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Angiogenic CXC Chemokine Expression During Differentiation of Human Mesenchymal Stem Cells Towards the Osteoblastic Lineage

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Abstract The potential role of ELR$^+$ CXC chemokines in early events in bone repair was studied using human mesenchymal stem cells (hMSCs). Inflammation, which occurs in the initial phase of tissue healing in general, is critical to bone repair. Release of cytokines from infiltrating immune cells and injured bone can lead to recruitment of MSCs to the region of repair. CXC chemokines bearing the Glu-Leu-Arg (ELR) motif are also released by inflammatory cells and serve as angiogenic factors stimulating chemotaxis and proliferation of endothelial cells. hMSCs, induced to differentiate with osteogenic medium (OGM) containing ascorbate, β-glycerophosphate (β-GP), and dexamethasone (DEX), showed an increase in mRNA and protein secretion of the ELR$^+$ CXC chemokines CXCL8 and CXCL1. CXCL8 mRNA half-life studies reveal an increase in mRNA stability upon OGM stimulation. Increased expression and secretion is a result of DEX in OGM and is dose-dependent. Inhibition of the glucocorticoid receptor with mifepristone only partially inhibits DEX-stimulated CXCL8 expression indicating both glucocorticoid receptor dependent and independent pathways. Treatment with signal transduction inhibitors demonstrate that this expression is due to activation of the ERK and p38 mitogen-activated protein kinase (MAPK) pathways and is mediated through the $G_{\alpha i}$-coupled receptors. Angiogenesis assays demonstrate that OGM-stimulated conditioned media containing secreted CXCL8 and CXCL1 can induce angiogenesis of human microvascular endothelial cells in an in vitro Matrigel assay. J. Cell. Biochem. 103: 812–824, 2008.

Key words: ELR$^+$ CXC chemokines; human mesenchymal stem cells; osteoblastic differentiation; dexamethasone; HMEC-1 angiogenesis

Mesenchymal stem cells (MSCs) (bone marrow stromal cells) have the potential to differentiate into a number of cell types including chondrocytes and osteoblasts thus sparking a great interest in their use in regenerative medicine for cartilage and bone. A number of culture medium additives, such as 1,25-dihydroxyvitamin D_3 [Jaiswal et al., 1997; D'Ippolito et al., 2002; Foster et al., 2005] in human mesenchymal stem cells (hMSCs) or retinoic acid in a murine model of mesenchymal stem cells (C3H10T1/2) differentiation [Gazit et al., 1993], have been used to engage these MSC models towards the osteogenic lineage. Exogenous glucocorticoids in combination with ascorbic acid and an organic phosphate such as β-glycerophosphate (β-GP) have also been widely used to differentiate hMSCs into osteoblasts under in vitro culture conditions [Jaiswal et al., 1997; D'Ippolito et al., 2004; Kim et al., 2005]. However, this mechanism of hMSC differentiation by glucocorticoids has yet to be discerned. In fact, glucocorticoid administration to differentiated osteoblasts has been shown to not only result in a decrease in cell proliferation, but also an inhibition of collagen type 1 and osteocalcin (OCN) expression, both markers of osteoblast differentiation [Stromstedt et al., 1991; Delany et al., 1994].

Glucocorticoids have been used in chronic inflammatory conditions such as rheumatoid

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arthritis to inhibit the inflammatory response mediated by pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα), in addition to chemokines released from polymorphonuclear leucocytes and mononuclear cells. While bone destruction brought on by increased osteoclastic activity seen with the induction of pro-inflammatory cytokines can be tempered by glucocorticoids, long term use of glucocorticoids can also result in bone loss primarily due to a decrease in bone formation with inhibition of osteoblastic proliferation and differentiation [for reviews see references Schacke et al., 2002; Rhen and Cidlowski, 2005].

During both endochondral bone formation and fracture repair, vascularization of the tissue is required for replacement of cartilage with mineralized bone [Street et al., 2002]. One of the initial steps involved in wound healing in general is an early inflammatory response after hemostasis has been established. This inflammatory response is coordinated by chemotactic cytokines (chemokines) whose function is to attract immune cells, polymorphonuclear leucocytes and monocytes/macrophages, to the wound site [Charo and Ransohoff, 2006]. The chemokine superfamily includes over 40 members in four families which are distinguished by the location of two cysteine residues at the amino terminal of the molecule [Baggiolini et al., 1997; Rollins, 1997]. Chemokines within the families are expressed at temporally distinct times due to differential regulation; although, they are functionally redundant. Members of the CXC chemokines family are also classified by the presence or absence of a Glu-Leu-Arg (ELR) motif located at the N-terminal region of the proteins. ELR^+ CXC chemokines are present very early during inflammation, attract neutrophils to the injury site [Engelhardt et al., 1998; Gillitzer and Goebeler, 2001], and exhibit angiogenic activity [Strieter et al., 1995; Belperio et al., 2000; Bernardini et al., 2003]; whereas, chemokines lacking the ELR motif are angiostatic [Strieter et al., 1995]. The prototypical representative of the ELR^+ CXC chemokine family is CXCL8, also known as interleukin-8 (IL-8). Several reports have demonstrated that human osteoclasts and osteoblasts can express chemokines, including CXCL8 and CXCL1 [Chaudary et al., 1992; Rothe et al., 1998; Lisignoli et al., 1999, 2002].

In this study, we demonstrate that the synthetic glucocorticoid, dexamethasone (DEX), can increase alkaline phosphatase (ALP) expression in hMSCs consistent with osteogenic differentiation, yet surprisingly also increased the production by hMSCs of CXCL8 and CXCL1, chemokines generally associated with a pro-inflammatory response. This is the first report to show that hMSCs can be stimulated by DEX to elaborate chemokines of the CXC family containing the ELR motif and that the production of these ELR^+ CXC chemokines is via the action of DEX.

MATERIALS AND METHODS

Cell Culture

hMSCs and complete growth medium (HMSCGM) was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). hMSCs from several donors were used (Table I). Cells were subcultured at 60–70% confluence in T75 flasks at 37°C under 95% air/5% CO_2 atmosphere, and used between passages 2–7. For differentiation towards the osteoblastic lineage, hMSCs were treated every 3–4 days with osteogenic medium (OGM) which consisted of HMSCGM supplemented with 50 mM ascorbic acid-2-phosphate (A-2-P), 10 mM β-GP, and 10^{-7} M DEX (Sigma-Aldrich, St. Louis, MO) following the protocol supplied from Cambrex. The human microvascular endothelial cell line HMEC-1 was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, GA) and propagated in MCDB131 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Ogden, UT), 10 ng/ml hEGF (Invitrogen), 1 μg/ml hydrocortisone (Sigma-Aldrich), and 1% glutamine, penicillin, and streptomycin (Sigma-Aldrich).

Time Course Experiments

hMSCs were plated at 5,000–10,000 cell/cm^2 in HMSCGM in 24-well plates and allowed to

TABLE I. Donor Characteristics From hMSCs Obtained From Cambrex

Lot #	Age (years)	Race	Sex
1F2155	24	A	M
1F1061	19	C	F
3F0551	26	B	M
4F0218	21	A	M
4F1560	23	B	F

A, Asian; C, Caucasian; B, Black; M, Male; F, Female.

attach for 4 h prior to treatment with OGM medium. In some experiments the glucocorticoid receptor inhibitor, mifepristone (MP; Sigma-Aldrich), was added at various timepoints to each well at a final concentration of 1 μM. Each experiment was done at least three times in triplicate and the supernatants saved for ELISA. For the OGM removal study, OGM was added and at specific timepoints, OGM was removed and replaced with HMSCGM for the remainder of the experiment. Signal transduction inhibitors (Calbiochem, San Diego, CA) and controls were also used at the following concentrations: $G_{\alpha i}$ pathway, pertussis toxin (PTX) at 100 ng/ml; extracellular receptor kinase (ERK) pathway, U0124 and U0126 (15 μM) and PD98059 (19 μM); p38 pathway, SB202474, SB202190, SB203580 (15 μM); jun N-terminal kinase (JNK) pathway, JNK Inhibitor Negative Control and JNK Inhibitor II (10 μM). Total RNA and protein supernatants were isolated using the Qiagen RNeasy Miniprep kit (Qiagen, Inc., Valencia, CA) at the indicated timepoints. Initially differentiation was demonstrated by von Kossa and Alizarin Red staining at 28 days and by semi-quanitative RT-PCR at 7 days. Subsequent differentiation was monitored by the expression of the reporter gene ALP using both mRNA detection and enzymatic activity analyses as previously described [Jarrahy et al., 2005].

Alkaline Phosphatase Assay

hMSCs plated in 24-well plates were washed once with cold PBS. Cells were lysed in 0.2% NP-40, 1 mM $MgCl_2$ solution (100 μl) by three cycles of freezing/thawing at −80°C. Following lysis, cells were scraped from the well and centrifuged at 13,000 rpm for 5 min at 4°C in a microfuge to remove cellular debris. The supernatant (15 μl) was mixed with 85 μl of freshly prepared p-nitrophenyl-phosphate (PNPP) substrate (8 mg/ml, Sigma, St. Louis, MO) in a 96-well plate and incubated at 37°C for 1 h. The enzymatic reaction was terminated with the addition of 150 μl of 0.5 N NaOH. The absorbance of p-nitrophenol (PNP) at 415 nm was determined spectrophotometrically. The OD values were normalized against protein concentration of the same lysates (50 μl).

von Kossa and Alizarin Red Staining

hMSCs were incubated in HMSCGM or OGM medium in 24-well plates for 28 days. Cells were washed once with PBS and fixed with 10% neutral buffered formalin (Sigma) for 30 min before staining. For Von Kossa staining, cells were rinsed twice with distilled water, covered with 0.5 ml of 5% silver nitrate, and exposed to UV light for 5 min. The cells were rinsed again with distilled water and treated with 2.5% sodium thiosulfate for 5 min. For Alizarin Red staining, fixed cultures were rinsed once with distilled water and then stained for 1–2 min with 2% Alizarin Red solution (pH 4.1). After staining, wells were photographed using an Olympus D-435 digital camera.

Quantitative RT-PCR

Semi-quantiative RT-PCR was performed using the PTC-200 Peltier Thermo Cycler (MJ Research, Inc., Incline Village, NV) and the One-Step RT-PCR kit from Qiagen using RNA (50 ng) from 7 days HMSCGM and OGM cultures. Reactions were performed as follows: reverse transcription at 50°C for 30 min, 95°C for 15 min; followed by 25 or 32 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. PCR products were run out on 1% agarose gels and visualized with ethidium bromide staining. See Table II for Primer sequences. Relative mRNA levels of various genes were quantitated by real-time RT-PCR using the Opticon Continuous Fluorescence System (MJ Research,) and the SYBR Green RT-PCR kit from Qiagen. Reactions were performed in triplicate: reverse transcription at 50°C for 30 min, 95°C for 15 min; followed by 50 cycles of denaturing at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Relative changes in gene expression were calculated in relation to 18S levels and the reference timepoint using the $2^{-\Delta\Delta C(T)}$ method [Livak and Schmittgen, 2001].

Media Component Analysis

hMSCs were plated at 5,000–10,000 cell/cm^2 in HMSCGM in 24-well plates and allowed to attach for 4 h prior to treatment for 10 days with HMSCGM, OGM, or HMSCGM supplemented with one of the OGM medium components, 50 mM A-2-P, 10 mM β-GP, and 10^{-7} M DEX either individually or in combination. Each experiment was done at least three times in triplicate and the supernatants saved for ELISA.

ELISA

CXCL8 and CXCL1 protein levels in supernatants were determined with the human

TABLE II. Primer Sequences Used in Semi-Quantitative and Real-Time RT-PCR

Gene of interest	Oligonucleotide sequence	GenBank accession #
ALP sense	5′-AAGCCGGTGCCTGGGTGGCCAT-3′	NM_000478
ALP antisense	5′-ACAGGAGAGTCGCTTCAGAG-3′	
BSP sense	5′-CCAGAGGAAGCAATCACCAAA-3′	NM_004967
BSP antisense	5′-TTGAGAAAGCACAGGCCATTC-3′	
OCN sense	5′-TGCAGAGTCCAGCAAAGGT-3′	NM_199173
OCN antisense	5′-TCCTGCTTGGACACAAAGG-3′	
18S rRNA sense	5′-CCGCAGGTTCACCTACTG-3′	NW_926741
18S rRNA antisense	5′-CGGGTCATAAGCTTGCCTG-3′	
CXCL8 sense	5′-GCCTTCCTGATTTCTGCAGC-3′	NM_000584
CXCL8 antisense	5′-TCCAGACAGAGCTCTCTTCC-3′	
CXCL1 sense	5′-TAGCCACACTCAAGAATGGGCGGAAAGCTT-3′	NM_001511
CXCL1 antisense	5′-TGGCCATTTGCTTGGATCCGCCAGCCT-3′	

ALP, alkaline phosphatase; BSP, bone sialoprotein; OCN, osteocalcin.

CXCL8/IL-8 and human CXCL1/GROα DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN) using protocols supplied with the kit. Supernatant samples were run in duplicate and compared to CXCL8 and CXCL1 standard curves.

TNFα/IL-1β Treatment of hMSCs

hMSCs plated in 24-well plates with HMSCGM or OGM medium for 3 days were treated with TNFα and IL-1β (10 ng/ml each) for 4 h prior to RNA isolation. Some wells also contained the glucocorticoid receptor inhibitor MP at 1 μM. Total RNA was analyzed for CXCL8 and 18S expression as indicated above.

mRNA Stability Study

hMSCs were cultured for 7 days in 24-well plates in HMSCGM or OGM. The transcription inhibitor actinomycin D was added (5 μg/ml) and total RNA isolated at various times up to 24 h. Relative levels of mRNA were compared to the 0 min timepoints. Half-life values were determined using GraphPad Prism version 3.00 for Windows (GraphPad Software, Inc., San Diego, CA).

Western Blot Analysis

hMSCs were plated at 5,000–10,000 cell/cm^2 in 35 mm dishes and treated for 7 days with OGM medium in the presence of ERK or p38 signal transduction inhibitors or negative controls. Cells were washed with cold PBS and solubilized in 200 μl PhosphoSafe Extraction Reagent (EMD Chemicals, Gibbstown, NJ) for 5 min at room temperature. Lysates were analyzed by SDS–PAGE and transferred to polyvinylidene difluoride membrane. Blots were probed with specific antibodies (Cell Signaling Technology, Danvers, MA) and immunoreactive proteins detected with the ECL kit (GE Healthcare Bio-sciences, Piscataway, NJ). Protein levels were quantitated using the BIOQUANT system (BioQuant Image Analysis Corp., Nashville, TN).

Angiogenesis Assays

Angiogenesis assays were conducted as previously described [Jarrahy et al., 2005]. Conditioned medium (1:8 dilutions in serum free medium) from HMSCGM and OGM-treated cultures (day 3–7 supernatant) was mixed 1:1 with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) on ice, and 50 μl of each mixture plated per well in a 96-well plate. After allowing the mixture to solidify at 37°C for 30 min, a 200 μl aliquot of HMEC-1 (2×10^4 cells) in serum-free medium was added to each well. Cells were incubated at 37°C for 4 h, observed by phase contrast microscopy and images of HMEC-1 cells on Matrigel were captured by digital camera, and subsequently evaluated for tube formation by quantification of pattern recognition and the number of branching points per field of view (10× magnification). Each field of view was given a numerical value for each pattern using the following criteria: cells isolated or in sheet like monolayer (0); cells begin to migrate and align (1); capillary tubes visible. No sprouting (2); sprouting of capillary tubes (3); closed polygons begin to form (4); complex mesh like structure developed (5). Branching points were also counted and the values of five fields of view averaged. Each experiment was done at least three times with three replicates per condition.

Statistics

Data is reported as the mean value ± SD. Values were analyzed by ANOVA and the Bonferroni method for multiple comparisons between pairs or by Student's *t*-test using GraphPad Prism statistical software and are considered significant if $P < 0.05$ in comparison to negative controls.

RESULTS

Temporal Expression of Genes

hMSCs treated with OGM differentiate towards the osteoblastic lineage as demonstrated by von Kossa and Alizarin Red staining at 28 days (Fig. 1A). mRNA levels of three bone markers were also analyzed at day 7 by semi-quantitative RT-PCR. Two of the bone markers, ALP, and bone sialoprotein (BSP) are upregulated at day 7 in OGM-treated cells in comparison to HMSCGM-treated cells (Fig. 1B). OCN mRNA could be detected but was not upregulated at day 7. It has been reported that OCN mRNA levels do not increase even at later timepoints during hMSC osteogenic differentiation unless additional medium supplements ($VitD_3$) are added [Schepmoes et al., 1991; Cheng et al., 1996; Catelas et al., 2006]. We confirmed this result by addition of $VitD_3$ to the medium and observed increased levels of OCN mRNA at day 7 (data not shown). ALP mRNA expression is significantly increased starting at 5 days post-treatment and continues throughout the time course (Fig. 1C). The maximum level of expression increased 22× (day 7) over that detected at HMSCGM day 0 (1×). ALP enzymatic activity could also be detected in the

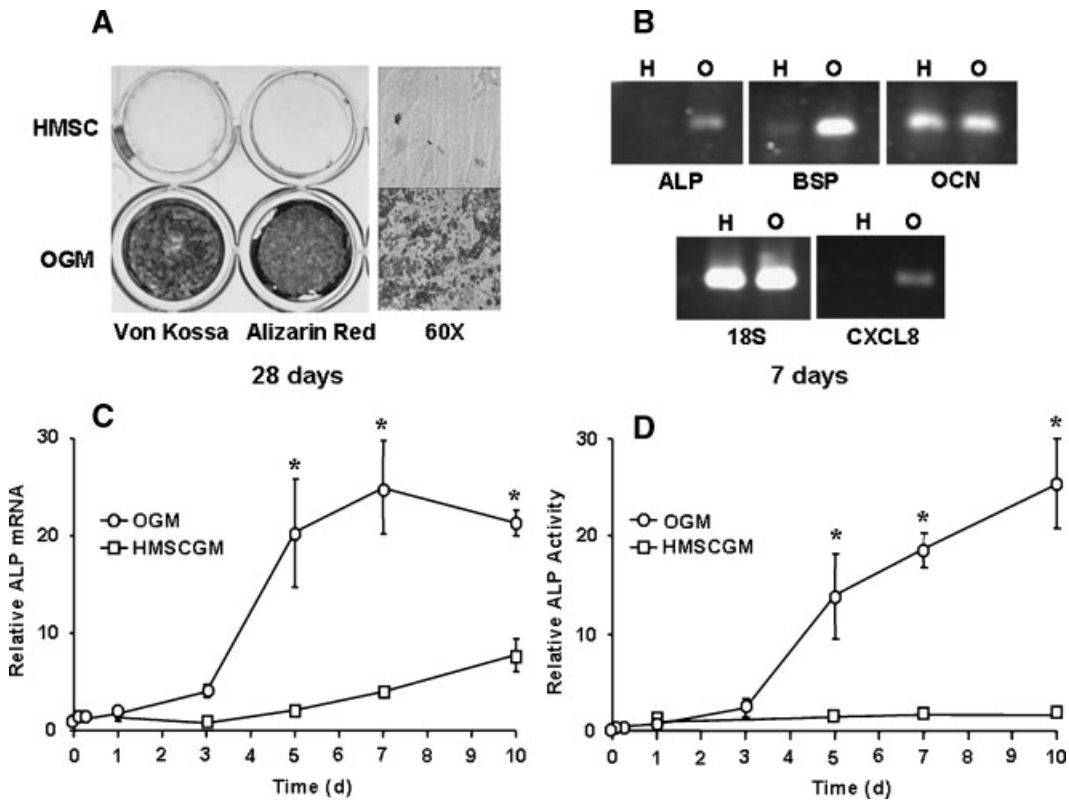

Fig. 1. Differentiation of human mesenchymal stem cells (hMSC) towards the osteoblastic lineage. **A**: hMSCs were treated with basal growth medium (HMSCGM) or osteogenic medium (OGM) for 28 days and visualizing at 60× magnification or stained with von Kossa or Alizarin Red and photographed with a digital camera. **B**: mRNA levels of the bone markers alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN) were detected by semi-quantiative RT-PCR in RNA from HMSCGM (H) or OGM (O) 7 day cultures. CXCL8 is also detected at day 7 in OGM-treated cells. 18S levels are similar in both conditions. **C**: Relative levels of ALP mRNA in HMSCGM- and OGM-treated cells were determined by real-time RT-PCR over a period of 10 days, normalized to 18S levels, and expressed in relation to the reference timepoint (0 h levels) using the $2^{-\Delta\Delta C(T)}$ method. **D**: Relative ALP activity was determined and expressed in relation to 0 h levels. *Indicates significant increase for OGM treatment compared to HMSCGM at each timepoint ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

medium starting at day 5 and increased to 25× at day 10 (Fig. 1D).

Treatment of hMSCs with OGM also significantly induced mRNA expression of CXCL8 (Figs. 1 B and 2 A) and CXCL1 (Fig. 2B). In the presence of OGM, CXCL8 levels were significantly higher at day 5 (15×) and continued to increase to 110× by day 10. In some experiments there was an earlier transient increase in CXCL8 mRNA at 6 h that returned to baseline by 24 h (day 1) which could not be consistently reproduced and may be a consequence of changing media at time 0. Levels of CXCL1 were increased 4× and 12× at day 7 and day 10, respectively. CXCL8 mRNA stability was also examined in the presence of OGM for 7 days prior to treatment with actinomycin D. In the absence of OGM, CXCL8 mRNA rapidly degraded with a half-life of 16 min. Culturing in OGM for 7 days resulted in a stabilization of the CXCL8 mRNA and an increase of the half-life to 62 min (data not shown).

Secreted CXCL8 and CXCL1 protein could also be detected in the medium by ELISA starting at day 5 and reached maximum levels at day 10 (Fig. 2C,D). CXCL8 levels were significantly higher at day 5 through day 10 (6–11 ng/ml). Likewise CXCL1 protein levels in the media also increased from day 5 to day 10 (6–15 ng/ml).

DEX Regulates CXCL8 Expression in hMSC

To ascertain the component(s) in OGM that was responsible for the expression of ALP and CXCL8, hMSCs were cultured for 10 days in HMSCGM containing one or more of the OGM components (A-2-P, β-GP, or DEX). Significant levels of ALP activity were induced when DEX was present in the medium individually or in combination with the other components (Fig. 3A). CXCL8 mRNA and protein secretion were also induced only when DEX was present in the medium (Fig. 3B,C). No significant increase in ALP activity or CXCL8 mRNA was found with A-2-P or β-GP stimulation alone or A-2-P and β-GP in combination without DEX. However, A-2-P and β-GP in combination did significantly decrease CXCL8 protein secretion (Fig. 3C). The expression of CXCL8 mRNA was dose-dependent on DEX in hMSCs with maximum CXCL8 mRNA expressed at 100 nM DEX (Fig. 4).

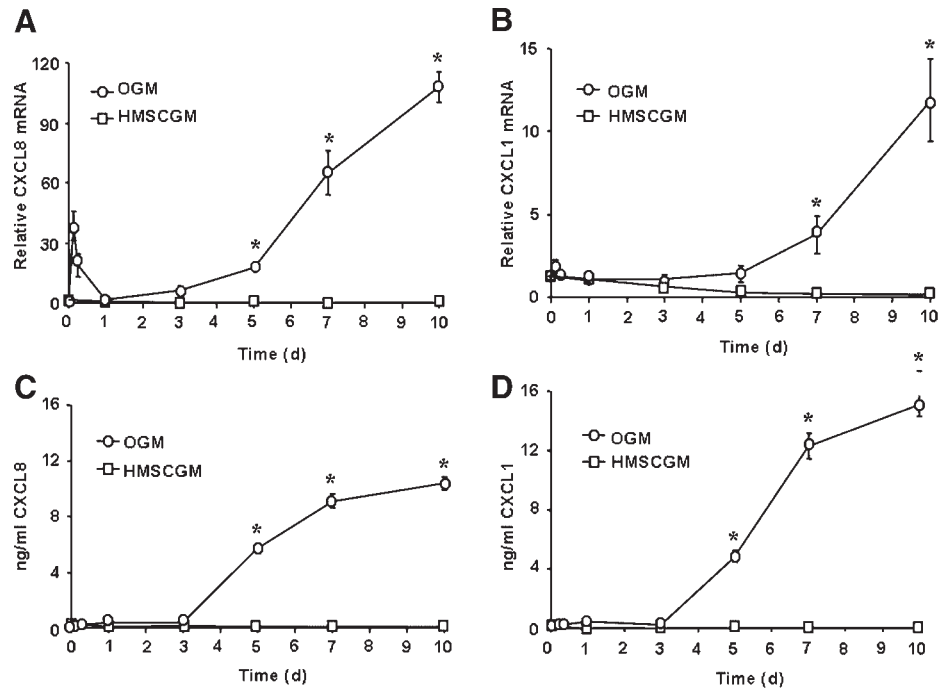

Fig. 2. ELR$^+$ CXC chemokine mRNA and protein secretion induced in hMSCs treated with osteogenic medium (OGM). Real-time RT-PCR analysis of human (**A**) CXCL8 and (**B**) CXCL1 in hMSCs treated with OGM for up to 10 days. Relative changes in expression were normalized to 18S levels and 0 h levels. **C**,**D**: CXCL8 and CXCL1 protein levels secreted into the medium were determined using the DuoSet ELISA kits from R&D systems. *Indicates significant increase for OGM treatment compared to HMSCGM at each timepoint ($P < 0.05$).

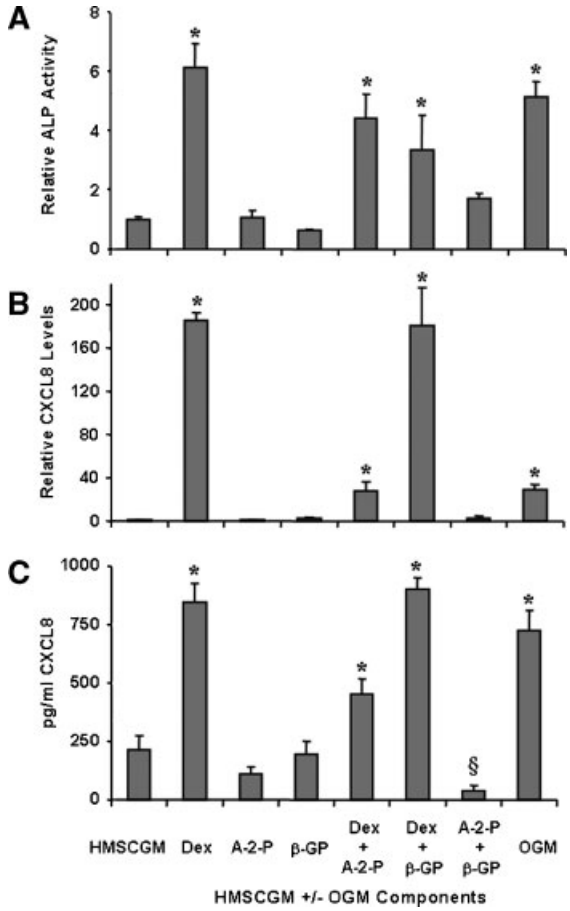

Fig. 3. Determination of the osteogenic media component(s) required for the induction of alkaline phosphatase (ALP) activity, CXCL8 mRNA induction, and CXCL8 secretion. **A**: ALP activity in relation to HMSCGM levels, (**B**) CXCL8 mRNA normalized to 18S and expressed in relation to HMSCGM levels, and (**C**) CXCL8 protein secreted into the supernatant measured after 10 days in HMSCGM +/− osteogenic medium components. *Indicates levels are significantly increased compared to HMSCGM levels for each graph ($P < 0.05$). §Indicates protein secretion levels are significantly decreased compared to HMSCGM protein levels ($P < 0.05$).

To test whether hMSCs are responsive to typical glucocorticoid-induced repression of pro-inflammatory cytokine expression, hMSCs were cultured in HMSCGM or OGM medium for 3 days prior to treatment with TNFα and IL-1β for 4 h (Fig. 5). OGM significantly induced CXCL8 mRNA expression (8×) in comparison to HMSCGM alone but at a much lower level than in HMSCGM medium containing 10 ng/ml TNFα/IL-1β (350×). Addition of TNFα/IL-1β in the presence of OGM medium resulted in a significant decrease in CXCL8 mRNA expression in comparison to CXCL8 mRNA expression in HMSCGM medium containing TNFα/IL-1β

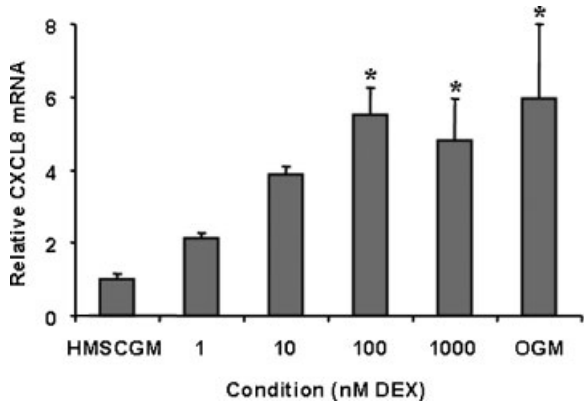

Fig. 4. Dexamethasone dose-dependent induction of CXCL8 mRNA expression. hMSC cultures were treated with HMSCGM containing 1, 10, 100, or 1,000 nM dexamethasone or with OGM medium for 10 days. Relative changes in CXCL8 mRNA levels were normalized to 18S levels and expressed relative to CXCL8 levels in HMSCGM. *Indicates significantly increased for CXCL8 levels compared to the HMSCGM condition ($P < 0.05$).

but was still significantly higher than in OGM medium alone (150×). Addition of the glucocorticoid receptor inhibitor MP had no effect on the ability of TNFα/IL-1β stimulation to induce CXCL8 expression (Fig. 5).

Experiments were also conducted in which OGM was added and then removed at various

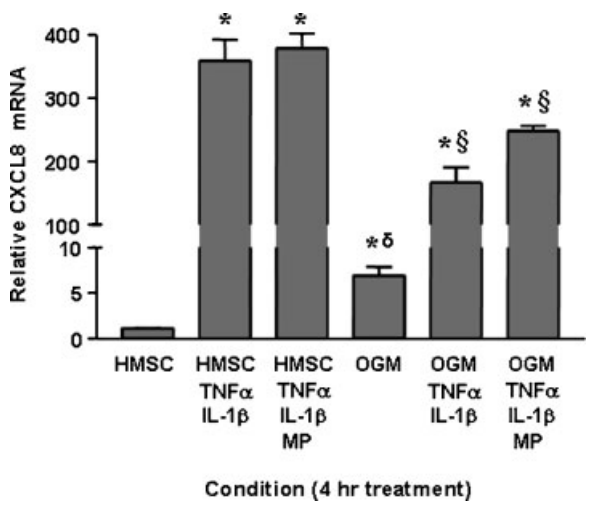

Fig. 5. CXCL8 expression in response to TNFα/IL-1β stimulation in OGM-treated cells. Cells were treated with osteogenic medium (OGM) for 3 days prior to TNFα/IL-1β stimulation (10 ng/ml each) for 4 h. CXCL8 levels were determined and normalized to 18S levels and expressed in relation to HMSC levels. *Indicates significantly increased compared to HMSCGM levels ($P < 0.01$). δIndicates levels in OGM alone were significantly decreased compared to levels induced with pro-inflammatory cytokines ($P < 0.05$). §Indicates significant decrease compared to HMSCGM TNFα/IL-1β levels ($P < 0.01$).

timepoints during differentiation to test whether DEX needed to be present continuously for CXCL8 expression (Fig. 6). Prior to day 7 (7 days in OGM then 3 days in HMSCGM), removal of DEX resulted in levels of CXCL8 comparable to undifferentiated cells (HMSCGM). Similar levels of CXCL8 induction were detected when DEX was removed at day 7 or when it was still present in the medium at day 10, suggesting that DEX does not need to be present at day 10 to get maximal levels of CXCL8 expression.

MP was also added at various times during OGM differentiation to determine whether the effects of DEX on CXCL8 induction were mediated through the glucocorticoid receptor. hMSCs grown in basal HMSCGM media exhibit a fibroblastic phenotype with elongated spindle-shaped cells, having well-defined cell membranes, and becoming packed tightly next to each other. Treatment with OGM for 10 days results in cell rounding with jagged and less defined cell borders (Fig. 7A). Addition of the glucocorticoid receptor inhibitor, MP, at early times while the cells were cultured in OGM (days 0, 1, 3 with inhibitor) resulted in the HMSCGM cell morphology. Addition of MP at later times (days 5 and 7) resulted in the OGM phenotype seen with osteogenic differentiation of hMSCs. CXCL8 mRNA expression was significantly inhibited at all times by addition of MP in comparison to CXCL8 mRNA levels in OGM (Fig. 7B). However, levels of CXCL8 mRNA were still significantly higher when MP was added at day 7 (3 days with inhibitor).

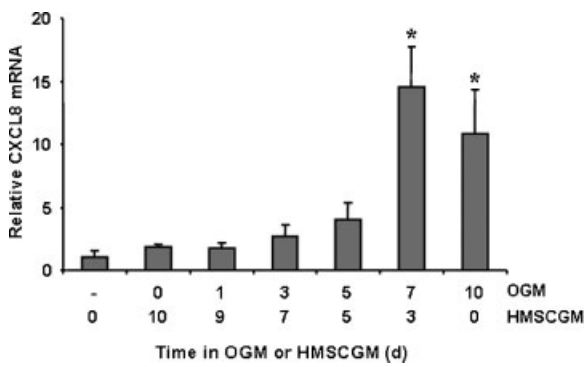

Fig. 6. Effect of addition and then removal of dexamethasone (DEX) on hMSC cultures. Osteogenic medium (OGM) containing DEX was added for 1,3,5,7, or 10 days before replacement with HMSCGM. Levels of CXCL8 mRNA were determined at day 10 using the $2^{-\Delta\Delta C(T)}$ method with normalization to 18S levels and are expressed in relation to HMSCGM levels (without any OGM addition). *Indicates values that are significantly different from HMSCGM levels.

Signal Transduction Pathways That Regulate CXCL8 mRNA Stimulation in OGM

To investigate signal transduction pathways involved in OGM-stimulated CXCL8 mRNA expression, various inhibitors were added to the culture media in OGM for 7 days prior to determination of CXCL8 mRNA levels (Fig. 8A). Treatment of hMSCs with the $G_{\alpha i}$ inhibitor, PTX, suppressed CXCL8 induction. Since $G_{\alpha i}$ can signal through the mitogen-activated protein kinase (MAPK) pathway [Gao et al., 2001; Fan et al., 2004] and since pro-inflammatory cytokine-stimulated CXCL8 mRNA expression is known to be mediated by both MAPK and nuclear factor-κB (NF-κB) [Mukaida et al., 1990; Hwang et al., 2004; Mitsuyama et al., 2004], specific inhibitors of these pathways were also tested. OGM-stimulated CXCL8 mRNA expression was regulated by ERK since PD98059, a MEK1 inhibitor, and U0126, a MEK1/MEK2 inhibitor, both inhibited CXCL8 mRNA expression by about 60% in comparison to U0124, an ERK negative control. Western analysis using phospho-ERK and pan-ERK specific antibodies indicated that the level of phospho-ERK was very low in the presence of U0126 (undetectable) and PD98059 (6%) at day 7 (Fig. 8B) in comparison to the negative control U0124 (set at 100%). Inhibition of p38 using SB202190 or SB203580 also resulted in an approximately 50% decrease in CXCL8 mRNA expression. The negative control for the p38 inhibitors, SB202474 did not significantly inhibit CXCL8 mRNA expression. Levels of phospho-p38 at day 7 in OGM were undetectable by western analysis (data not shown). JNK inhibition did not affect CXCL8 mRNA expression. Addition of a pyrrolidine derivative of dithiocarbamate (PDTC) to inhibit NF-κB resulted in death of the cultures by day 7 (data not shown).

Endothelial Tube Formation Is Stimulated by Conditioned Medium From hMSCs Grown in OGM

The functionality of CXCL8 and CXCL1 production by hMSCs stimulated to differentiate along the osteogenic lineage was examined using in vitro angiogenesis assays and conditioned medium. Microvascular endothelial cells

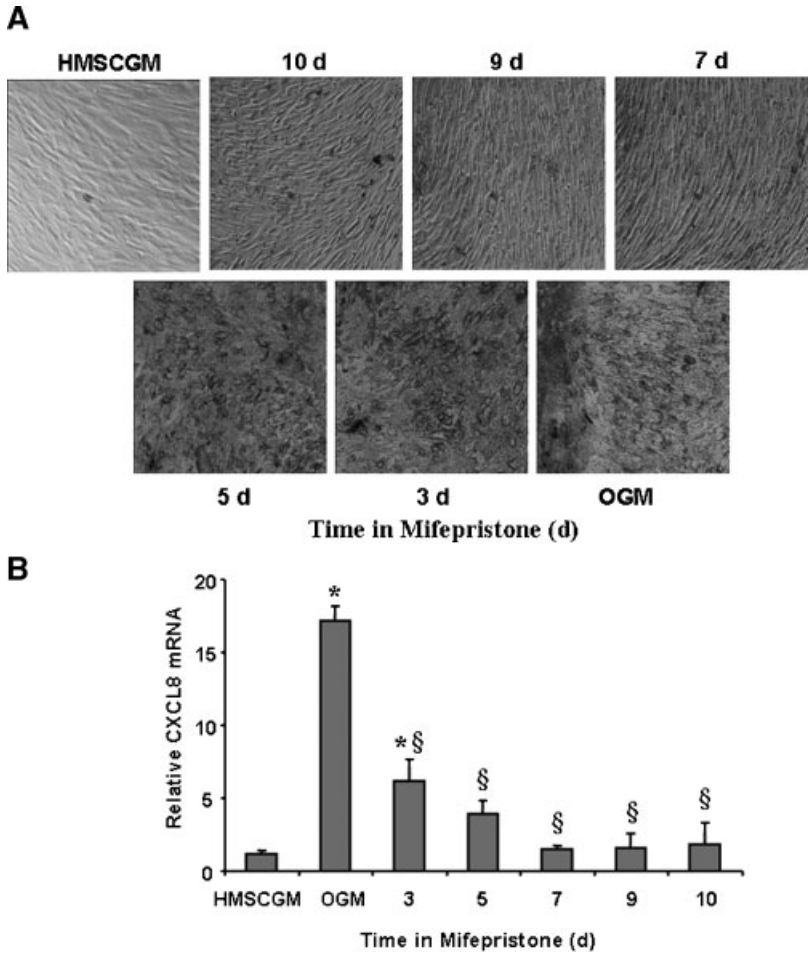

Fig. 7. Glucocorticoid receptor influence on hMSC cell morphology and CXCL8 induction at day 10 in OGM. hMSC cultures were treated with OGM continuously for 10 days. The glucocorticoid receptor inhibitor, mifepristone was added at 1 μM at various timepoints throughout the experiment (days 0, 1, 3, 5, and 7 corresponding to 10, 9, 7, 5, and 3 days in mifepristone, respectively). **A**: Cultures were examined at 10× magnification and photographed for comparison of HMSCGM and OGM cultures. **B**: CXCL8 mRNA levels in each culture were determined at day 10, normalized to 18S levels, and expressed in relation to HMSCGM levels. *Indicates significantly increased levels compared to HMSCGM ($P < 0.05$). §Indicates significantly decreased levels compared to OGM ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(HMEC-1) readily form tube-like structures when grown on Matrigel containing OGM conditioned medium in comparison to conditioned medium from hMSCs grown in HMSCGM (Fig. 9A). HMEC-1 cells plated on OGM-stimulated conditioned medium had an average pattern recognition score of 3.8 after 4 h indicating the formation of closed polygons; whereas, HMSCGM conditioned medium had an average score of 2.3 indicating capillary tubes starting to form in some wells but without spouting. Likewise, OGM conditioned medium stimulated an average of 31 branching points, whereas HMSCGM conditioned medium generated only 15 branching points per field of view (Fig. 9B).

DISCUSSION

Several reports have demonstrated that human osteoclasts and osteoblasts can express chemokines, including CXCL8 and CXCL1 [Chaudary et al., 1992; Rothe et al., 1998; Lisignoli et al., 1999, 2002]. It has also been reported that the production of CXCL8 in inflammatory arthritic pathologies can stimulate an influx of hematopoietic cells (e.g., polymorphonuclear leucocytes) leading to subsequent joint destruction and bone resorption [for reviews see references Szekanecz et al., 2003; Fournier et al., 2006]. However, the expression of ELR^+ CXC chemokines during osteogenesis has not been reported.

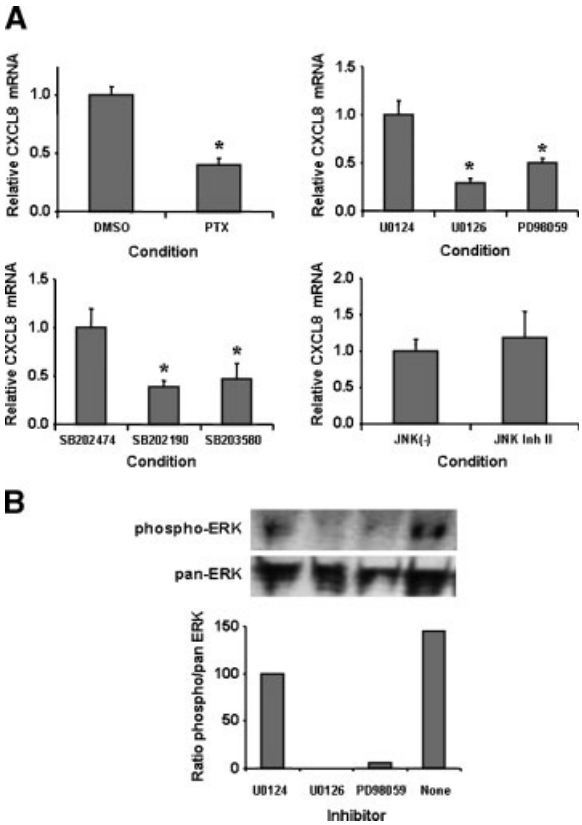

Fig. 8. **A**: Effect of signal transduction inhibitors on OGM-stimulated CXCL8 induction at day 7. hMSC cultures were treated with signal transduction inhibitors for 30 min prior to addition of OGM for 7 days (continuously with inhibitors). Inhibition of $G_{\alpha i}$ with pertussis toxin (PTX, 100 ng/ml), ERK with U0126 (15 μM) and PD98059 (19 μM), p38 pathways with SB202190 and SB203580 (15 μM) and Jun N-terminal kinase (JNK) pathways with the JNK II inhibitor (10 μM). CXCL8 levels were determined at 7 days and normalized to 18S levels and expressed in relation to negative control levels (DMSO, U0124, SB202474, or JNK(−)). *Indicates significantly decreased levels in comparison to the negative control for each pathway ($P < 0.05$). **B**: Western blot analysis of phospho- and pan-ERK in the presence of ERK inhibitors at day 7 in OGM medium. Phospho- and pan-ERK were detected with the phosph-p44/42 MAPK (Thr202/Tyr204) and p44/42 MAPK antibodies from Cell Signaling Technologies and quantitated using the BIOQUANT system. The ratio of phospho/pan ERK was calculated and is reported in relation to the level in the presence of the ERK negative control U0124 (100%).

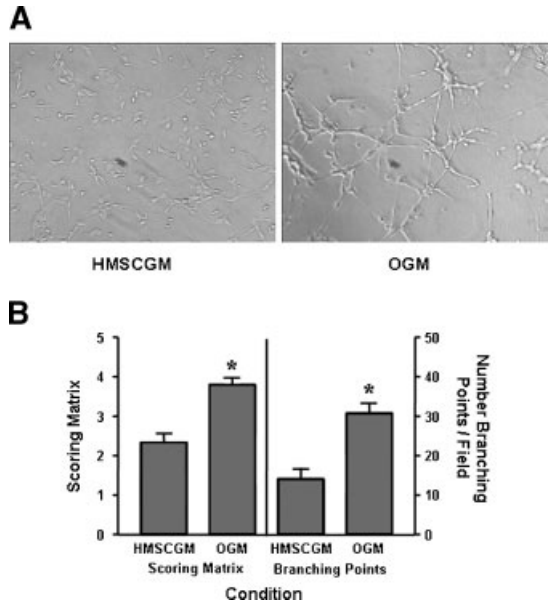

Fig. 9. Angiogenic potential of OGM-treated conditioned medium. Conditioned medium from HMSCGM or OGM-treated cells was collected at days 3–7, mixed 1:1 with growth factor-reduced Matrigel and plated in a 96-well plate. HMEC-1 cells were added to each well (2×10^4 cells/well). Cells were incubated at 37°C for 4 h, observed by phase contrast microscopy and images captured by digital camera. **A**: Human microvascular endothelial cells (HMEC-1) cells form a mesh-like structure when placed on Matrigel containing OGM-conditioned medium over a period of 4 h (10× magnification). **B**: Quantification of pattern scoring matrix and branching points per field of view. *Indicates significant increase compared to HMSCGM ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Our results demonstrate that the expression of the CXCL8 and CXCL1 ELR^+ CXC chemokines is upregulated when hMSCs are grown in medium enabling differentiation along the osteogenic lineage in comparison to hMSCs grown in medium that does not foster osteogenic differentiation. Furthermore, we have found that the glucocorticoid, DEX, used in the differentiation medium is the primary stimulator of CXCL8 production; whereas, the other components found in the differentiation medium, A-2-P and β-GP, do not contribute to the stimulation of CXCL8 mRNA or protein. In fact, A-2-P in combination with DEX actually decreases the amount of CXCL8 mRNA and protein expressed. Removal of DEX from the medium prior to day 7 resulted in complete lack of CXCL8 mRNA induction. DEX-dependent stimulation of CXCL8 in hMSCs was found to be only partially dependent on the glucocorticoid receptor. There is complete inhibition of CXCL8 production by hMSCs when MP, a glucocorticoid receptor antagonist, is added at the initiation of osteogenic differentiation in OGM. However, there is only partial inhibition when MP is added at day 7 (~30% of OGM levels suggesting both glucocorticoid receptor-dependent and receptor-independent pathways of CXCL8 induction.

Traditional agonists of CXC chemokine production include IL-1 and TNFα. While mRNA

expression of CXCL8 was stimulated by IL-1 in combination with TNFα approximately 350-fold in hMSCs grown in non-osteogenic differentiation medium, addition of OGM containing DEX did inhibit the combined IL-1/TNFα stimulation of CXCL8. Stimulation of CXCL8 by these pro-inflammatory cytokines has been shown to be regulated in large part through the NF-κB pathway. This pathway is sensitive to inhibition by glucocorticoids [McKay and Cidlowski, 1999; Bosscher et al., 2003]. Thus, the present data are consistent with DEX partial suppression of IL-1 and TNFα-stimulated CXCL8 production by hMSCs. However, it is interesting that there may be a DEX-stimulated CXCL1 and CXCL8 pathway either independent of the NF-κB signaling pathway involved in pro-inflammatory cytokine stimulation of CXCL8 or overcome when NF-κB mediated CXCL8 production is induced.

We observed that A-2-P inhibited the DEX-stimulated increase in CXCL8 mRNA by approximately 80% and CXCL8 protein by 50%. It has been shown that in other cell types such as endothelial, monocytic, and malignant cell line models, that ascorbic acid can inhibit TNFα-stimulated NF-κB through a variety of mechanisms including the inhibition of IκBα phosphorylation, degradation through inhibition of IκB kinase, and prevention of nuclear translocation of NF-κB [Bowie and O'Neill, 2000; Carcamo et al., 2002; Han et al., 2004]. Thus, it is possible that in hMSCs, DEX can stimulate CXCL8 mRNA expression in part through an NF-κB-mediated pathway. The role of NF-κB in OGM-stimulated CXCL8 regulation is currently under investigation.

OGM-stimulated CXCL8 expression is mediated through the MAPK pathways, ERK1/2 and p38. ERK/1/2 inhibition decreased CXCL8 mRNA expression by 60% while p38 inhibition alone accounted for approximately a 50% inhibition of CXCL8 mRNA expression. The JNK/SAPK (stress-activated protein kinase) MAPK pathway was not involved in OGM-stimulated CXCL8 mRNA expression. It has also been demonstrated that ERK activation can mediate secretion of CXCL8 in macrophages [Bhattacharyya et al., 2002]. In other cell systems, CXCL8 mRNA transcription was shown to be stimulated by JNK and the NF-κB signaling pathways while p38 increased CXCL8 mRNA stability or mRNA transcription [Holtmann et al., 1999; Li et al., 2003; Saatian et al., 2006]. We observed that the half-life of CXCL8 mRNA was prolonged in the presence of OGM compared to that in HMSCGM. While not directly tested, it is possible that the p38 MAPK pathway may be mediating CXCL8 mRNA stability in hMSCs.

In addition to having MSCs migrate to the area where bone is to be re-established and then differentiate into osteoblasts, the development of an adequate blood supply to nourish the newly forming bone as well as the elaboration of growth factors from endothelial cells that may influence osteogenesis is also important in bone repair. Both CXCL8 and CXCL1 are CXC chemokines having the ELR motif that has been shown to be related to the angiogenic ability of these chemokines [Strieter et al., 1995; Bernardini et al., 2003]. Binding of these ELR^+ CXC ligands to the CXC receptor 2 on endothelial cells conveys the angiogenic response which includes proliferation and migration of endothelial cells and tube formation [Murdoch et al., 1999; Addison et al., 2000; Heidemann et al., 2003]. Our observation that hMSCs are stimulated to produce CXCL8 and CXCL1 when differentiated towards the osteogenic lineage by DEX is suggestive of an angiogenic purpose for the elaboration of these chemokines. Moreover, OGM-conditioned medium containing CXCL1 and CXCL8 could induce tube formation of HMEC-1 cells in only 4 h; whereas, HMSCGM-conditioned medium could not do so within this timeframe.

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