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VEGF-A Expression in Osteoclasts Is Regulated by NF-κB Induction of HIF-1α

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ABSTRACT

Large osteoclasts (10+ nuclei), predominant in rheumatoid arthritis and periodontal disease, have higher expression of proteases and activating receptors and also have increased resorptive activity when compared to small (2–5 nuclei) osteoclasts. We hypothesized that large and small osteoclasts activate different signaling pathways. A Signal Transduction Pathway Finder Array™ was used to compare gene expression of large and small osteoclasts in RAW 264.7-derived osteoclasts. Expression of vascular endothelial growth factor A (*Vegfa*) was higher in large osteoclasts and this result was confirmed by RT-PCR. RT-PCR further showed that RANKL treatment of RAW cells induced *Vegfa* expression in a time-dependent manner. Moreover, VEGF-A secretion in conditioned media was also increased in cultures with a higher proportion of large osteoclasts. To investigate the mechanism of *Vegfa* induction, specific inhibitors for the transcription factors NF-κB, AP-1, NFATc1, and HIF-1 were used. Dimethyl bisphenol A, the HIF-1α inhibitor, decreased *Vegfa* mRNA expression, whereas blocking NF-κB, AP-1, and NFATc1 had no effect. Furthermore, the NF-κB inhibitor gliotoxin inhibited *Hif1α* mRNA expression. In conclusion, VEGF-A gene and protein expression are elevated in large osteoclasts compared to small osteoclasts and this increase is regulated by HIF-1. In turn, *Hif1α* mRNA levels are induced by RANKL-mediated activation of NF-κB. These findings reveal further differences in signaling between large and small osteoclasts and thereby identify novel therapeutic targets for highly resorptive osteoclasts in inflammatory bone loss. J. Cell. Biochem. 110: 343–351, 2010.

KEY WORDS: OSTEOCLASTS; VEGF; INFLAMMATION; ARTHRITIS

Osteoclasts (OCs) are multinucleated bone resorbing cells formed by fusion of mononuclear precursors. Under inflammatory conditions, such as rheumatoid arthritis and periodontal disease, OCs are larger and contain more nuclei compared to normal controls [Makris and Saffar, 1982; Takano et al., 2004]. We demonstrated that large OCs (10+ nuclei) have an increased expression of proteases, such as MMP9 and cathepsin K, and activating receptors, such as RANK, IL-1RI, and TNF-R1, whereas small OCs (2–5 nuclei) expressed higher levels of the decoy receptor IL-1RII [Trebec et al., 2007]. Moreover, large OCs had an increased resorptive activity in response to IL-1β compared to small OCs [Trebec et al., 2007]. We hypothesized that different signaling pathways are activated in large and small OCs. To test this hypothesis, we used a Signal Transduction Pathway Finder Array™ to compare gene expression of large and small OCs. The array confirmed activation of NF-κB and AP-1 signaling pathways and also revealed a significant increase in vascular endothelial growth factor A (VEGF-A) expression in large OCs compared to small OCs.

VEGFs are a family of growth factors involved in angiogenesis/vasculogenesis and lymphangiogenesis [Olsson et al., 2006]. In mammals, the VEGF family consists of five glycoproteins designated as VEGF-A, B, C, D, and placental growth factor (PlGF). Mouse VEGF-A has several splice variants: VEGF-A120, VEGF-A144, VEGF-A164, VEGF-A188, and VEGF-A205 [Tischer et al., 1991], with VEGF-A120, VEGF-A164, and VEGF-A188 reported to be present in bone [Maes et al., 2002]. VEGFs bind to VEGF receptors (VEGF-Rs), members of the receptor tyrosine kinase superfamily that include VEGF-R1, VEGF-R2, VEGF-R3, and the neuropilin co-receptors (NP-1 and NP-2).

The major inducer of VEGF-A expression is hypoxia [Shweiki et al., 1992; Brahimi-Horn and Pouyssegur, 2007]. Low oxygen levels (hypoxia) activate hypoxia inducible factor 1 (HIF-1), a

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heterodimeric transcription factor consisting of α (inducible) and β (ubiquitous) subunits. HIF-1α contains an oxygen-dependent degradation domain and is degraded under non-hypoxic conditions. During hypoxia, HIF-1α is stabilized and is free to translocate to the nucleus, bind its dimerization partner HIF-1β, and initiate gene expression of its target genes (e.g., VEGF-A, VEGF-R2, glucose transporters GLUT1 and GLUT3) [Semenza, 2003]. Under normoxic conditions there are other factors that can regulate HIF-1α. For example, FGF, TGFβ [Fukuda et al., 2002], LPS [Blouin et al., 2004], reactive oxygen species (ROS) [Bonello et al., 2007], IL-1β [Frede et al., 2005], and TNF-α [Haddad and Land, 2001; van Uden et al., 2008] are known to stabilize HIF-1α and/or elevate its gene transcription.

Here we show for the first time that in OCs, RANKL regulates *Hif1α* mRNA expression under normoxic conditions. Moreover, RANKL induction of *Hif1α* expression, and consequently the expression of the HIF-1 target gene *Vegfa*, is mediated by NF-κB. We also show that this signaling pathway and upregulation of VEGF-A expression is specific to large OCs.

MATERIALS AND METHODS

MATERIALS

The RAW 264.7 cell line (RAW) was obtained from American Type Culture Collection (ATCC, catalog # TIB-71). Dulbecco's modified Eagle's medium, α-MEM, antibiotics (penicillin/streptomycin, fungizone) and sterile fetal bovine serum (Gibco #12318-028) were obtained from Invitrogen. Antibodies to VEGF-A (sc-507), VEGF-C (sc-9047), and HRP-conjugated secondary antibodies were obtained from Santa Cruz. Monoclonal antibody to GAPDH was obtained from Abcam. Gliotoxin (cat # 317715) was obtained from Calbiochem, cyclosporin A (C1832) and curcumin (C7727) were obtained from Sigma. Tanshinone IIA (GR-339) and dimethyl bisphenol-A (GR-339) were from Enzo Lifesciences.

DIFFERENTIATION AND ISOLATION OF LARGE AND SMALL OCs FROM THE RAW 264.7 CELL LINE

Populations of at least 75% large (10+ nuclei) or small (2–5 nuclei) OCs were obtained as described in Trebec et al. [2007]. Prior to protein and/or RNA isolation, the remaining mononuclear cells were removed by gently washing the dishes with 3 × 10 ml phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) STAINING

Culture dishes were washed with PBS and then fixed with formalin for 10 min. Staining for TRAP was carried out according to the protocol described in BD Biosciences Technical Bulletin # 445 with minor modifications as previously described [Trebec et al., 2007].

MICROARRAY

Large and small OCs were generated from RAW cells in 100-mm cell culture dishes, and RNA was isolated using the GEArray Total RNA Isolation Kit (SA Biosciences, cat # GA-013) according to manufacturer's instructions. One to three micrograms of total RNA was then amplified and labeled with biotin-16-UTP (Roche) in vitro for 4–12 h using the GEArray Truelabeling-AMP 2.0 kit (SA Biosciences, GA-030). Two micrograms of labeled RNA was then hybridized to a GEArray Signal Transduction Pathway Finder Array™ (SA Biosciences, EMM-014) overnight (~16 h). Arrays were visualized by chemiluminescence and analyzed using GEArray Expression Analysis Suite 2.0 (SA Biosciences). The arrays were analyzed, corrected for background, and then normalized to GAPDH. Gene expression differences of at least 1.5-fold were examined.

RT-PCR

RNA from large and small OCs used in the array was subjected to RT-PCR to confirm the presence/absence of genes identified in the array. To test the effect of inhibitors on gene expression, cells were incubated in the presence or absence of RANKL, and the inhibitors for 3 h, and then RNA from large and small OCs was isolated using TRIzol following manufacturer's instructions as previously described [Trebec et al., 2007]. The following concentrations of the inhibitors were used: gliotoxin (NF-κB inhibitor) 30 ng/ml, cyclosporin A (NFAT inhibitor) 1 μg/ml, tanshinone IIA (c-Fos inhibitor) 20 μg/ml, curcumin (AP1 and NF-κB inhibitor) 10 μM, and dimethyl bisphenol-A (HIF-1α inhibitor) 50 μM. Primers for *Vegfa*, *Vegfc*, *Gapdh*, *Nfatc1*, *IL-1β* and *Hif1α* are summarized in Table I. RNA was treated as previously described [Trebec et al., 2007]. PCR reactions were performed in 50 μl volumes using HotStarTaq polymerase (Qiagen). The initial activation was performed at 95°C for 15 min, followed by 45 s at 94°C for denaturation, 1 min at the annealing temperature, 1 min at 72°C for extension, and 10 min at 72°C for a final extension. PCR products were separated on 1.8% agarose gels and visualized using GeneSnap software (Syngene). The band intensities were analyzed by either

TABLE I. Primer Sequences Used in RT-PCR

Gene name	Primer sequences	T_a (°C)/number of cycles	References
Vegfa	5′-GGACCCTGGCTTTACTGCTGTACC-3′ 5′-TCACCGCCTTGGCTTGTCACA-3′	60/30	Veillette and von Schroeder [2004]
Vegfc	5′-AGACTCAATGCATGCCACG-3′ 5′-TTGAGTCATCTCCAGCATCC-3′	51/33	Weich et al. [2004]
Hif1α	5′-TGCTCATCAGTTGCCACTTCC-3′ 5′-CGCTGTGTGTTTTGTTCTTTACCC-3′	60/34	Albina et al. [2001]
Gapdh	5′-TGCCAGCCTCGTCCCGTAGAC-3′ 5′-CCTCACCCCATTTGATGTTAG-3′	60/25	Haynes et al. [1999]
Nfatc1	5′-CAACGCCCTGACCACCGATAG-3′ 5′-GGCTGCCTTCCGTCTCATAGT-3′	60/25	Kwak et al. [2006]
IL1β	5′-CTACCTGTGTCTTTCCCGTGG-3′ 5′-CCAGCAGGTTATCATCATC-3′	60/30	Haynes et al. [1999]

GeneTools (Syngene) or Quantity One® band analysis software with rolling disk background correction (Biorad) and then normalized to *Gapdh*.

PROTEIN ISOLATION

Protein was harvested from RAW-cell-derived OCs using the RIPA buffer method as previously described [Trebec et al., 2007]. Briefly, cells were washed with cold PBS without Ca^{2+} and Mg^{2+} and then lysed in 1 ml of RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X, 1% SDS, 0.5% sodium deoxycholate) containing complete protease inhibitor cocktail (Sigma, P8340). The whole cell lysates were incubated on ice for 20 min with vortexing every 10 min, then centrifuged at 14,000 rpm at 4°C for 15 min. Protein concentrations were determined using Pierce BCA kit (Pierce cat # 23225).

IMMUNOBLOTTING

Fifty micrograms of protein from OCs were separated on 12% mini SDS–PAGE gels. Protein was transferred to nitrocellulose as previously described [Trebec et al., 2007]. The membranes were then blocked in Tris-buffered saline Tween-20 (TBS-T) buffer containing 5% milk (w/v) for 1 h. The blots were incubated with primary antibodies in blocking solution (5% milk in TBS-T) overnight at 4°C, washed three times in TBS-T buffer, incubated with appropriate secondary antibody in blocking solution at room temperature for 1 h, washed again, and then developed using ECL reagents (Promega). Images were captured using ChemiDoc XRS HQ2 (Biorad) and analysis was carried out using Quantity One® Software with rolling disk background correction.

VEGF ELISA

RAW cells were incubated in the presence of RANKL 150 ng/ml for 7 days and conditioned media were collected every 24 or 48 h with media changes at days 3 and 5. After collecting conditioned media, corresponding cells were fixed and TRAP stained for counting. VEGF-A levels were measured using the Quantikine Mouse VEGF

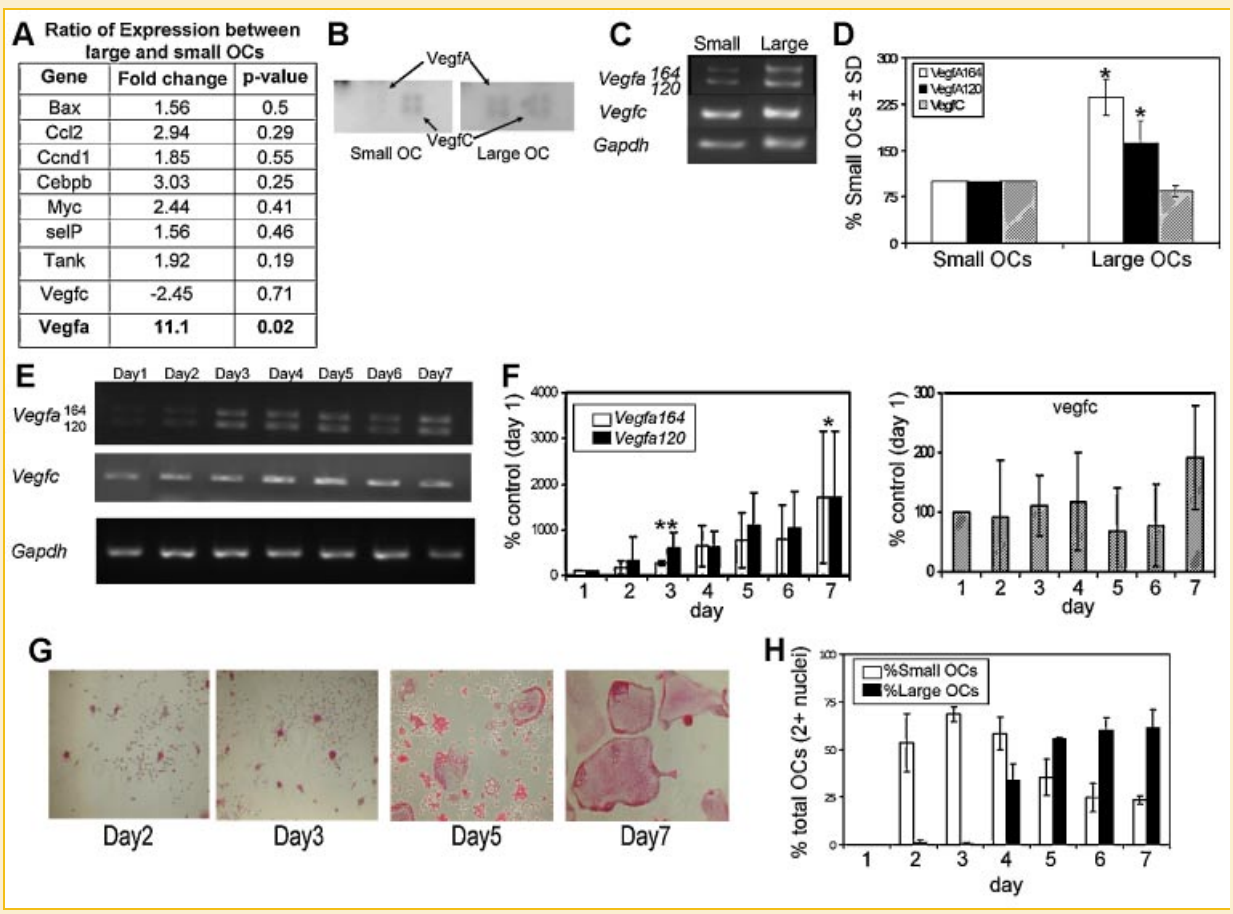

Fig. 1. Large OCs express higher levels of *Vegfa* compared to small OCs. Enriched populations of large (10+ nuclei) and small (2–5 nuclei) OCs were generated from RAW 264.7 cells in the presence of sRANKL (150 ng/ml). mRNA was isolated and hybridized to a Signal Transduction Pathway Finder Array™ (SA Biosciences). Gene expression was normalized to *Gapdh* expression with the cutoff set at 1.5-fold difference in expression; n = 3. Statistical analysis was assessed using GEArray Expression Analysis Suite (SA Biosciences) and considered significant when $P < 0.05$. (A) Quantified results of fold differences in expression in large OCs compared to small OCs; (B) typical presentation of *Vegfa* and *Vegfc* genes on the array; (C) semi-quantitative RT-PCR to verify *Vegfa* and *Vegfc* expression in large and small OCs; (D) quantification of three combined experiments expressed as a percentage of small OCs ± SD; (E) Time course of OC differentiation: mRNA was collected every 24 h and expression of *Vegfa* and *Vegfc* was assessed using RT-PCR; (F) quantification of RT-PCR results, four combined experiments expressed as a percentage of control (day 1) ± SD; $^{*}P < 0.05$ versus day 1, $^{**}P < 0.05$ versus day 7; (G) time course of TRAP stained OCs; (H) quantification of combined experiments shown in (G) expressed as a percent of total OCs, n = 4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Immunoassay (R&D Systems, MMV00) according to the manufacturer's instructions. Briefly, samples were warmed to room temperature (RT) and centrifuged at 500*g* for 5 min to pellet cell debris. Fifty microliters of sample/standard was added to wells with diluents and incubated at RT for 2 h. Plates were then washed 5× with wash buffer and then incubated with VEGF conjugate for 2 h at RT. After washes, the plates were incubated with the substrate solution for 30 min at RT, the reaction was terminated with stop solution, and the absorbance was measured at 450 nm. Similar results were seen in both 24 and 48 h collections.

STATISTICS

Statistical analysis was done using SPSS 17.0 for Windows by Student's *t*-test or one-way ANOVA. Data were considered statistically significant when $P < 0.05$.

RESULTS

LARGE OCs EXPRESS HIGHER LEVELS OF VEGFA COMPARED TO SMALL OCs

We previously demonstrated that large and small OCs express distinct sets of markers [Trebec et al., 2007] and have different resorptive abilities [Lees et al., 2001; Trebec et al., 2007]. Based on these results, we hypothesized that the signaling pathways in large and small OCs are differentially activated. To test this hypothesis, we used RNA from RAW 264.7 (RAW) cell-derived OCs, and a Signal Transduction Pathway Finder Array™ to compare activation of major signaling pathways in large and small OCs. The array analysis revealed that a number of molecules were differentially expressed, particularly the expression of *Vegfa* which was elevated 11-fold in large OCs (Fig. 1A,B). Other molecules, such as *Tank* (TRAF family member-associated NF-κB activator, NF-κB pathway), *Ccl2* (C-C motif chemokine 2, NF-κB pathway), *c-Myc* (NF-κB pathway), and *Cebpb* (CCAAT/enhancer-binding protein β, IL-6 pathway) also met the 1.5-fold cutoff criteria but were not statistically significant when data from three arrays were combined (Fig. 1A). *Vegfc* levels, on the other hand, appeared to be higher in small compared to large OCs, but this was also not statistically significant. RT-PCR confirmed greater expression of *Vegfa* in large OCs and found no difference in *Vegfc* expression (Fig. 1C,D). To ask whether *Vegfa* and *Vegfc* expression levels change over the course of OC differentiation, mRNA was collected every 24 h post-RANKL addition and analyzed by RT-PCR (Fig. 1E,F). Expression of *Vegfa* (*Vegfa120* and *Vegfa164*) was virtually absent at the early differentiation time points (days 1 and 2), but increased at the later time points (days 3 to 7), while *Vegfc* expression remained unchanged during the course of differentiation (Fig. 1E,F). The increase in *Vegfa* expression coincided with the higher proportion of large OCs in cultures (Fig. 1G,H). *Vegfa* levels at day 7, when the majority of cells had 10+ nuclei, were significantly higher than *Vegfa* levels at day 3, when the majority of cells were small OCs. In summary, these results show that *Vegfa* gene expression is significantly upregulated in large OCs compared to small OCs.

VEGF-A SECRETION IS INCREASED IN LARGE OCs-ENRICHED CULTURES

We next wished to assess whether increased gene expression was reflected in increased protein expression. As VEGF-A is both a

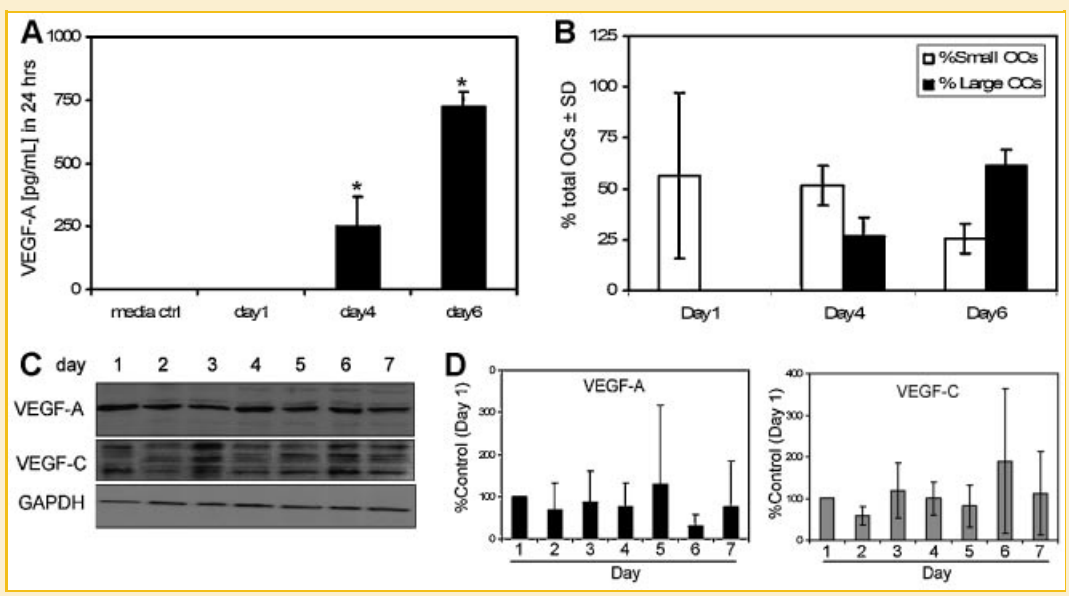

Fig. 2. VEGF-A secretion is increased in large OCs-enriched cultures. RAW cells were differentiated with RANKL and conditioned medium was collected every 24 h with media changes at days 3 and 5. Secreted VEGF-A was measured using an ELISA. (A) ELISA results from three combined experiments; n = 8, $^{*}P < 0.001$ versus day 1. (B) TRAP staining of the cells in corresponding wells, the results are expressed as proportion of small and large OCs ± SD, n = 3. (C) RAW cells were differentiated with RANKL for up to 7 days, cells were lysed and protein was collected every 24 h. Fifty micrograms of protein was loaded on a 12% SDS–PAGE gel and probed for VEGF-A and VEGF-C, representative experiment; n = 3. (D) VEGF-A and VEGF-C protein expressions were quantified and normalized to GAPDH expression. Quantification of three combined experiments is shown, expressed as a percentage of control (day 1) ± SD.

secreted [Houck et al., 1991; Otrock et al., 2007] and cell-associated [Houck et al., 1991] cytokine, we assessed levels of VEGF-A both in the conditioned media and in whole cell lysates over the course of OC differentiation. Secreted VEGF-A increased starting at day 4 (Fig. 2A) and this increase coincided with a higher proportion of large OCs in the corresponding cultures (Fig. 2B). For cell-associated VEGF-A and VEGF-C, immunoblotting demonstrated that there were no significant differences over the course of differentiation (Fig. 2C,D). These results indicate that in agreement with increased gene expression, large OCs express more VEGF-A, and this increased protein is secreted.

NF-κB, NFATc1, AND c-Fos INHIBITORS DO NOT DIRECTLY AFFECT VEGFA GENE EXPRESSION

To elucidate the molecular mechanism of RANKL-induced *Vegfa* expression, several transcription factors involved in osteoclastogenesis and in VEGF regulation were examined. The *Vegfa* promoter has bindings sites for transcription factors Sp1, Sp2, AP-1, AP-2, NF-κB, p53, T-cell factor (TCF), and HIF-1 [Shima et al., 1996; Silins et al., 1997; Pages and Pouyssegur, 2005; Clifford et al., 2008]. As AP-1, NF-κB, and NFATc1 play key roles in OC differentiation [Takayanagi, 2005] these transcription factors were examined. Enriched populations of large and small OCs were treated with gliotoxin (NF-κB inhibitor), curcumin (c-Fos and NF-κB inhibitor), tanshinone IIA (c-Fos inhibitor), and cyclosporin A (NFATc1 inhibitor) in the presence or absence of RANKL for 3 h. The decrease in mRNA expression of *Trap*, *IL1β*, and *Nfatc1* confirmed inhibition of NFATc1, NF-κB, and c-Fos, respectively. Inhibition of NF-κB, NFATc1, or c-Fos had no effect on *Vegfa120* (Fig. 3A,B), *Vegfa164* (data not shown), or *Vegfc* gene expression (data not shown); whereas c-Fos and NF-κB inhibitor curcumin significantly inhibited *IL1β* and *Nfatc1* expression, confirming inhibition of these signaling pathways (Fig. 3C). Similar results were observed in small OCs (data not shown). These results demonstrate that NFATc1, NF-κB, and c-Fos do not directly regulate *Vegfa* and *Vegfc* expression.

THE TRANSCRIPTION FACTOR HIF-1 REGULATES RANKL-INDUCED VEGFA EXPRESSION IN LARGE OCs

HIF-1 is a key inducer of VEGF genes [Levy et al., 1995]. It is induced by hypoxic conditions [Shweiki et al., 1992], by increased metabolic demands on the cell [Song et al., 2009] and by cytokines, such as IL-1β [Murata et al., 2006] and TNF-α [Haddad and Land, 2001; van Uden et al., 2008]. To test the involvement of HIF-1 in RANKL-induced *Vegf* gene expression, OCs were treated with the HIF-1α inhibitor dimethyl bisphenol A (BpA). BpA significantly inhibited *Vegfa120* and *Vegfa164* expression in large OCs (Fig. 4A,B). This effect was not observed in small OCs (data not shown). As expected from previous reports [Chilov et al., 1997], *Vegfc* levels were not affected by HIF-1α inhibition (Fig. 4A,B). These results suggest that

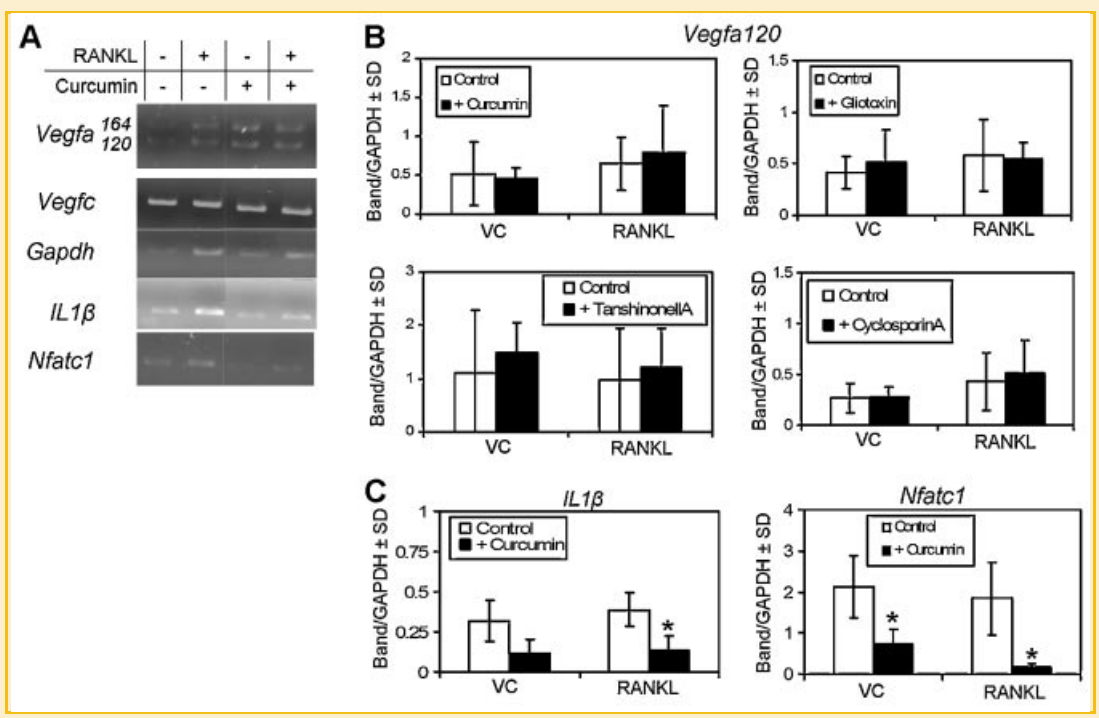

Fig. 3. NF-κB, NFATc1, and c-Fos inhibitors do not have a direct effect on *Vegfa* gene expression. Large and small OCs generated from RAW cells were pretreated with inhibitors for 30 min at the following concentrations: gliotoxin 30 ng/ml, cyclosporinA 1 μg/ml, tanshinone IIA 20 μg/ml, and curcumin 10 nM. RANKL (50 ng/ml) or vehicle control (VC) was then added and the cells were incubated for an additional 3 h. RNA was then isolated and semi-quantitative RT-PCR was performed. (A) Results from a typical experiment in large OCs are shown. For quantification of RT-PCR, band intensities were measured and normalized to *Gapdh*; n = 3. (B) *Vegfa120* expression in large OCs: combined data from three independent experiments for each inhibitor. (C) Quantification of *IL1β* and *Nfatc1* expression to confirm inhibition of NF-κB and AP-1 (c-Fos), respectively, normalized to *Gapdh* ± SD; n = 3, $^{*}P < 0.05$.

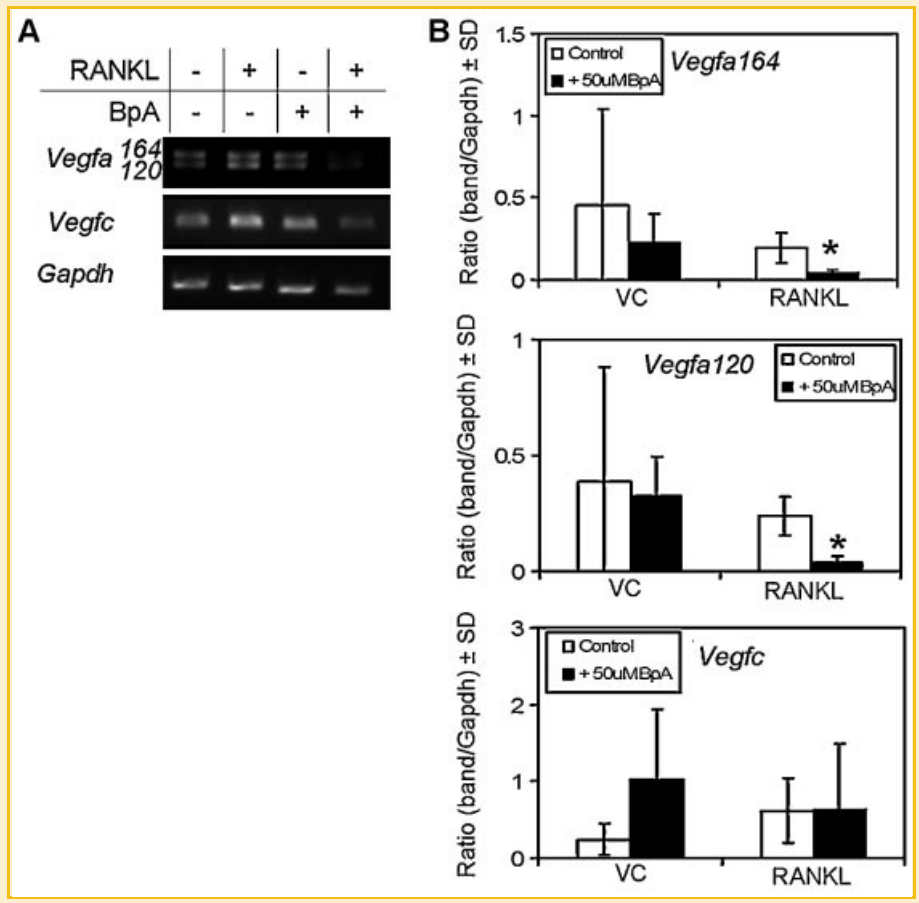

Fig. 4. The transcription factor HIF-1 regulates RANKL-induced *Vegfa* expression in large OCs. Large and small OCs generated from RAW cells were pretreated with 50 μM of dimethyl bisphenol A (BpA) for 30 min. RANKL (50 ng/ml) or vehicle control (VC) was added to cells for an additional 3 h. mRNA was isolated and semi-quantitative RT-PCR was performed. (A) Results from a single representative experiment on large OCs. (B) Quantification of *Vegfa* and *Vegfc* expression from combined experiments in large OCs, expressed as a ratio of band intensity normalized to *Gapdh* (*band/Gapdh*) ± SD; n = 3. $^{*}P < 0.05$ versus control.

HIF-1 is the transcription factor involved in RANKL-induced *Vegfa* expression in large OCs but not small OCs.

NF-κB REGULATES RANKL-INDUCED HIF-1α EXPRESSION IN LARGE OCs

As the previous figure suggested that HIF-1 is the transcription factor involved in RANKL-induced *Vegfa*, it was important to verify that RANKL induces *Hif1α* expression in OCs. Figure 5A,B shows that RANKL upregulates *Hif1α* mRNA expression over the course of OC differentiation peaking at day 5. We next addressed which RANKL signaling pathway is inducing *Hif1α*. As the *Hif1α* promoter contains NF-κB, AP-1, and NFATc1 binding sites [Minet et al., 1999; Frede et al., 2006; Walczak-Drzewiecka et al., 2008], we asked whether *Hif1α* gene expression in OCs is regulated by any of these transcription factors. The cells were treated with the inhibitors mentioned previously and *Hif1α* expression levels were assessed. The NF-κB inhibitor gliotoxin inhibited RANKL-induced *Hif1α* expression compared to vehicle control (Fig. 5C,D), while neither the NFATc1 inhibitor cyclosporin A (Fig. 5C,D) nor the AP-1 inhibitor tanshinone IIA (data not shown) had any effect. None of these inhibitors had any effect in small OCs (Fig. 5E,F and data not shown). These results indicate that NF-κB regulates RANKL-induced *Hif1α* expression in large but not small OCs.

DISCUSSION

VEGFs are a family of growth factors known for their role in angiogenesis. In bone, VEGFs are secreted by osteoblasts, OCs, chondrocytes, and endothelial cells and are important for normal bone development, as well as for bone repair [Wang et al., 1997; Niida et al., 1999; Zhang et al., 2008]. OCs have been shown to secrete VEGF-A and VEGF-C and to express VEGF receptors, such as VEGF-R1 and VEGF-R2 [Tombran-Tink and Barnstable, 2004; Yang et al., 2008; Zhang et al., 2008]. Here we present evidence that VEGF-A is preferentially expressed by large OCs and we further show that VEGF-A expression is induced by a non-hypoxic pathway via NF-κB activation of *Hif1α*.

The importance of VEGF-A in osteoclastogenesis was highlighted when the osteopetrotic phenotype in the *op/op* mouse (ablated for M-CSF) was rescued by a single injection of human recombinant VEGF-A, showing that VEGF-A could substitute for M-CSF *in vivo*

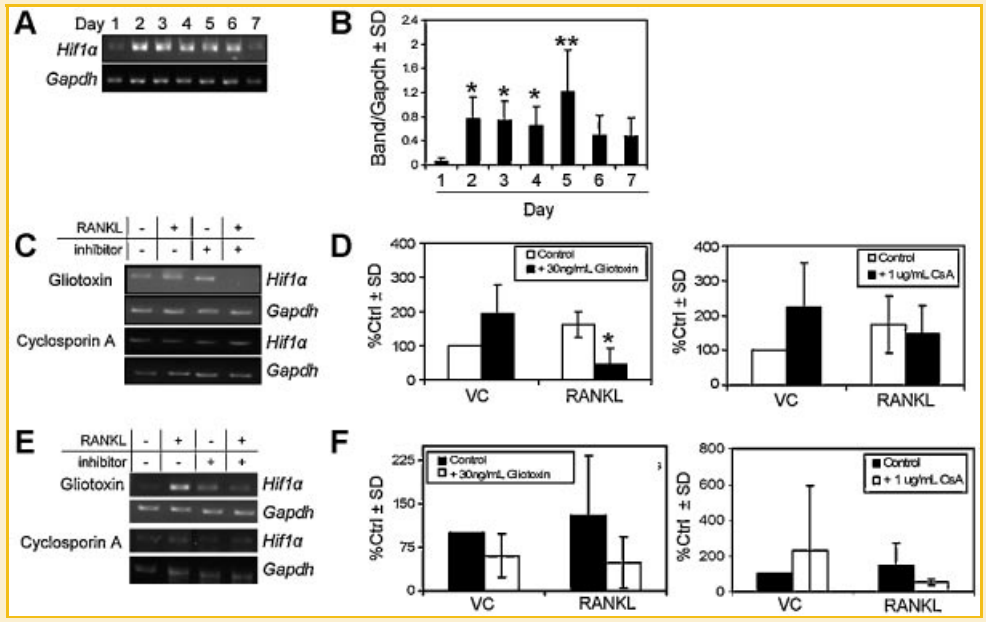

Fig. 5. NF-κB regulates *Hif1α* expression in large OCs. (A) Time course of osteoclast differentiation: mRNA was collected every 24 h and expression of *Hif1α* was assessed using RT-PCR. Results were quantified and normalized to *Gapdh*. The combined results from three experiments are shown in (B). $^{*}P < 0.05$; $^{**}P < 0.01$ versus day 1. OCs were pretreated with inhibitors for 30 min and then incubated with RANKL or control media for another 3 h. mRNA was isolated and *Hif-1α* expression was assessed by semi-quantitative RT-PCR. (C) Representative RT-PCR experiment: *Hif1α* expression in large OCs treated with gliotoxin or CsA; (D) quantification of *Hif1α* expression in large OCs, expressed as a percent of control (VC) ± SD; n = 3. (E) Representative RT-PCR experiment: *Hif1α* expression in small OCs treated with gliotoxin or CsA; (F) quantification of *Hif1α* expression in small OCs, expressed as a percent of control (VC) ± SD; n = 3. CsA = cyclosporinA.

[Niida et al., 1999]. *In vitro*, VEGF-A treatment enhances OC survival, differentiation, and bone resorption [Dai and Rabie, 2007; Knowles and Athanasou, 2008; Yang et al., 2008]. To date, VEGF-A has been shown to be produced by osteoblasts, chondrocytes, and endothelial cells and it acts as a paracrine molecule to recruit OCs to the site of remodeling, to stimulate OC differentiation, and to promote OC resorption [Niida et al., 1999; Maes et al., 2002; Sipola et al., 2006; Knowles and Athanasou, 2008]. Data presented here reveal for the first time that RANKL induces OC VEGF-A expression and secretion, suggesting that VEGF-A can also act in an autocrine fashion, possibly to enhance OC survival and activity levels. Moreover, we show that *Vegfa* gene expression and the amount of secreted VEGF-A increase during osteoclastogenesis (Figs. 1 and 2). Our results appear to contradict findings by Zhang et al. [2008] who showed that in spleen-derived OCs only *Vegfc*, and not *Vegfa*, was upregulated by RANKL. This discrepancy is likely due to differences in incubation time points: Zhang et al. measured *Vegfa* gene expression levels up to 3 days, while here we show expression upregulated after 3 days (Fig. 1E,F). This is confirmed by our results showing that VEGF-A secretion is also upregulated at later time points (after day 4 and up to day 7; Fig. 2A and data not shown). We also observed the presence of *Vegfc* in our culture system; however, the levels of *Vegfc* did not change over the course of differentiation (Fig. 1), corresponding to data described by Zhang et al.

As the mechanism of RANKL-induced *Vegfa* expression has not been described, it was not clear which signaling pathway was involved. *Vegfa* expression is regulated by HIF-1; however, the promoter region also contains binding sites for other transcription factors, such as Sp1, Sp2, AP-1, AP-2, NF-κB, p53, and TCF [Shima et al., 1996; Chilov et al., 1997; Silins et al., 1997; Minet et al., 1999; Pages and Pouyssegur, 2005; Clifford et al., 2008]. To elucidate which transcription factor is regulating *Vegfa* expression in OCs, the cells were treated with specific inhibitors. We show that NF-κB, AP-1, and NFATc1, the key transcription factors involved in osteoclastogenesis, were not directly involved in RANKL-induced *Vegfa* expression, whereas HIF-1α was the transcription factor mediating RANKL-induced *Vegfa* expression (Figs. 3 and 4).

HIF-1 is a hypoxia-inducible factor and, as the name implies, is usually induced by hypoxia; however, other factors, such as inflammatory cytokines TNF-α [Haddad and Land, 2001; van Uden et al., 2008] and IL-1β [Frede et al., 2005], are also known to activate this transcription factor. Since all experiments were performed under normoxic conditions, we wished to determine if RANKL, a member of TNF superfamily, could also induce *HIF-1α* gene expression. We show that RANKL is capable of inducing *Hif1α* expression via activation of NF-κB (Fig. 5). Moreover, peak *HIF-1α* expression (day 5) coincides with cultures enriched in large OCs, indicating that HIF-1α and consequently VEGF-A play a role in later stages of osteoclastogenesis, possibly by promoting cell survival and enhancing bone resorption. As VEGF-A was secreted into the media, it is also possible that it plays a role in recruiting endothelial cells and osteoblasts to the sites of resorption, to promote angiogenesis and bone formation, thus completing the bone remodeling cycle. It is possible that under inflammatory conditions when the majority of OCs are large, inflammatory cytokines such as TNF-α and IL-1β further enhance VEGF-A secretion and, therefore, OC resorptive

activity and survival, contributing to the vicious cycle observed in rheumatoid arthritis. Supporting this hypothesis, VEGF-A has been reported to be elevated in rheumatoid arthritis [Malemud, 2007; Yoo et al., 2008].

Further studies are required to elucidate the role of VEGFs in OCs as well as the pathways activated in OC signaling. Recent studies investigating anti-VEGF therapy in arthritic mouse models are promising [Miotla et al., 2000; Yoo et al., 2008; Choi et al., 2009]; however, the effects on bone erosion need to be better assessed. Thus, targeting VEGF-A in inflammatory bone loss could provide a novel therapeutic target specifically aimed at the hyperactive OCs characteristically seen in these conditions while still maintaining a normal bone remodeling cycle.

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