid triangles represent OASF-treated eBM-MSCs and * represent statistically significant fold increases ($P \leq 0.05$).

use of abattoir cadaver limbs prevents knowledge of accurate case history (treatment or clinical signs).

It could also be argued that the 10% dilution of SF employed for the stimulations of eBM-MSCs in culture resulted in dilution effects and subthreshold concentrations of IL-1β. A 10% dilution was employed to facilitate manipulation and mixing due to the natural high viscosity of SF. Other groups have previously reported significant effects of SF at these (and lower) dilutions, on various biological processes such as the transcriptional activity, migration and differentiation of cultured MSCs[10,19,34–36]. If it had been possible to use 100% SF, the observed effects may have been enhanced or additional effects may have been identified. IL-1β stimulation was employed to compensate for this dilution, as it is known to be present in OASF[2]. The lack of robust differences between the groups of NSF and OASF may also be due to the small n values ($n = 3$) in the study design.

Other investigators have recently observed an up-regulation of IL-6 on exposure of MSCs to 20% OASF[10]. IL-6 is a pleiotropic cytokine with some pro-inflammatory actions but also involved in regenerative processes and regulation of metabolism[37,38]. There are a number of possible explanations for the apparent discrepancy between our results and the recent findings of Leijs *et al.*, who observed a twofold up-regulation of IL-6 following 48 h of stimulation. It may be explained by the different timepoints assessed and also possible differences in joint fluid composition related to the stage of the OA disease process. We investigated multiple timepoints up to 24 h whereas Leijs and colleagues studied only one timepoint at 48 h[10].

OASF induces a BM-MSC anabolic cytokine profile

The gene expression of the anabolic cytokines VEGF, TGF-β1 and IGF-1 were investigated in the study herein as they are all believed to have a positive anabolic role for joint repair[39]. VEGF is a potent angiogenic factor that possess anti-apoptotic effects and that promotes the neovascularization of injured sites[9,40–43]. The up-regulation of VEGF observed on exposure to OASF suggests that OASF may promote a healing response through the mechanisms outlined above. MSCs' capacity to up-regulate VEGF in the presence of disease has already been demonstrated in a murine model of ischemia[44]. This modest, yet significant, change in VEGF expression was not induced by stimulation with NSF, indicating that eBM-MSCs respond differentially to NSF compared to OASF.

Conclusion

In conclusion, the present findings add to the very limited knowledge of the behavior of BM-MSCs in response to changes in SF composition that occur in OA. BM-MSCs can adopt a pro-inflammatory or anabolic phenotype depending on environmental cues. Although the pro-inflammatory cytokine IL-1β, a key player in OA degradative events, induced a BM-MSC pro-inflammatory phenotype, OASF, on the other hand, promoted VEGF expression without any pro-inflammatory cytokine induction.

Author contribution

Raphaël Vézina Audette: Analysis and interpretation of the data, Drafting of the article, Obtaining of funding, Collection and assembly of data.

Anouk Lavoie-Lamoureux: Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Statistical expertise, Collection and assembly of data.

Jean Pierre Lavoie: Conception and design, Critical revision of the article for important intellectual content, Provision of study materials or patients, Obtaining of funding.

Sheila Laverty: Conception and design, Provision of study materials or patients, Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding.

Raphaël Vézina Audette (raphael.vezina-audette@umontreal.ca) and Sheila Laverty (Sheila.Laverty@umontreal.ca) take responsibility for the integrity of the work as a whole, from inception to finished article.

Competing interest

None of the authors has received any financial contribution from commercial sources for this work, nor do we have other financial interests that would create a potential conflict of interest with regards to the work.

Role of funding source

The study sponsors had no involvements in the study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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