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Osteocyte apoptosis regulates osteoclast precursor adhesion via osteocytic IL-6 secretion and endothelial ICAM-1 expression

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ABSTRACT

Osteocyte apoptosis precedes osteoclast resorption, and may act as a critical signal to trigger bone remodeling. While osteoclast precursors are known to travel via the circulation, the specific mechanisms by which they accumulate at remodeling sites are unclear. We hypothesized that osteocyte apoptosis mediates osteoclast precursor adhesion to vascular endothelium by regulating osteocytic secretion of IL-6 and soluble IL-6 receptor (sIL-6R) to promote endothelial ICAM-1 expression. We found that conditioned media from TNF-α-induced apoptotic MLO-Y4 osteocytes promoted RAW264.7 osteoclast precursor adhesion onto D4T endothelial cells ($P<0.05$). Blocking osteocyte apoptosis with a pan-caspase inhibitor (ZVAD-FMK) reduced osteoclast precursor adhesion to baseline levels ($P<0.001$). Endothelial cells treated with apoptotic osteocyte conditioned media had elevated surface expression of ICAM-1 ($P<0.05$), and blocking ICAM-1 abolished apoptosis-induced osteoclast precursor adhesion. Apoptotic osteocyte conditioned media contained more IL-6 ($P<0.05$) and sIL-6R ($P<0.05$) than non-apoptotic osteocyte conditioned media. When added exogenously, both IL-6 and sIL-6R were required for endothelial activation, and blocking IL-6 reduced apoptosis-induced osteoclast precursor adhesion to baseline levels ($P<0.05$). Therefore, we conclude that osteocyte apoptosis can promote osteoclast precursor adhesion to endothelial cells via ICAM-1; this is likely through increased osteocytic IL-6 and sIL-6R secretion, both of which are indispensible to endothelial activation.

Introduction

Osteocytes are hypothesized to be mechanosensors that are responsible for translating mechanical forces experienced within bone into chemical signals that regulate osteoblasts and osteoclasts in bone remodeling. Recent studies have shown that osteocyte apoptosis, whether induced by weightlessness [1] or fatigue loading [2–4], precedes osteoclast resorption *in vivo*, suggesting that osteocyte apoptosis triggers osteoclast precursor recruitment to initiate bone resorption.

Osteoclasts are formed by the fusion of mono-nucleated osteoclast precursors that are derived from the hematopoietic lineage [5]. Similar to leukocytes, osteoclast precursors travel via the circulation to reach their target site. However, the specific mechanisms by which osteoclast precursors transport from the vasculature to the bone-lining surface of the remodeling site are unclear. Through immuno-histological evidence, Andersen et al. reported that osteoclast precursors might be delivered directly to the remodeling site via new blood vessels [6]. Consistent with this, we found that apoptotic osteocyte conditioned media promotes angiogenesis [7], suggesting a mechanism by which apoptotic osteocytes may direct new blood vessel growth to aid site-specific osteoclast precursor recruitment.

Once closer to the remodeling site, osteoclast precursors extravasate the vasculature by adhering to [8] and transmigrating [9] across the endothelium to enter the remodeling site. Kindle et al. showed that pretreatment of endothelial cells with the inflammatory cytokines IL-1α and TNF-α upregulated endothelial intercellular adhesion molecule 1 (ICAM-1) expression [9], which promoted osteoclast precursor adhesion. We found that apoptotic osteocyte conditioned media contains elevated levels of IL-6 [7], a known pro-inflammatory cytokine and inducer of leukocyte recruitment [10]. This finding suggested that osteocyte apoptosis may regulate endothelial inflammation via IL-6 to promote osteoclast precursor adhesion. IL-6 is produced by osteoblasts [11] in bone, and regulates bone turnover [11–13] by modulating the differentiation of osteoblasts [14,15] and osteoclasts [11,16]. IL-6 mediates its biological activity by binding to its membrane-bound receptor, IL-6R. The IL-6/IL-6R complex associates with a homodimer of the transmembrane signal-transducing element gp130 to trigger intracellular signaling [17]. While expression of gp130 is ubiquitous [18], the expression of membrane-bound IL-6R

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is limited to hepatocytes and leukocyte subpopulations [17]. However, formation of a soluble form of IL-6R (sIL-6R) via shedding [10,19,20] or mRNA splicing [21] allows for IL-6 to exert biological functions on cells that do not express membrane-bound IL6-R [22–25]. As TNF-α treated osteoblasts have previously been demonstrated to shed IL-6R [26], we hypothesized that TNF-α induced osteocyte apoptosis promotes osteoclast precursor adhesion by regulating osteocytic secretion of IL-6 and sIL-6R and mediating endothelial ICAM-1 expression.

Methods

Cell culture

MLO-Y4 cells (gift from Dr. Lynda Bonewald, University of Missouri-Kansas City) served as a model for osteocytes and were cultured on rat-tail collagen type 1 (BD Biosciences) coated glass slides in α-MEM (Gibco/Invitrogen) with 2.5% fetal bovine serum (Hyclone; FBS), 2.5% calf serum (Hyclone; CS), and 1% penicillin/streptomycin (Gibco/Invitrogen; P/S). RAW264.7 monocyte cells (AATC) served as a cell model for osteoclast precursors and were cultured in DMEM (Gibco/Invitrogen) with 10% FBS (Hyclone) and 1% P/S. Mouse embryonic stem cell-derived D4T cells (gift from Dr. Peter Zandstra, University of Toronto) served as a model for endothelial cells [7]. D4T cells were cultured in IMDM + L-glutamine (Gibco/Invitrogen) supplemented with 5% certified FBS (Gibco/Invitrogen) and 1% P/S.

Conditioned medium

Conditioned media was collected as previously described [7]. Briefly, MLO-Y4 cells were cultured under apoptotic (10 ng/mL TNF-α) or normal culture conditions (0 ng/mL TNF-α) for 6 h (n = 3). Cells were rinsed with phosphate buffered saline (PBS) prior to incubating with fresh supplemented α-MEM media for 2 h to make apoptotic and non-apoptotic osteocyte conditioned media, respectively.

In some cases, apoptosis was blocked by pre-treating the osteocytes with ZVAD-FMK (0.1 μM, R&D systems) or DMSO as vehicle control for 1 h at 37 °C prior to TNF-α treatment. All conditioned media were stored at −20 °C until further use.

Adhesion assay

CellTracker Green (Invitrogen) labeled-osteoclast precursors [7] were added to confluent endothelial cells after treatment with the various conditions for 18 h. After a one-hour incubation, the culture was rinsed gently with PBS to remove non-adhered osteoclast precursors. Adhered cells were viewed under a fluorescence microscope (Olympus 1X71) and imaged (QImaging Q16443) at five evenly spaced 0.7 mm^2-sized viewpoints per sample (n = 6).

For blocking experiments, rat anti-mouse IL-6 antibody (5 μg/mL, R&D Systems) was added to conditioned media and incubated for 1 h at 37 °C prior to treatment of endothelial cells. Mouse ICAM-1 antibody (5 μg/mL, R&D Systems) was added to endothelial cells and incubated for 1 h at 37 °C after conditioned media treatment.

For the IL-6 and sIL-6R supplementation assays, endothelial cells were treated with normal cell culture media alone, or media supplemented with IL-6 (10 ng/mL, Cedarlane), sIL-6R (50 ng/mL, R&D systems), or both for 18 h.

Flow cytometry

ICAM-1 expression was measured by flow cytometry after endothelial cells were treated with apoptotic or non-apoptotic osteocyte conditioned media or supplemented with IL-6 (10 ng/mL) and sIL-6R (50 ng/mL) for 18 h. Briefly, endothelial cells were gently detached with 0.05% Trypsin + 0.02% EDTA (Gibco/Invitrogen) and blocked with PBS containing 1% bovine serum albumin (BSA) for 10 min at 4 °C. Endothelial cells were incubated with phycoerythrin (PE)-conjugated rat anti-mouse ICAM-1 (1.25 μg/mL, E-Biosciences), or PE-conjugated rat IgG (isotype control, E-biosciences) for 20 min at 4 °C. After labeling, endothelial cells were rinsed 3 times with PBS + 1% BSA, and stained with 7-Aminoactinomcyin D (7-AAD, Invitrogen). Fluorescence was measured by a BD FACSCanto flow cytometer (BD Biosciences), and data was analyzed with BD FACSDiva software (BD Biosciences).

Osteocyte conditioned media characterization

IL-6 and sIL-6R concentrations in conditioned media were measured with a mouse IL-6 ELISA (Quantikine, R&D Systems) and sIL-6R ELISA (Duoset, R&D Systems), respectively, according to manufacturer's protocols. Mouse IL-6 and sIL-6R standards ranging from 0 to 500 pg/mL and 0 to 3000 pg/mL, respectively, were used. Serum-supplemented α-MEM media was used for baseline measurement of IL-6 and sIL-6R in the culture media. IL-6 and sIL-6R concentrations were normalized to total cell lysate protein concentration measured by a colorimetric assay (Pierce 660 nm, Thermo Scientific). Briefly, after conditioned media were collected, cells were lysed in 1× lysis buffer (Cell Signaling Technology) for 30 min. Cell lysates were centrifuged at 4 °C at 18.8 *g* for 15 min, and the supernatants of the respective samples were collected and stored at −20 °C. Samples were incubated with Pierce reagent for 5 min and absorbance was read at 660 nm. BSA ranging from 0 to 2000 μg/mL was used as a standard.

Statistical analysis

All experiments were repeated at least twice. Two tailed Student's t-tests and one-way ANOVA were used to determine statistical significance. The Student–Newman–Keuls was used as a post-hoc test.

Results

Osteocyte apoptosis promotes osteoclast precursor adhesion

Osteoclast precursor adhesion to endothelial cells treated with apoptotic osteocyte conditioned media was significantly greater than to endothelial cells treated with non-apoptotic osteocyte conditioned media ($P<0.001$, Fig. 1). TNF-α induced osteocyte apoptosis was caspase-dependent (Supplementary Fig. 1), and conditioned media from TNF-α treated osteocytes in which apoptosis was blocked with ZVAD-FMK had no effect on endothelial adhesion of osteoclast precursors (Fig. 1). This confirmed that osteocyte apoptosis was required for conditioned media-enhanced endothelial adhesion of osteoclast precursors.

Apoptotic osteocyte conditioned media treatment elevates endothelial ICAM-1 expression

As ICAM-1 has been shown to mediate osteoclast precursor adhesion to the endothelium [9], we used flow cytometry to measure ICAM-1 surface expression in endothelial cells treated with apoptotic osteocyte conditioned media (Fig. 2A). Compared with endothelial cells treated with non-apoptotic osteocyte conditioned media, we observed an increasing trend of ICAM-1 positive endothelial cells ($P = 0.10$, Fig. 2B) and significantly higher mean fluorescence intensity ($P<0.05$, Fig. 2C) after apoptotic osteocyte conditioned media treatment. Treatment of endothelial cells with an ICAM-1 blocking antibody abolished osteoclast precursor adhesion induced by apoptotic conditioned media ($P<0.05$, Fig. 3), confirming the functional role of ICAM-1.

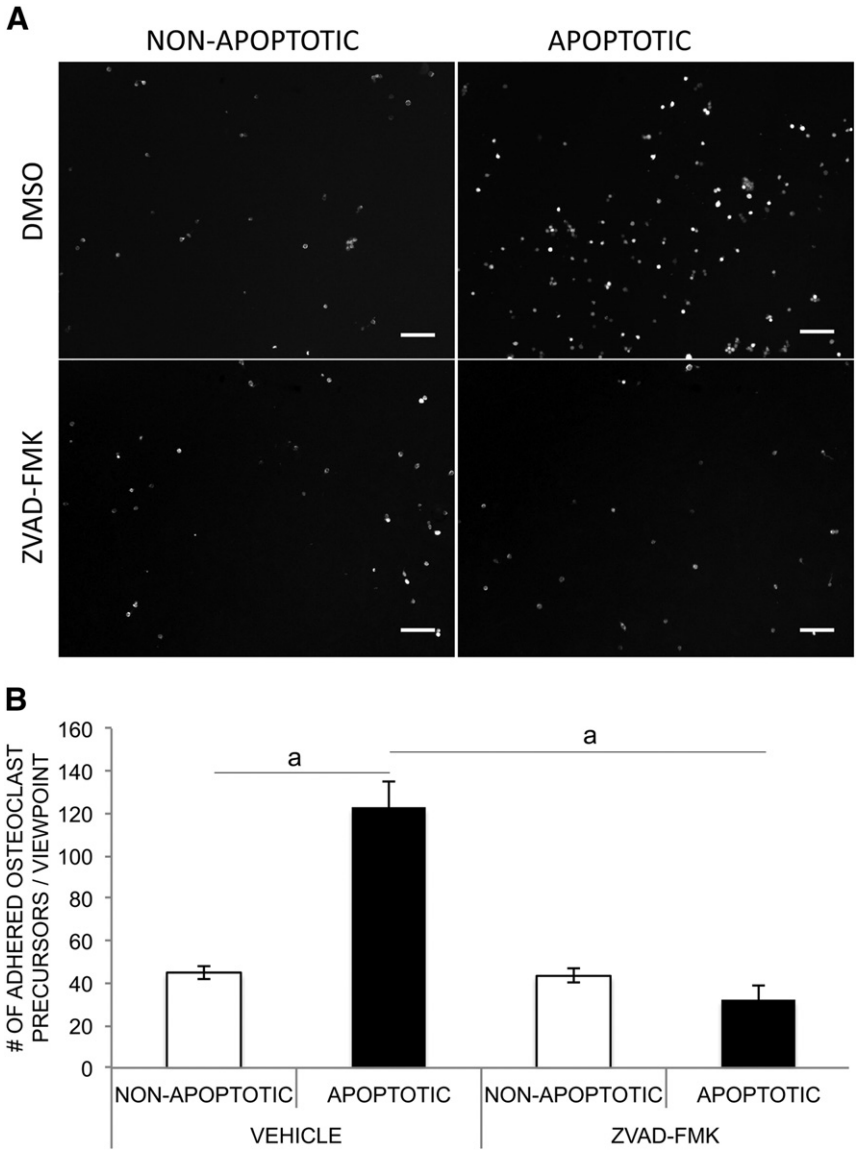

Fig. 1. Blocking apoptosis inhibits apoptotic osteocyte conditioned media-induced osteoclast precursor adhesion. A. Conditioned media collected from osteocytes pre-treated with vehicle (DMSO) or a pan-caspase inhibitor (ZVAD-FMK) to block TNF-α-induced osteocyte apoptosis was used to determine whether osteocyte apoptosis mediates osteoclast precursor adhesion on endothelial cells. Shown are CellTracker Green labeled-osteoclast precursors that have adhered onto an unlabelled monolayer of endothelial cells for 1 h. Endothelial cells were pre-incubated with conditioned media for 18 h prior to the addition of osteoclast precursors. (Scale bar = 100 μm) B. Blocking osteocyte apoptosis caused significantly lower osteoclast precursor adhesion onto the endothelium compared to apoptotic osteocyte conditioned media alone. (a, $P<0.001$).

Apoptotic osteocyte conditioned media contains elevated IL-6 and sIL-6R levels

To investigate whether osteocyte apoptosis mediates osteocytic IL-6 and sIL-6R expression, we used quantitative ELISAs to measure IL-6 and sIL-6R concentrations in the various conditioned media. Apoptotic osteocyte conditioned media contained significantly more IL-6 ($P<0.05$, Fig. 4A) and sIL-6R ($P<0.05$, Fig. 4B) than the other conditioned media conditions. Blocking osteocyte apoptosis with ZVAD-FMK partially reduced IL-6 levels ($P<0.001$, Fig. 4A) and fully reduced IL-6R levels in the conditioned media to control conditions ($P<0.005$, Fig. 4B).

IL-6 mediates osteoclast precursor adhesion on endothelium via ICAM-1 and requires the presence of sIL-6R

As endothelial cells lack surface receptors to bind IL-6 [25,27], we tested whether sIL-6R and IL-6 were both required for endothelial activation and osteoclast precursor adhesion. Treatment of endothelial cells with IL-6 or sIL-6R alone had no effect on osteoclast precursor adhesion ($P>0.05$, Fig. 5A). However, treatment with sIL-6R and IL-6 together significantly increased osteoclast precursor adhesion ($P<0.05$, Fig. 5A) and mean fluorescence intensity expression of ICAM-1 ($P<0.05$, Fig. 5B). Blocking IL-6 in apoptotic osteocyte conditioned media reduced osteoclast precursor adhesion to control levels ($P<0.05$, Fig. 5C).

Discussion

The initiating events in bone remodeling include osteocyte apoptosis and osteoclast precursor adhesion to the endothelium. However, the relationship between these two processes has never been investigated. In this study, we demonstrated that osteocyte apoptosis triggers the activation of endothelial cells to promote osteoclast precursor adhesion.

In our study, osteocyte apoptosis was required for conditioned media-induced osteoclast precursor adhesion, as blocking apoptosis with ZVAD-FMK eliminated enhanced adhesion (Fig. 1). The TNF-α

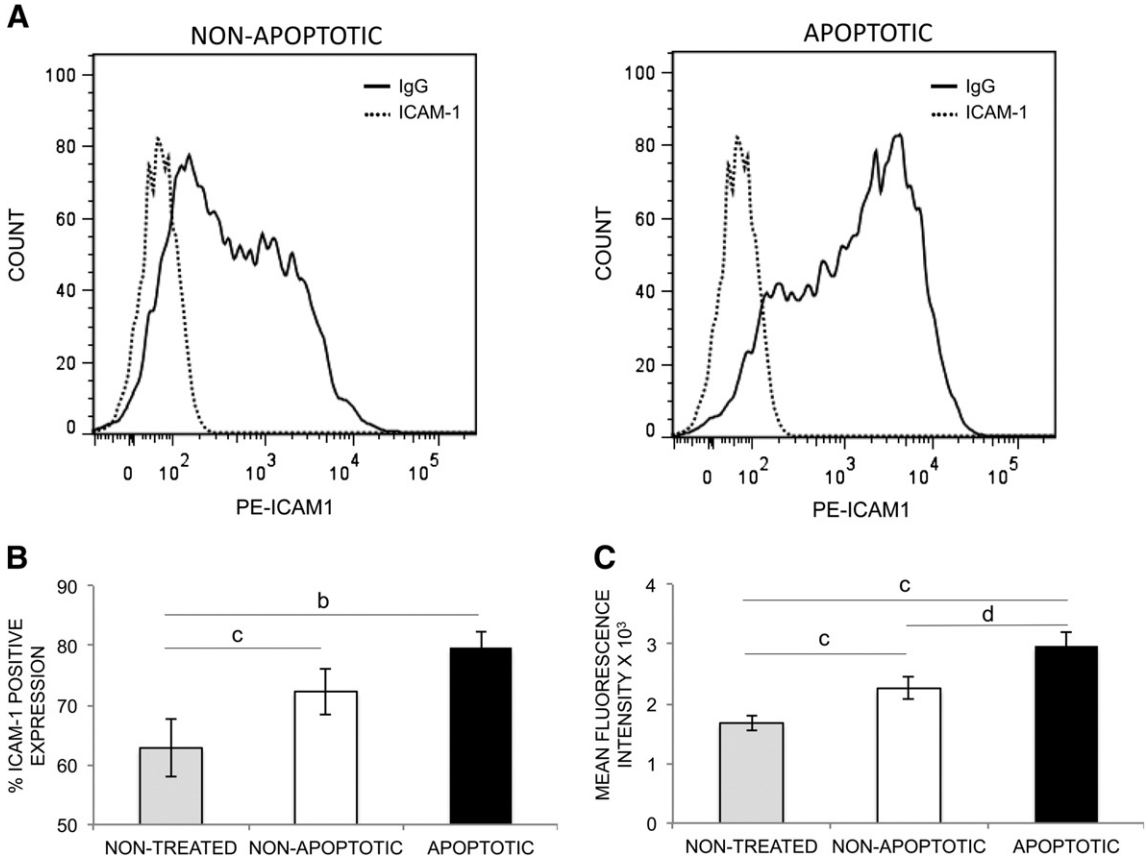

Fig. 2. Apoptotic osteocyte conditioned media promotes endothelial ICAM-1 expression. A. Flow cytometry was used to measure the amount of PE-conjugated ICAM-1 (x-axis) on the surface of endothelial cells after apoptotic or non-apoptotic osteocyte conditioned media treatment. B. Conditioned media treatment increased endothelial ICAM-1 expression (b, $P<0.005$, c, $P<0.05$). Also, an increasing trend of ICAM-1 positive cells was observed after apoptotic osteocyte conditioned media treatment ($P=0.10$). C. Apoptotic osteocyte conditioned media treatment significantly increased the mean fluorescence intensity measurement of ICAM-1 on endothelial cells compared to non-apoptotic osteocyte conditioned media treatment (d, $P<0.01$). Treatment of conditioned media also significantly increased the mean fluorescent intensity of endothelial ICAM-1 compared to controls (c, $P<0.05$).

treatment we used induces apoptosis in approximately 50% of the osteocyte population [7]. Thus, the elevated levels of IL-6 and sIL-6R in the apoptotic osteocyte conditioned media (Fig. 4A, B) may have come from healthy or apoptotic cells. Osteoblasts increase expression of IL-6R when treated with TNF-α [26], so it is conceivable that osteocytes treated with TNF-α to undergo apoptosis similarly shed IL-6R leading to the elevated sIL-6R expression in our system. This is supported by the fact that blocking apoptosis reduced sIL-6R secretion to control levels (Fig. 4B). Interestingly, others have also shown that induction of apoptosis by TNF-α to a portion of the population promoted upregulation of IL-6 expression [28]. In our system, blocking osteocyte apoptosis only partially attenuated IL-6 secretion into the conditioned media (Fig. 4A), suggesting that IL-6 expression may have been stimulated in the viable osteocyte population by TNF-α through an apoptosis-independent pathway.

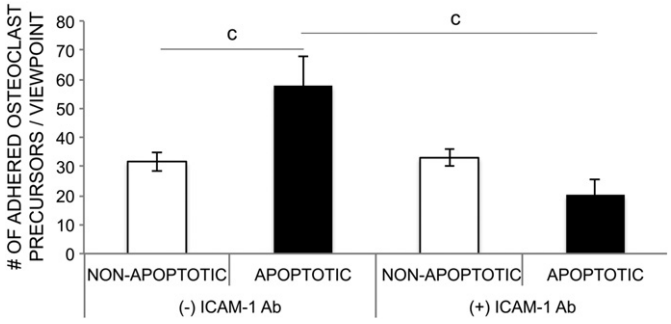

Fig. 3. ICAM-1 blocking abolishes apoptotic osteocyte conditioned media-induced osteoclast precursor adhesion. Treatment of endothelial cells with an ICAM-1 blocking antibody reduced osteoclast precursor adhesion to baseline levels (c, $P<0.05$).

Despite only partial attenuation of IL-6 secretion when osteocyte apoptosis was blocked, osteoclast precursor adhesion to the conditioned medium-treated endothelium was reduced to control levels (Fig. 1). In cells that express the gp130 subunit and not membrane bound IL-6R, IL-6 effects are mediated by trans-signaling with the IL-6/sIL-6R complex [22–25]. For instance, Modur et al. showed that IL-6 alone was unable to stimulate human umbilical vein endothelial cells for neutrophil adhesion, whereas treatment with both IL-6 and sIL-6R effectively activated the endothelium [25]. Similarly, we found that both IL-6 and sIL-6R were required to increase osteoclast precursor adhesion to the endothelium, while treatment with either alone had no effect (Fig. 4C). This was further verified by the abolishment of apoptotic conditioned medium-enhanced adhesion when IL-6 was blocked with an antibody (Fig. 5); therefore, sIL-6R alone was insufficient to promote osteoclast precursor adhesion. Altogether, our data indicate that osteocyte apoptosis regulates the osteocytic secretion of IL-6 and sIL-6R, and that both IL-6 and sIL-6R are necessary to activate the endothelium to allow for osteoclast precursor adhesion.

To our knowledge, our study is the first to identify that cellular populations containing apoptotic cells express IL-6 and sIL-6R to activate the endothelium for phagocytic cell recruitment. Cellular apoptosis has been traditionally considered to elicit a neutral immune response, as the process of programmed cell death involves a "quiet" process of chromatin fragmentation, DNA condensation,

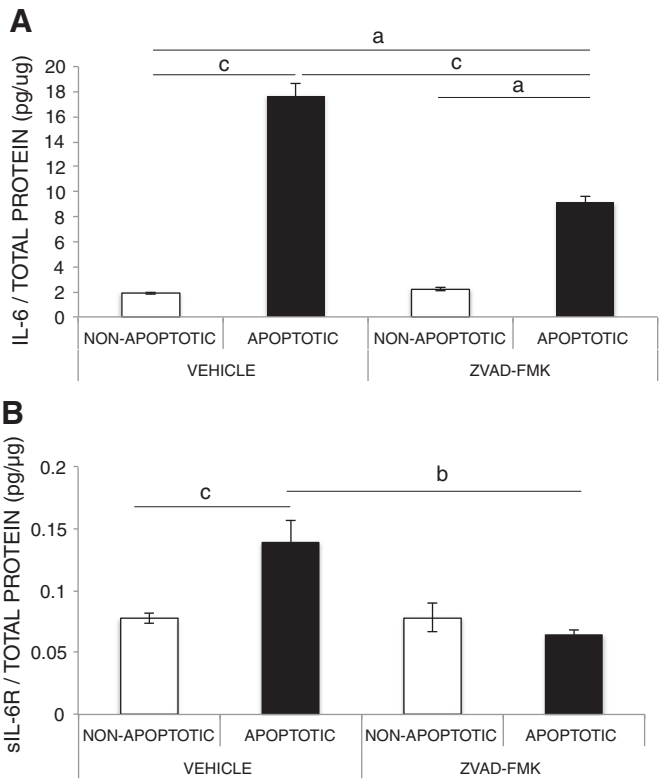

Fig. 4. Apoptotic osteocyte conditioned media contains elevated IL-6 and sIL-6R. A. Apoptotic osteocyte conditioned media contained significantly more IL-6 (c, $P<0.05$). Conditioned media collected from apoptotic osteocytes pre-treated with ZVAD-FMK contained less IL-6 compared to apoptotic conditioned media (c, $P<0.05$), but more IL-6 than non-apoptotic osteocytes pre-treated with or without ZVAD-FMK (a, $P<0.001$) B. Apoptotic osteocyte conditioned media contained significantly more sIL-6R compared to non-apoptotic osteocyte conditioned media (c, $P<0.05$). Blocking osteocyte apoptosis with ZVAD-FMK reduced sIL-6R expression to baseline levels. (b, $P<0.005$).

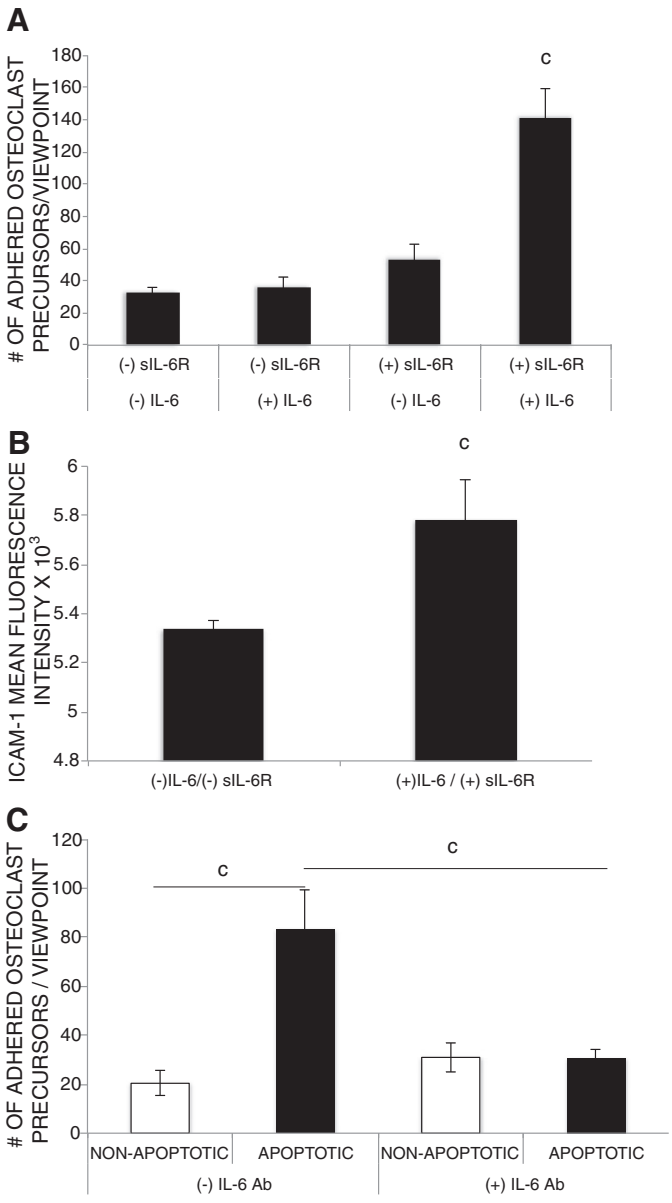

Fig. 5. Both IL-6 and sIL-6R are required for endothelial activation and osteoclast precursor adhesion. Blocking elevated levels of IL-6 in apoptotic osteocyte conditioned media abolishes osteoclast precursor adhesion. A. Endothelial cells pre-treated with IL-6 (10 ng/mL) and sIL-6R (50 ng/mL) adhered significantly more osteoclast precursors than those treated with IL-6 or sIL-6R alone (c, $P<0.05$ respectively). B. Treatment of both IL-6 and sIL-6R induced increased mean fluorescence intensity of ICAM-1 (c, $P<0.05$). C. Blocking IL-6 in apoptotic osteocyte conditioned media reduced osteoclast precursor adhesion (c, $P<0.05$) to levels that were similar to those observed on endothelial cells treated with non-apoptotic osteocyte conditioned media.

nuclear fragmentation, and eventual blebbing into apoptotic bodies for engulfment by professional phagocytes. However, recent evidence suggests that apoptosis is a key regulator of inflammatory responses [29,30]. For instance, phagocytes engulfing apoptotic cells have shown to increase secretion of anti-inflammatory cytokines IL-10 [31,32] and TGF-β [33,34] and decrease the secretion of the pro-inflammatory cytokine TNF-α [33]. In contrast, apoptotic cells themselves have shown the capacity to secrete pro-inflammatory cytokines such as IL-1β [35], and IL-18 [36]. Interestingly, the inefficient clearance of apoptotic cells can lead to the propagation of autoimmune diseases that involve chronic inflammation, including rheumatoid arthritis [37,38], systemic lupus erythematosus [37], and cystic fibrosis [39]. These studies suggest that apoptotic cells may be the gatekeepers of the immune response, by releasing a small but sufficient amount of inflammatory cytokines to recruit phagocytic cells. Upon binding and engulfment of the apoptotic cells, phagocytes release anti-inflammatory cytokines to abrogate the recruitment process. Thus, our observations that osteocyte apoptosis activates the endothelium and promotes osteoclast precursor recruitment may not be specific to bone cells, but may be a more general mechanism linking apoptosis to inflammation. Additional work is needed to test this hypothesis *in vivo* and in other tissues.

Functional activation of the endothelium by IL-6/sIL-6R appeared in our experiments to be mediated solely through ICAM-1, as antibody blockade of ICAM-1 completely abolished apoptotic osteocyte conditioned media-enhanced osteoclast precursor adhesion (Fig. 3). The dependency of osteoclast precursor adhesion on ICAM-1 was reported previously [9,40]. Endothelial ICAM-1 binds onto leukocyte-function-associated antigen-1 (LFA-1) expressed on the surface of osteoclast precursors that result in subsequent osteoclast precursor recruitment and osteoclast differentiation [41]. The majority of the endothelial cells in our cultures expressed a basal level of ICAM-1, even under normal medium conditions (Fig. 2), consistent with reports in the literature [28,42]. Treatment with apoptotic osteocyte conditioned media resulted in a trend of more ICAM-1 positive endothelial cells (Fig. 2B) and a significantly higher mean fluorescence intensity (Fig. 2C) compared with normal media and non-apoptotic conditioned media treatments. This suggests that the apoptotic conditioned medium treatment increased both the number of endothelial cells expressing ICAM-1 and the surface expression of ICAM-1 on each cell, resulting in greater osteoclast precursor adhesion (Fig. 1). Our observations further verified the causal relationship between IL-6/sIL-6R signaling and ICAM-1 expression that others have shown [27,40], as we demonstrated that treatment of

endothelial cells with both IL-6 and sIL-6R induces an increase in ICAM-1 surface expression (Fig. 5B).

It has been hypothesized that homing of osteoclast precursor cells is achieved via osteocyte communication with bone lining cells, which in turn activate the nearby endothelium to provide an "area code" for the recruitment of circulating cells [43]. Transport of IL-6 from osteocytes to the endothelium is feasible, as IL-6 is 21–29 kDa [44] and solutes as large as 43 kDa can be transported from the osteocytic lacunar-canalicular system to vasculature via diffusion [45]. Our *in vitro* data suggest that osteocytes are capable of communicating directly with endothelial cells via IL-6 to direct osteoclast precursor recruitment and do not require bone lining cells for this process. *In vivo* studies are required to confirm this alternative hypothesis suggested by our data. Bone lining cells may play additional roles in osteoclast precursor recruitment and differentiation that are not recapitulated in our co-culture model, but could be incorporated in future studies.

In conclusion, we found that osteocyte apoptosis plays a critical role in regulating osteoclast precursor adhesion, as osteocytic populations containing apoptotic osteocytes secrete elevated levels of IL-6 and sIL-6R that together promote endothelial ICAM-1 expression and osteoclast precursor adhesion. Further studies aimed at studying the effect of osteocyte apoptosis on osteoclast precursor recruitment may lead to better treatments to control the imbalance of osteoclast activity in diseases such as osteoporosis and osteopetrosis.

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Disclosures/conflicts of interest

The authors have nothing to disclose, and have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bone.2011.09.052.

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