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Apoptotic Osteocytes Regulate Osteoclast Precursor Recruitment and Differentiation In Vitro

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ABSTRACT

Fatigue loading causes a spatial distribution of osteocyte apoptosis co-localized with bone resorption spaces peaking around microdamage sites. Since osteocytes have been shown to regulate osteoclast formation and activity, we hypothesize that osteocyte apoptosis regulates osteoclastogenesis. In this study, we used serum-starvation to mimic reduced nutrient transport in microdamaged bone and induce apoptosis in MLO-Y4 osteocyte-like cells; conditioned medium was used to apply soluble factors released by apoptotic osteocytes (aOCY) to healthy non-apoptotic MLO-Y4 cells. Osteoclast precursor (RAW264.7 monocyte) migration and differentiation were assessed in the presence of conditioned media (CM) from: (A) aOCY, (B) osteocytes treated with apoptosis conditioned medium (i.e., healthy osteocytes in the presence of apoptosis cues; apoptosis CM-treated osteocytes (atOCY)), and (C) osteocytes treated with non-apoptosis conditioned medium (i.e., healthy osteocytes in the absence of apoptosis cues; non-apoptosis CM-treated osteocytes (natOCY)). Receptor activator for nuclear factor-κB ligand (RANKL), macrophage colony stimulating factor (M-CSF), vascular endothelial growth factor (VEGF) and osteoprotegerin (OPG) mRNA, and protein expression were measured. Our findings indicate that soluble factors released by aOCY and atOCY promoted osteoclast precursor migration (up to 64% and 24% increase, respectively) and osteoclast formation (up to 450% and 265% increase, respectively). Osteoclast size increased up to 233% in the presence of aOCY and atOCY CM. Recruitment, formation and size were unaltered by natOCY. RANKL mRNA and protein expression were upregulated only in aOCY, while M-CSF and VEGF increased in atOCY. Addition of RANKL-blocking antibody abolished aOCY-induced osteoclast precursor migration and osteoclast formation. VEGF and M-CSF blocking antibodies abolished atOCY-induced osteoclastogenesis. These findings suggest that aOCY directly and indirectly (through atOCY) initiate targeted bone resorption by regulating osteoclast precursor recruitment and differentiation. J. Cell. Biochem. 112: 2412–2423, 2011.

KEY WORDS: OSTEOCYTE APOPTOSIS; OSTEOCLAST PRECURSOR RECRUITMENT; OSTEOCLAST FORMATION; BONE REMODELING; TARGETED BONE RESORPTION

Bone adapts and undergoes remodeling in response to mechanical stimuli. It is able to repair microdamage caused by fatigue loading through targeted remodeling, a process believed to be orchestrated by osteocytes [Lanyon, 1993; Noble, 2005; O'Brien et al., 2005], which are the major mechanosensory cells in bone. However, the mechanisms underlying osteocyte-regulated targeted bone remodeling are not well understood.

Osteocyte apoptosis has been observed in vivo in microdamage [Noble, 2005; Cardoso et al., 2009] and mechanical disuse [Aguirre et al., 2006]. Microdamage is thought to compromise osteocyte integrity by physically damaging the cells [Taylor et al., 2003], oxidative stress [Kikuyama et al., 2002; Kogianni et al., 2004; Noble, 2005], or disruption of blood flow and/or fluid flow in the lacunar–canalicular system, thereby reducing nutrient and oxygen supply to osteocytes and altering their mechanical and chemical environment [Tami et al., 2002]. Regions of osteocyte apoptosis have been shown to be accompanied by increased local bone turnover and targeted bone resorption [Verborgt et al., 2000; Clark et al., 2005; Gu et al., 2005; Noble, 2005; Cardoso et al., 2009]. Bone resorption spaces coinciding with microdamage-induced osteocyte apoptosis and empty lacunae suggest that osteoclasts target specific regions to phagocytose damaged osteocytes [Savill, 1998]. Together, these

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studies suggest that apoptotic osteocytes (aOCY) are potential regulators of targeted bone remodeling.

Interestingly, away from microdamage sites (2.0–3.0 mm), osteocytes do not undergo apoptosis [Verborgt et al., 2002] and intracortical resorption spaces are not observed [Cardoso et al., 2009]. Furthermore, the ability of osteocytes to produce VEGF-A, which is an important factor in stimulating receptor activator for nuclear factor-κB ligand (RANKL) expression and, thus, osteoclast formation, has been indicated to decrease with distance away from microdamage sites [Herman et al., 2007]. This has led to the speculation that both apoptotic osteocytes in the immediate vicinity of microdamage, as well as healthy osteocytes within some distance from microdamage, may play an important role in regulating osteoclast recruitment and activation [Verborgt et al., 2000]. While osteocyte apoptosis has been observed to spatially correlate with localized bone resorption in vivo, it is unknown whether the mechanism by which apoptotic osteocytes regulate osteoclastogenesis is direct (i.e., directly impacting osteoclast precursors) or indirect (i.e., by acting upon neighboring healthy osteocytes, which in turn impact osteoclast precursors). It is also unclear which cytokines mediate these regulatory mechanisms.

In this study, we hypothesize that apoptotic osteocytes play a critical role in regulating osteoclast precursor recruitment and differentiation. Specifically, we hypothesize that this regulation can be *direct* (i.e., cytokines released by apoptotic osteocytes directly acting on osteoclast precursors) as well as *indirect* (i.e., by signaling to neighboring healthy osteocytes that direct recruitment and differentiation). However, it is difficult to differentiate between the response of healthy and apoptotic osteocytes around sites of microdamage in vivo. Therefore, we developed a novel in vitro conditioned medium-based model (see Materials and Methods Section) to allow us to delineate the potential regulatory pathways mentioned above.

Various proteins have been shown to be involved in osteoclast precursor recruitment, differentiation and activation. Among them, receptor activator for nuclear factor-kB ligand (RANKL), osteoprotegerin (OPG), macrophage colony stimulating factor (M-CSF) [Boyce and Xing, 2007], and vascular endothelial growth factor (VEGF) [Sipola et al., 2006; Yang et al., 2008] have been shown to play important roles in osteoclast regulation. Because these cytokines have also been shown to be expressed by osteocytes under various conditions including bone microdamage [Bonewald, 2006; Herman et al., 2007; You et al., 2008; Lau et al., 2010], we speculate that they may be involved in osteocyte apoptosis regulated targeted bone remodeling.

In our conditioned media (CM) system we examine the role of apoptotic osteocytes in regulating osteoclast precursor recruitment and differentiation. We investigate the presence of these four cytokines (RANKL, OPG, M-CSF, and VEGF) in osteocyte CM to determine their potential role in osteocyte apoptosis-mediated regulation of osteoclast precursor recruitment and osteoclast formation.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

We serum-starved osteocytes to induce apoptosis, thus mimicking nutrient-deprived apoptotic osteocytes in the vicinity of microdamage (aOCY). Serum starvation was chosen as the apoptosis inducer as it more closely represents microdamage-induced reduced nutrient transport, and does not involve an inflammatory response like other pro-apoptosis cytokines (e.g., TNF-α [Tan et al., 2006]). Furthermore, this method has been previously used by others to induce apoptosis in osteocytes [Bakker et al., 2004] and osteoblasts in vitro [Pagel et al., 2003].

To investigate the indirect effect of aOCY on osteoclast formation and recruitment, conditioned medium from aOCY was applied to healthy osteocytes in vitro [apoptosis CM-treated osteocytes (atOCY)] to represent healthy osteocytes in the vicinity of apoptosis cues in vivo (Fig. 1). CM from aOCY and atOCY were applied to osteoclast precursors to test the direct (aOCY) and indirect (atOCY) effects of aOCY on osteoclast precursor recruitment and osteoclast formation.

CELL CULTURE

MLO-Y4 osteocyte-like cells (gift of Dr. Lynda Bonewald, University of Missouri-Kansas City) were cultured in alpha-MEM medium (Invitrogen) supplemented with 5% FBS (Hyclone), 5% calf serum (Hyclone), and 1% penicillin–streptomycin (Invitrogen). RAW264.7 (ATCC), an established monocyte cell line used for inducing osteoclast formation in vitro [Collin-Osdoby et al., 2003], were maintained in DMEM medium (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin–streptomycin (Invitrogen).

APOPTOSIS INDUCTION AND CONDITIONED MEDIA (CM)

Apoptosis was induced in MLO-Y4 cells by serum-starvation (alpha-MEM with 0% FBS, 0% calf serum, and 1% penicillin–streptomycin) for 24 h to simulate aOCY under microdamage conditions. As a control, MLO-Y4 cells were maintained in serum-supplemented medium (alpha-MEM with 5% FBS, 5% calf serum, and 1% penicillin–streptomycin) in parallel with the starved cells. Cells were subsequently cultured in serum-supplemented medium (alpha-MEM with 5% FBS, 5% calf serum, and 1% penicillin–streptomycin), and conditioned medium was collected after 2 h (i.e., early response to apoptosis) or 24 h (i.e., late response to apoptosis).

To determine the effect of aOCY on neighboring healthy osteocytes (i.e., atOCY), we incubated normally cultured (healthy) MLO-Y4 cells with the previously obtained aOCY conditioned medium for 6 h, representing an early response to cues secreted by aOCY. Conversely, to mimic healthy osteocytes in the absence of apoptosis cues (i.e., non-apoptosis CM-treated osteocytes (natOCY)) a similar procedure was performed as for atOCY but using conditioned medium from non-apoptotic osteocytes (see Table I for CM definitions). After the 6 h incubation, the existing CM were replaced with serum-supplemented alpha-MEM (alpha-MEM with 5% FBS, 5% calf serum, and 1% penicillin–streptomycin), and new CM were obtained after 2 h (i.e., early response to apoptosis) or 24 h (i.e., late response to apoptosis) from atOCY and natOCY (Fig. 1).

TUNEL STAINING

DNA fragmentation in aOCY was identified using TUNEL staining using a TACS Blue Label In Situ Apoptosis Detection kit (Trevigen). Nuclear fast red was used to counterstain. Cells treated with TACS

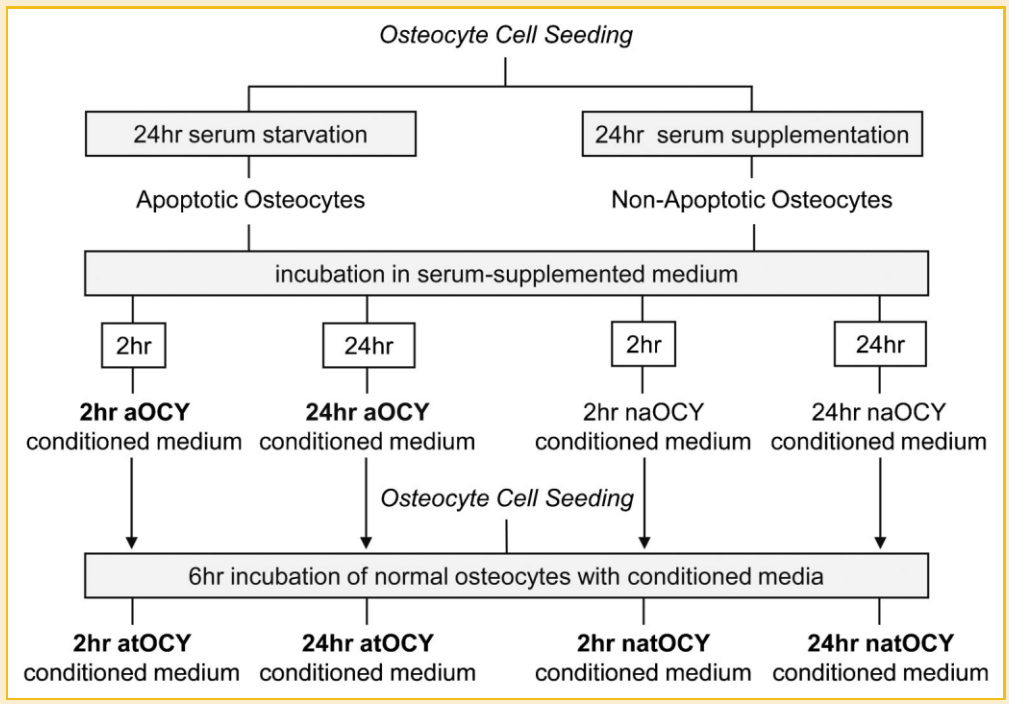

Fig. 1. Apoptosis induction and conditioned medium collection flowchart. MLO-Y4 cells are serum-starved for 24 h prior to collecting 2 or 24 h apoptotic osteocyte (aOCY) conditioned medium. This conditioned medium is then incubated with new normally cultured MLO-Y4 cells for 6 h to treat cells with soluble factors from apoptotic osteocytes. Finally, 2 or 24 h conditioned medium from osteocytes treated with aOCY conditioned medium is obtained. A similar procedure is performed in parallel for non-apoptotic osteocytes except cells were incubated in serum-supplemented medium instead of serum-starvation.

nuclease served as a positive control for TUNEL, whereas cells not labeled with TdT enzyme were negative controls. Apoptosis was quantified by imaging five random fields of view per sample under 100× magnification and counting the number of TUNEL-positive cells relative to the total number of cells per image.

OSTEOCLAST PRECURSOR MIGRATION

RAW264.7 cells were stained with 5 μm CellTracker Green dye (Invitrogen) for 30 min prior to seeding (5×10^4 cells/ml) in Boyden chambers with 8.0 μm pore size PET membranes (Millipore). The bottom chambers were filled with a 1:1 mixture of conditioned medium and 100 ng/ml sRANKL in serum-supplemented DMEM; control groups received either 0 or 100 ng/ml sRANKL in supplemented DMEM. The purpose of adding 100 ng/ml sRANKL (the optimized minimum concentration for monocyte migration) was to provide a baseline stimulus for monocyte migration to supplement the conditioned medium, which is otherwise insufficient to induce migration alone (data not reported). Cells were allowed to migrate through the membranes for 6 h then fixed with 3.7% formaldehyde. Non-migrated cells in the top chamber were removed with a cotton swab, and membranes were imaged at 200× magnification using fluorescent microscopy to quantify the number of migrated cells.

OSTEOCLAST FORMATION

To induce osteoclast formation, RAW264.7 cells were seeded on tissue culture plates and cultured in 5 ng/ml sRANKL (R&D Systems) in serum-supplemented DMEM for 2 days. On day 3, conditioned medium was added to the cells in a 1:1 ratio with 5 ng/ml sRANKL in serum-supplemented DMEM. This concentration of sRANKL was the optimized minimum baseline stimulus (not reported) to induced osteoclast formation for supplementing but without masking the effect of CM. Negative control received serum-supplemented DMEM alone, while the addition of 5 ng/ml soluble RANKL served as a positive control.

On day 7, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using an Acid Phosphatase Leukocyte kit (Sigma). Osteoclasts were quantified by imaging five fields of view under 200× magnification and counting the number of TRAP-positive cells with three or more nuclei. Osteoclast size was

TABLE I. Conditioned Medium Groups

	Definition	Models for...
aOCY	Apoptotic osteocyte	Apoptotic osteocytes at the microdamage site
atOCY	Apoptosis CM-treated osteocytes	Healthy osteocytes in the presence of cues from apoptotic osteocytes near the microdamage
natOCY	Non-apoptosis CM-treated osteocytes	Healthy osteocytes in the presence of cues from healthy osteocytes in the absence of microdamage

determined by counting the number of nuclei per cell, where large osteoclasts were defined as cells having more than five nuclei.

BLOCKING EXPERIMENTS

Neutralizing antibodies specific for RANKL, M-CSF, and VEGF (R&D Systems) were used to block specific proteins in CM. Antibodies at 1 μg/ml were incubated with CM at 37°C for 1 h prior to adding to cells. Osteoclast precursor migration and osteoclast formation experiments were subsequently performed as above.

PROTEIN MEASUREMENT

Levels of soluble proteins in osteocyte CM were measured using ELISA Quantikine immunoassays for RANKL, VEGF, and M-CSF (R&D Systems), respectively. Concentrations were normalized relative to total protein content in cell lysates using a colorimetric bicinichoninic acid (BCA) assay (Thermo Scientific).

QUANTITATIVE REAL-TIME PCR

mRNA expression levels of RANKL, OPG, M-CSF, and VEGF were measured using quantitative reverse transcription PCR. Total RNA was extracted from MLO-Y4 cells using RNeasy Mini kit (Qiagen) with DNase I (Fermentas) to remove contaminating DNA. cDNA was synthesized by reverse transcription of 1 μg RNA using SuperScript III (Invitrogen). Quantitative PCR was performed using mouse gene-specific primers (Eurofins MWG Operon) for the above proteins (Table II) and SYBR Green I Master Mix (Roche) in a thermal cycler (Mastercycler ep realplex^2 S, Eppendorf). Standards and samples were run in triplicate, and normalized to 18S housekeeping gene.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) with Tukey post hoc analysis was used to compare means of experimental groups using a significance level $\alpha = 0.05$. Analysis was performed using Statistical Analysis for Social Sciences software (SPSS v. 16.0). Experiments were conducted at least two times. Data is reported as means with standard deviations.

RESULTS

SERUM STARVATION INDUCES TERMINAL OSTEOCYTE APOPTOSIS

We incubated MLO-Y4 cells in serum-free medium for 24 h to induce 100% apoptosis, thereby ensuring that the observed effects are from aOCY only. TUNEL staining for DNA fragmentation (i.e., terminal apoptosis) indicated that subsequent incubation with serum-supplemented medium for 2 or 24 h after serum-starvation did not reverse apoptosis ($P \leq 0.001$), and all cells remained apoptotic (Fig. 2).

OSTEOCYTE APOPTOSIS SUPPORTS OSTEOCLAST PRECURSOR RECRUITMENT

We cultured RAW264.7 monocytes in Boyden chambers, with the bottom chamber filled with conditioned medium and 100 ng/ml sRANKL in growth medium in a 1:1 ratio for 6 h (Fig. 3). The negative control received no sRANKL or conditioned medium, while the positive control received 100 ng/ml sRANKL without conditioned medium, which has been previously shown to induce osteoclast precursor migration [Henriksen et al., 2003]. Apoptotic osteocyte conditioned medium (aOCY) collected at both 2 and 24 h induced significantly more migration than all other groups (48% and 64% increase relative to 100 ng/ml sRANKL control, respectively; $P \leq 0.001$). Twenty-four hour conditioned medium from atOCY induced 32% more RAW264.7 migration relative to natOCYnatOCY (Fig. 3B, $P \leq 0.009$).

INCREASED OSTEOCLAST FORMATION AND SIZE IN THE PRESENCE OF OSTEOCYTE APOPTOSIS

To determine the effect of osteocyte apoptosis on osteoclast formation, we applied a 1:1 mixture of conditioned medium and 5 ng/ml sRANKL-supplemented medium to RAW264.7 cells (Fig. 4A,B). Both 2 and 24 h aOCY CM significantly increased osteoclast formation relative to 2 and 24 h natOCY CM and sRANKL alone (450% and 226% increase relative to 5 ng/ml sRANKL control at 2 and 24 h, respectively; $P \leq 0.005$). atOCY also promoted osteoclast formation, with a 265% increase in 2 h conditioned medium and 123% increase in 24 h conditioned medium relative to the sRANKL-only control ($P \leq 0.005$).

Osteoclast size was evaluated based on the number of nuclei in each cell, where large osteoclasts were defined as having greater than five nuclei (Fig. 4C). Among the 2 h CM groups, aOCY and atOCY CM yielded the most number of large osteoclasts (233% and 148% increase relative to natOCY conditioned medium, respectively; $P \leq 0.02$), while the sRANKL-only control produced no large osteoclasts.

UPREGULATION OF RANKL PROTEIN SECRETION BUT NOT GENE EXPRESSION IN APOPTOTIC OSTEOCYTES

We measured protein and mRNA levels of RANKL and OPG to determine if apoptosis affected the expression of RANKL and OPG in osteocytes (Fig. 5). There was a 426% and 326% increase in RANKL protein secretion in 2 h aOCY CM over atOCY and natOCY CM (Fig. 5A; $P \leq 0.001$), and approximately 120% increase in soluble RANKL in 24 h aOCY over both atOCY and natOCY (Fig. 5B; $P \leq 0.001$). OPG protein levels were not detectable in CM using an ELISA immunoassay (not shown).

TABLE II. Mouse Gene-Specific Primers

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)	Product Size (bp)
RANKL	CAGCATCGCTCTGTTCCTGTA	CTGCGTTTTCATGGAGTCTCA	107
OPG	GGGGGCCGTGCAGAAGGAAC	CTTGCCTCGCTGGGCCACAT	106
M-CSF	TTGCTGACTGTTGGGGGCCT	CCTGGGTCAGGGAGCTGTCCTC	112
VEGF	GGAGAGCAGAAGTCCCATGAAGTGAT	CGCATCAGCGGCACACAGGA	163
18S	GAGAAACGGCTACCACATCC	CTTCTCAGCGCTCCGCCAGG	158

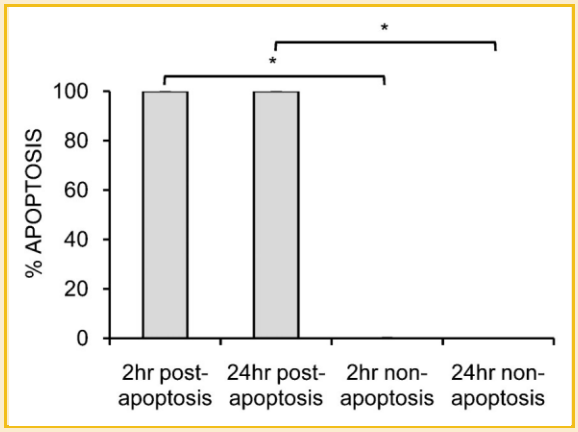

Fig. 2. Percentage of apoptosis after serum-starvation. TUNEL staining of osteocytes after incubation with serum-supplemented medium indicates 100% apoptosis relative to total number of cells (n = 4, $^{*}P < 0.001$); therefore, any effects observed by the 2 or 24 h aOCY condition groups are solely derived from apoptotic osteocytes.

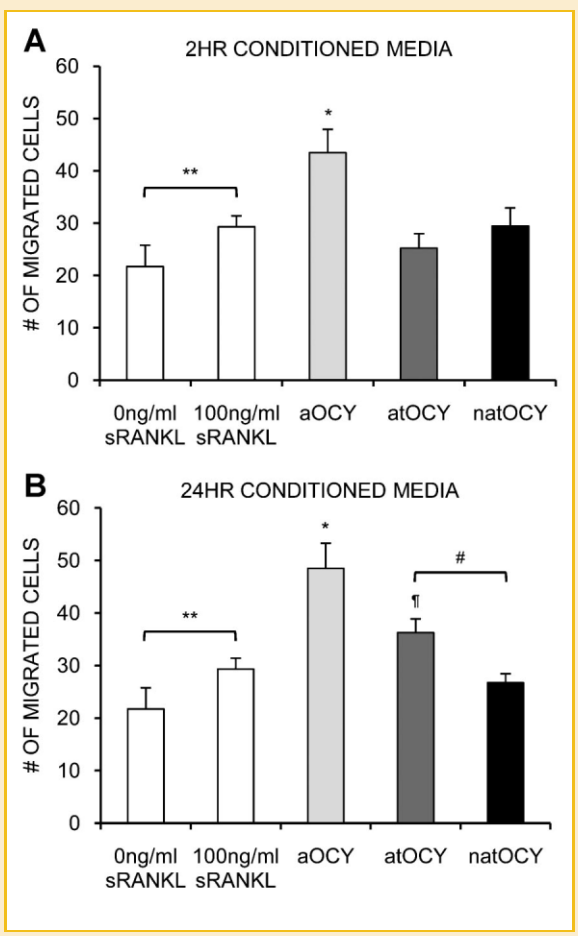

Fig. 3. Osteoclast precursor migration in the presence of conditioned medium. A: Two hour apoptosis conditioned medium increased RAW264.7 migration. B: Twenty-four hour apoptosis and apoptosis CM-treated osteocyte conditioned media both increased osteoclast precursor migration. $^{*}P < 0.001$ relative to 0 ng/ml sRANKL and 100 ng/ml sRANKL controls; $^{**}P < 0.001$, $^{\#}P = 0.009$, $^{\P}P < 0.001$ as indicated (n = 4).

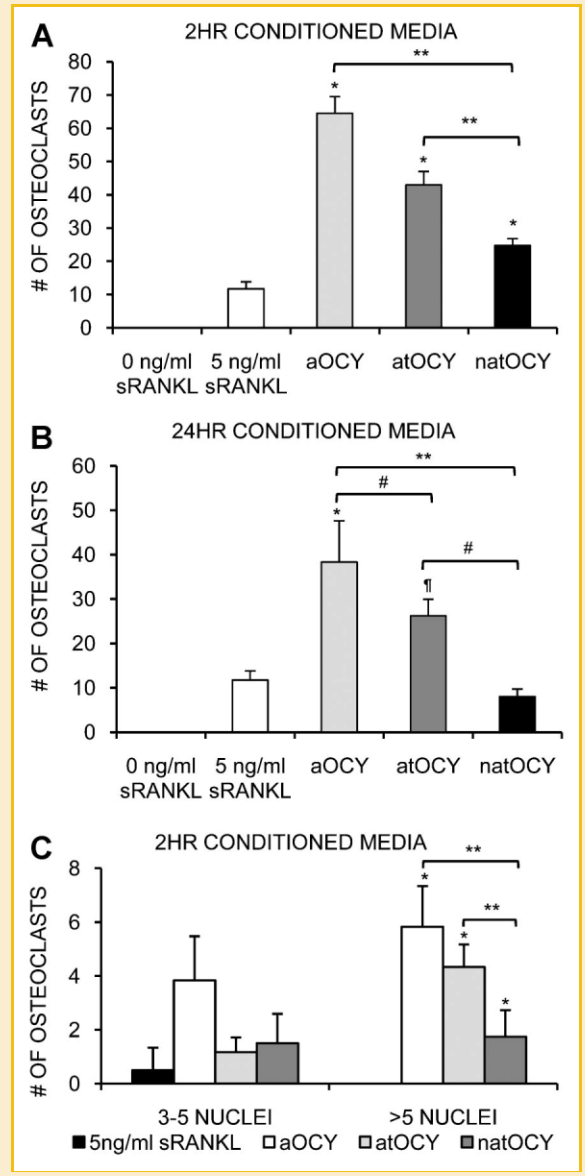

Fig. 4. Effect of conditioned medium on osteoclast formation. A: aOCY and atOCY both increased osteoclast formation with both (A) 2 h and (B) 24 h conditioned media (n = 4). C: aOCY and atOCY conditioned media promoted formation of large (greater than five nuclei) osteoclasts. $^{*}P \leq 0.001$ relative to 0 ng/ml sRANKL and 5 ng/ml sRANKL controls, $^{**}P \leq 0.005$ as indicated on figure, $^{\#}P = 0.02$, $^{\P}P = 0.05$ (n = 6).

At 2 h RANKL mRNA were expressed 97% and 123% higher in aOCY than atOCY and natOCY (Fig. 5C; $P \leq 0.001$), respectively. However, 24 h RANKL mRNA levels decreased in aOCY to similar levels as atOCY but lower than natOCY by 43% ($P \leq 0.001$). OPG 2 h mRNA expression was also higher in aOCY by 123% relative to atOCY and 151% relative to natOCY ($P \leq 0.001$); however, mRNA levels were similar among all three conditions at 24 h. As such, RANKL/OPG ratio was close to unity for all conditions at 2 h, but increased in natOCY by 85% and 65% relative to aOCY and atOCY, respectively ($P \leq 0.001$).

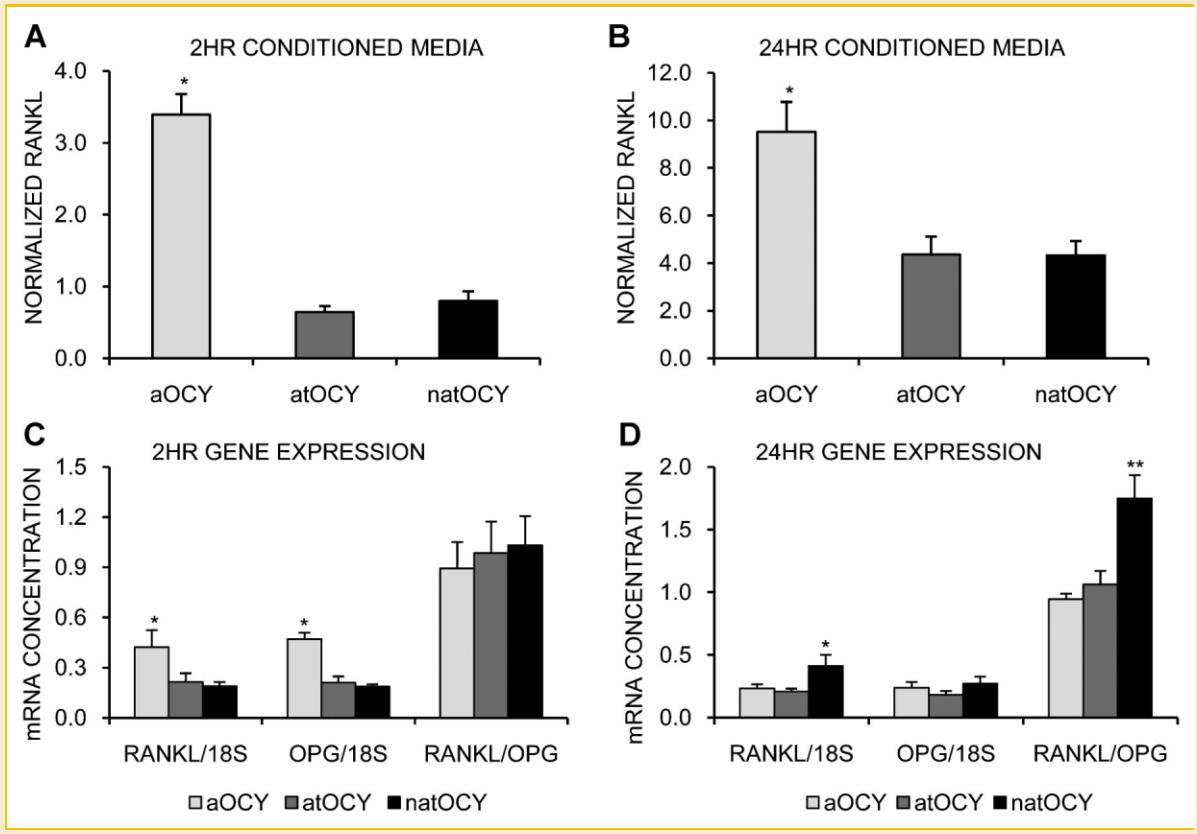

Fig. 5. RANKL and OPG expression in conditioned medium. Apoptotic osteocytes secreted the most amount of soluble RANKL in (A) 2 h and (B) 24 h conditioned media ($^{*}P \leq 0.001$ relative to atOCY and natOCY). C: RANKL and OPG mRNA levels were also highest in 2 h apoptotic osteocytes, but RANKL/OPG ratio was similar among all groups ($^{*}P < 0.01$ relative to aOCY and atOCY). D: mRNA expression tends at 24 h were reversed and non-apoptotic CM-treated osteocytes had higher RANKL and RANKL/OPG ratio ($^{*}P < 0.01$ relative to aOCY and atOCY, $^{**}P < 0.001$ relative to aOCY and natOCY). All experiments n = 4.

LOW M-CSF PROTEIN SECRETION BY APOPTOTIC OSTEOCYTES

M-CSF protein secretion and mRNA expression were measured to determine if M-CSF is a possible factor involved in osteocyte apoptosis regulation of osteoclast formation (Fig. 6). atOCY and natOCY osteocytes secreted 69% and 49% more M-CSF than aOCY at 2 h, respectively (Fig. 6A; $P \leq 0.001$). atOCY secreted 25% more M-CSF than natOCY, while there was no statistical difference between aOCY and natOCY at 24 h (Fig. 6B; $P = 0.01$). At 2 h M-CSF mRNA levels in atOCY were approximately 50% greater than atOCY and natOCY (Fig. 6C; $P \leq 0.001$). There was no difference in M-CSF mRNA expression at 24 h among all three condition groups (Fig. 6C).

VEGF SECRETION TIME AND SOURCE DEPENDANT

We measured VEGF levels in conditioned medium and mRNA expression in all three osteocyte groups (Fig. 7). At 2 h aOCY and atOCY CM contained 87% and 286% more soluble VEGF protein than natOCY conditioned medium, respectively (Fig. 7A; $P \leq 0.001$). However, at 24 h, VEGF in atOCY conditioned medium decreased by 81% relative to the 2 h level; aOCY secreted 471% and 12% more VEGF at 24 h than atOCY and natOCY, respectively (Fig. 7B; $P < 0.05$). There was a 92% and 78% increase in aOCY VEGF mRNA expression over atOCY and natOCY at 2 h, respectively (Fig. 7C; $P \leq 0.001$). At 24 h, while VEGF expression decreased in aOCY, mRNA levels were still 24% and 41% greater than in atOCY and natOCY, respectively ($P \leq 0.001$).

RANKL REGULATES aOCY-INDUCED OSTEOCLAST PRECURSOR RECRUITMENT AND OSTEOCLAST FORMATION

To confirm if the aOCY-induced increase in osteoclast precursor recruitment and differentiation are indeed due to the increased RANKL protein secretion, we applied a RANKL-blocking antibody to the CM prior to incubating with cells. RANKL-blocking antibody abolished the aOCY-induced increase in osteoclast precursor migration and osteoclast differentiation to similar levels as natOCY CM. Migration induced by 2 h aOCY conditioned medium was reduced by 30% and 42% by 24 h conditioned medium (Fig. 8A,B; $P \leq 0.002$). Osteoclast formation was reduced by 66% and 41% in the presence of antibody-treated 2 and 24 h CM, respectively (Fig. 8C,D; $P \leq 0.03$).

VEGF AND M-CSF NEUTRALIZATION ABOLISHED atOCY CONDITIONED MEDIUM-INDUCED OSTEOCLAST PRECURSOR MIGRATION AND OSTEOCLAST FORMATION

To confirm whether the increased VEGF and M-CSF protein expression by atOCY is responsible for the atOCY-induced

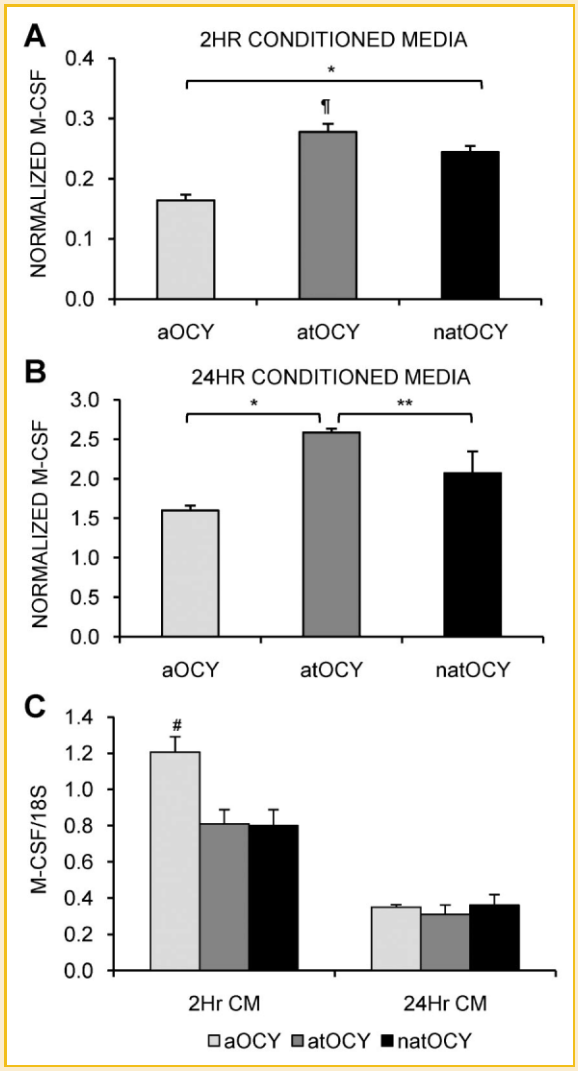

Fig. 6. M-CSF expression in conditioned medium. Protein concentrations of M-CSF in apoptosis CM-treated and non-apoptosis CM-treated conditioned media were greater than in apoptosis conditioned medium at both (A) 2 h and (B) 24 h time points. C: M-CSF mRNA expression was highest in apoptosis osteocyte conditioned medium at 2 h, but decreased at 24 h to similar levels as the other conditioned media groups. $^{\P}P \leq 0.001$ relative to aOCY and natOCY, $^{*}P \leq 0.001$ as indicated on figure, $^{**}P = 0.01$ relative to natOCY, $^{\#}P \leq 0.001$ relative to atOCY and natOCY ($n = 4$).

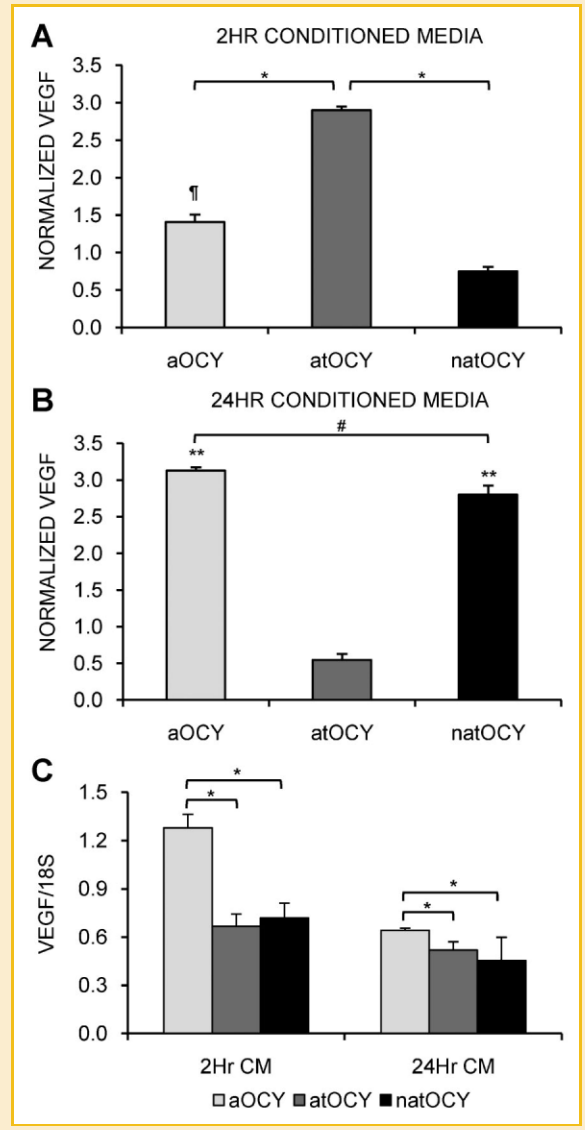

Fig. 7. VEGF expression in conditioned media. A: In 2 h conditioned media, apoptosis and apoptosis CM-treated osteocyte conditioned media had higher VEGF protein concentration than in non-apoptosis CM-treated osteocyte conditioned medium. B: At 24 h, VEGF protein in apoptosis CM-treated conditioned medium significantly decreased, however, apoptosis conditioned medium still expressed the highest concentration of VEGF. C: VEGF mRNA was highest in apoptotic osteocytes at both 2 h and 24 h. $^{\P}P \leq 0.001$ relative to natOCY, $^{*}P \leq 0.001$ as indicated on figure, $^{**}P \leq 0.001$ relative to atOCY, $^{\#}P = 0.05$ relative to natOCY ($n = 4$).

osteoclast precursor recruitment and osteoclast formation, we added specific VEGF and M-CSF antibodies to atOCY CM. VEGF neutralization significantly inhibited the effect of 2 and 24 h CM on osteoclast precursor migration by 63% and 34%, respectively (Fig. 9A,B; $P \leq 0.001$) to similar levels as natOCY. Blocking VEGF also decreased 2 and 24 h CM-induced osteoclast formation by 49% and 22%, respectively (Fig. 9C,D; $P \leq 0.003$).

Neutralization of M-CSF had a similar effect as VEGF-blocking, where osteoclast precursor migration was inhibited by 54% and 24% in the presence of 2 and 24 h atOCY CM, respectively ($P \leq 0.001$) to levels comparable to natOCY CM. Osteoclast formation showed a similar response in which the number of multi-nucleated osteoclasts was reduced by 31% and 28% in the presence of M-CSF-neutralized 2 and 24 h atOCY CM, respectively ($P \leq 0.001$).

Blocking VEGF and M-CSF together produced the same effect as blocking of individual proteins. Osteoclast precursor recruitment decreased by 60% and 22% in the presence of neutralized 2 and 24 h atOCY CM, respectively ($P \leq 0.003$). Osteoclast formation was also reduced by 56% and 17% in the presence of neutralized 2 and 24 h CM, respectively ($P \leq 0.01$).

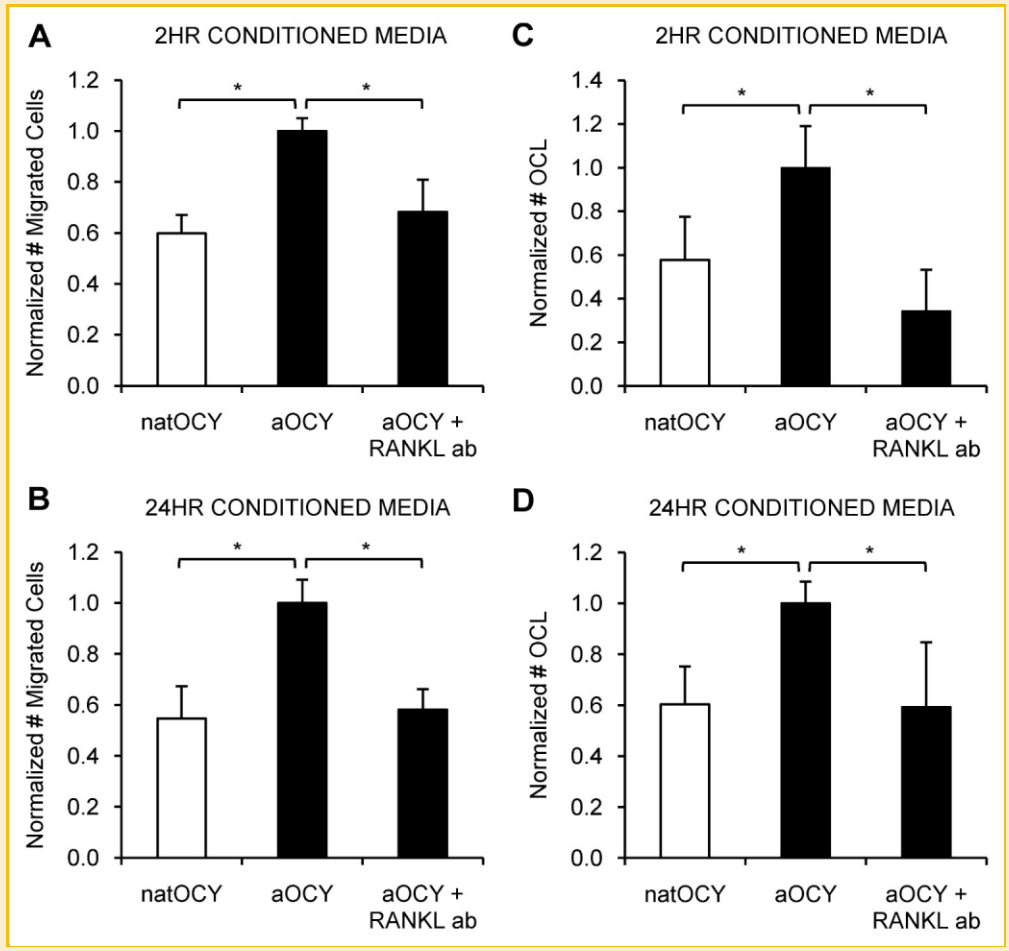

Fig. 8. Blocking RANKL abolished osteoclast precursor migration and differentiation induced by aOCY conditioned media. Adding a RANKL-blocking antibody decreased osteoclast precursor migration by (A) 30% with 2 h CM and (B) 42% with 24 h CM. Osteoclast formation decreased by (C) 66% in the presence of 2 h aOCY CM, and (D) 41% with 24 h CM. Values are normalized relative to aOCY (with no antibody) conditioned medium group. $^{*}P \leq 0.001$ relative to aOCY CM without RANKL antibody ($n = 4$).

DISCUSSION

One of the consequences of bone microdamage is a spatial distribution of aOCY that is localized around the site of microdamage but diminishes with distance [Verborgt et al., 2002]. Bone resorption spaces coincident with regions of osteocyte apoptosis suggest a relationship between osteocyte apoptosis and localized resorption [Verborgt et al., 2000; Gu et al., 2005; Noble, 2005]; however, it is not clear whether osteocyte apoptosis is directly or indirectly involved in this process. In this study, we aimed to investigate the role of osteocyte apoptosis on the initiation of osteoclast recruitment and differentiation. We found that aOCY play both a direct and indirect (by signaling to nearby healthy osteocytes) role by responding at the mRNA and protein level of several important regulating molecules, leading to the regulation of osteoclast precursor migration and differentiation.

To identify the regulating molecules involved in the increased osteoclast precursor migration and differentiation, we measured protein and mRNA expression of RANKL, OPG, M-CSF, and VEGF. Consistent with mechanical disuse studies in which osteocyte viability is compromised [Aguirre et al., 2006; You et al., 2008], apoptosis conditioned medium contained a significant increase in soluble RANKL. mRNA expression at 2 h post-apoptosis was also upregulated in aOCY; however, at 24 h, aOCY RANKL mRNA levels, as well as mRNA expression of other cytokines, decreased. This is consistent with the fact that RNA transcription is expected to cessate due to DNA fragmentation during later stages of apoptosis [Taylor et al., 2008]. At 24 h post-apoptosis, since mRNA transcription is stopped and protein modifications have been suggested to be inhibited (due to cleavage of translation initiation factors and ribosomal proteins [Lüthi and Martin, 2007]), we speculate that the increase in RANKL protein concentration may be a result of post-translational events such as the translation of different isoforms of RANKL [Ikeda et al., 2001] that may/may not require cleavage at the cell membrane [Hikita et al., 2006]. This could be further elucidated in future studies by staining the cell membrane for different isoforms of RANKL.

OPG protein levels in CM from MLO-Y4 cells were below the minimum threshold of ELISA assays, as was previously reported [Lau et al., 2010]; therefore, we were unable to measure OPG concentration. At the gene level, we found similar levels of OPG mRNA as RANKL, thereby rendering the RANKL/OPG ratio close to

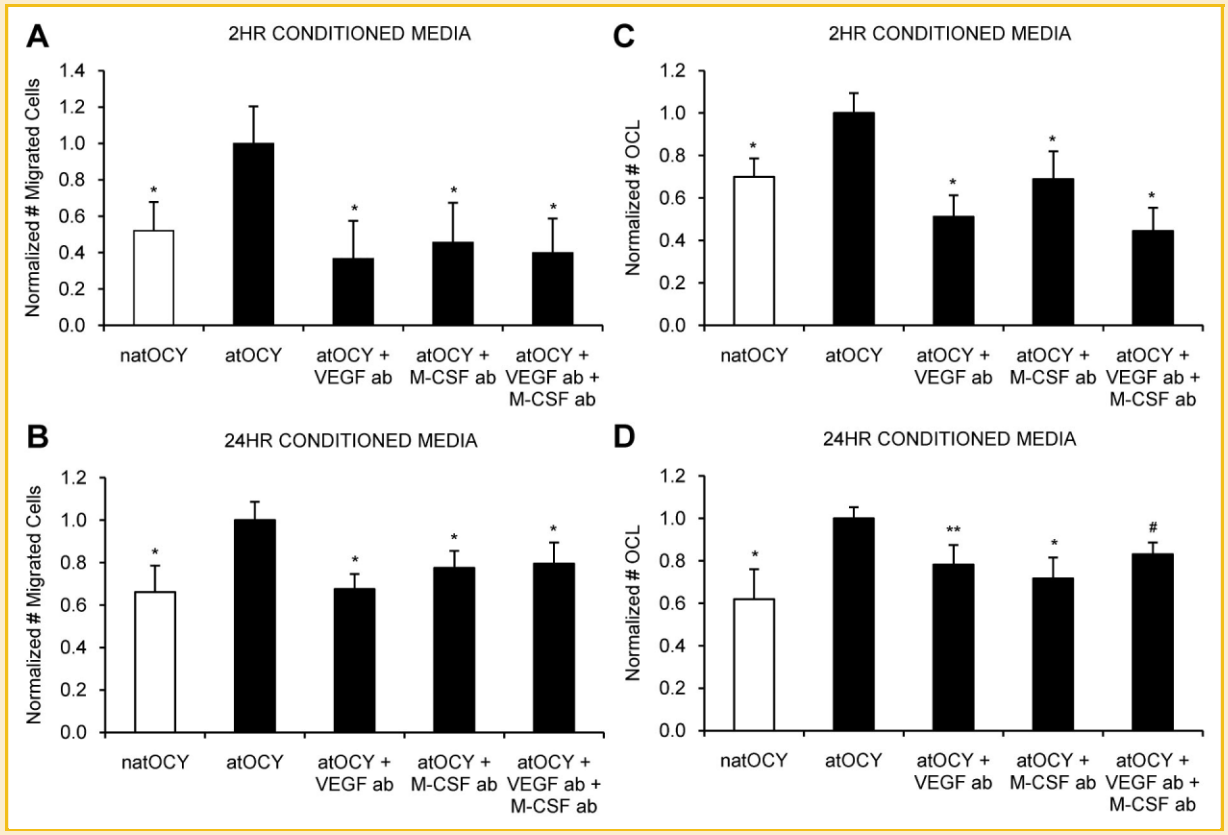

Fig. 9. Blocking VEGF and M-CSF abolished osteoclast precursor migration and differentiation induced by atOCY conditioned media. VEGF and/or M-CSF blocking antibodies reduced osteoclast precursor migration induced by atOCY by (A) approximately 60% with 2 h CM, and (B) 30% with 24 h CM. Osteoclast differentiation was reduced by (C) 45% with 2 h CM, and (B) 22% with 24 h atOCY CM. Values are normalized relative to atOCY (with no antibodies) conditioned medium group. $^{*}P < 0.003$, $^{**}P = 0.006$, $^{\#}P = 0.023$ all relative to atOCY CM without any antibodies ($n = 4$).

unity for all groups at both time points. Nevertheless, we speculate that the differences in osteoclastogenesis observed among condition groups may be due to the differences in RANKL protein levels. It, therefore, appears that aOCY use RANKL as a direct regulator of osteoclast formation.

M-CSF, which is necessary for osteoclast formation was lowly expressed in aOCY at 2 h, but increased to similar levels to natOCY at 24 h. Interestingly, mRNA expression in aOCY at 2 h was significantly greater than both atOCY and natOCY, but decreased to levels comparable to the atOCY and natOCY groups at 24 h. The transient increase in M-CSF secretion by aOCY may be a result of delayed protein cleavage [Deng et al., 1996] due to altered growth conditions (i.e., starvation), as changes in growth conditions have been previously implicated in transient M-CSF secretion [Caldwell et al., 1991]. It also appears that since atOCY have comparable M-CSF mRNA expression to natOCY but different protein expression, protein synthesis and post-translational modification may be favored in atOCY thus accounting for increased M-CSF protein secretion. This further suggests that M-CSF secretion may be a pathway through which aOCY indirectly regulate osteoclast formation (i.e., via atOCY).

VEGF has not only been implicated in angiogenesis but also in regulating osteoclast precursor recruitment, osteoclast formation and bone resorption [Sipola et al., 2006; Yang et al., 2008]. Indeed, we found that VEGF mRNA and protein expression were elevated in aOCY relative to the other condition groups, suggesting that VEGF directly regulates osteoclasts.

Interestingly, there was a transient decrease in VEGF protein secretion from atOCY; however, mRNA expression was relatively unchanged. This discrepancy may be due to a post-translational modification and/or membrane secretion step that may be inhibited at the later 24 h time point, which agrees with previously reported findings for VEGF and other proteins in the cases of hypoxia and tumor biology [Clemens et al., 2000; Kawagishi et al., 2010; Livingstone et al., 2010]. The transient VEGF secretion by atOCY, as well as previous findings by Verborgt et al. [2002] that show a decrease in osteocyte apoptosis and increase in anti-apoptosis signals away from microdamage sites, suggest that VEGF might be a pro-survival molecule secreted by osteocytes. VEGF has been shown to be an autocrine feedback regulator of osteoblast survival in response to apoptosis by stimulating expression of Bcl-2, an anti-apoptotic gene [Street and Lenehan, 2009]. This has been suggested to be mediated through the binding of VEGF protein to VEGF receptor 2 (VEGFR-2), which activates the MAP kinase cascade; this upregulates gene expression of heat shock protein 90 (Hsp90), which in turn binds to and stabilizes Bcl-2 [Dias et al., 2002]. Our transient VEGF secretion data leads us to speculate that healthy osteocytes in the presence of apoptosis cues (atOCY) initially upregulate VEGF

expression (i.e., at 2 h) as a Bcl-2 related survival mechanism, and that the subsequent decrease in soluble VEGF concentration (i.e., at 24 h) may be due to the binding of VEGF to VEGFR-2. VEGF staining of the cell membrane and measurement of Bcl-2 expression can be performed to verify this hypothesis.

aOCY conditioned medium treatment significantly increased osteoclast precursor migration at both 2 and 24 h, consistent with the increase in RANKL and M-CSF secretion by aOCY, suggesting that both RANKL and M-CSF may be responsible for the observed increase. VEGF was expected to also play a chemotactic role as per previous studies showing VEGF-induced osteoclast and osteoclast precursor migration [Barleon et al., 1996; Aldridge et al., 2005; Shibata et al., 2005]. However, our findings indicated that 2 h conditioned medium from atOCY did not promote osteoclast precursor migration despite there being an elevated level of VEGF secretion in conditioned medium. The discrepancy between our results and others' findings [Barleon et al., 1996; Aldridge et al., 2005; Shibata et al., 2005] may be due to the differences between the VEGF levels in our conditioned medium (absolute concentrations up to 815 pg/ml; not shown) and concentrations used in literature (1–100 ng/ml). In addition, these migration studies [Barleon et al., 1996; Aldridge et al., 2005; Shibata et al., 2005] use pure VEGF that is likely more potent than the VEGF found in our CM. Furthermore, other factors present may be present in CM that might mask the effect of VEGF. Nevertheless, our findings agree with in vivo work showing regions of bone resorption around aOCY, suggesting that osteoclast precursors are recruited to areas of apoptosis directly by aOCY and indirectly through healthy cells that sense apoptosis cues [Verborgt et al., 2000; Noble et al., 2003]. Differences in the migration response between aOCY and atOCY are likely due to a regulatory mechanism for localizing osteoclasts to apoptosis regions. However, further experiments need to be performed to verify this hypothesis.

CM from aOCY and atOCY (both 2 and 24 h) promoted more osteoclast formation than natOCY or controls. Upregulation of RANKL in CM is responsible for increased osteoclastogenesis by aOCY, as was observed by our neutralization experiments. Neutralization of RANKL abolished the increase in osteoclastogenesis induced by aOCY CM relative to natOCY CM. Although, there was low RANKL secretion by atOCY, increased osteoclast formation is likely due to the combined effect of M-CSF and VEGF based on results from our blocking experiments, where the increase in atOCY-induced osteoclastogenesis relative to natOCY treatment was abolished. Neutralization of VEGF and M-CSF individually had the same effect on osteoclastogenesis as the combined effect of VEGF and M-CSF inhibition. This suggests that VEGF and M-CSF act synergistically on osteoclast precursors, which agrees with previous findings by Yao et al. [2006] suggesting that a low dose of M-CSF is required to assist in VEGF-stimulated osteoclast formation. Our osteoclast size data is in agreement with osteoclast formation data from 2 h CM, in which aOCY and atOCY formed more large osteoclasts than natOCY or control. At 24 h, there was no significant difference among groups (not shown), which we speculate may be due to the presence of competing factors (e.g., TNF-α, sclerostin, PGE(2), COX-2, and Il-6 [Axmann et al., 2009; Yoshimatsu et al., 2009; Boudot et al., 2010; Gooi et al., 2010]) that may influence osteoclast size and/or also contribute to the overall osteoclastogenesis process.

The aim of our system was to model the in vivo events surrounding the role of osteocyte apoptosis in osteoclast regulation in response to microdamage. Several limitations exist in these studies: (1) Our in vitro model does not incorporate other consequences of microdamage, such as necrosis, hypoxia, and physical damage to osteocytes. Furthermore, although we observed DNA fragmentation (which is characteristic of apoptosis) in all serum-starved MLO-Y4 cells, we cannot rule out autophagy as an initial response to starvation that may influence osteoclast formation. In other words, as a result of autophagy, cellular components may be degraded and vacuolized [Fink and Cookson, 2005], and released in the conditioned medium thereby contributing to the observed effect. (2) While we are able to investigate the response of three different osteocyte populations (aOCY, atOCY, and natOCY), our system does not incorporate other bone cells that may participate in the regulation of osteoclasts and bone remodeling. For example, endothelial cells lining blood vessels [Aldridge et al., 2005] or bone lining cells at the bone surface [Parfitt, 1994] may act as potential regulators in the migration process. (3) Molecular transport in the lacunar–canalicular porosity of bone is based on diffusion and convection, which is slower than large scale in vitro systems, thereby rendering concentrations of soluble factors in vivo lower than in vitro. Transport can also be affected by the presence of microdamage in the bone matrix. (4) Since conditioned medium-based assays do not allow real-time bi-directional cell communication that normally takes place in vivo, more physiological studies should be performed using co-culture systems.

In summary, we presented an in vitro osteocyte apoptosis model to study the effect of osteocytic apoptosis on osteoclast regulation. Our findings suggest that osteocyte apoptosis promotes a pro-osteoclastogenic response at the mRNA and protein levels in aOCY and atOCY. Increased osteoclast precursor migration and differentiation into osteoclasts in the presence of aOCY and atOCY CM are likely due to increased soluble RANKL, M-CSF, and VEGF in CM. aOCY appear to regulate osteoclasts directly through RANKL secretion, and indirectly through atOCY by means of M-CSF and VEGF secretion. Findings from this study support our hypothesis, as well as previous in vivo data [Verborgt et al., 2000], that in the presence of microdamage aOCY directly and indirectly (through neighboring healthy osteocytes) initiate osteoclast precursor recruitment and differentiation.

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