RANKL Inhibits the Production of Osteoprotegerin from Smooth Muscle Cells under Basal Conditions and following Exposure to Cyclic Strain

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Keywords

RANKL · Osteoprotegerin · Calcification · Cyclic strain · Endothelium · Smooth muscle

Abstract

Receptor activator of nuclear factor- κ B ligand (RANKL) promotes vascular calcification, while osteoprotegerin (OPG) opposes it by blocking RANKL activity. It remains unclear which vascular cell populations produce and secrete OPG/RANKL. This study characterizes the production of OPG/RANKL from human aortic endothelial and smooth muscle cells (HAECs and HASMCs) under various conditions. HAECs and HASMCs were exposed to inflammatory stimuli (tumor necrosis factor- α or hyperglycemia) or physiological levels of hemodynamic (cyclic) strain. After 72 h, both cells and supernatant media were harvested for assessment of OPG/RANKL production. Based on initial findings, the experiments involving HASMCs were then repeated in the presence of exogenous RANKL. OPG was produced and secreted by HASMCs and (to a considerably lesser degree) HAECs under basal conditions. Inflammatory stimuli upregulated OPG production in both cell populations. Cyclic strain significantly upregulated OPG production in HASMCs. Neither cell pop-

ulation produced RANKL. Exposing HASMCs to exogenous RANKL inhibited basal OPG production and completely abrogated the strain-mediated upregulation of OPG. These data suggest that HASMCs are a significant source of OPG within the vasculature but that RANKL, once present, down-regulates this production and appears capable of preventing the “protective” upregulation of OPG seen with HASMCs exposed to physiological levels of cyclic strain.

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Introduction

Vascular calcification (VC) is a pathophysiological process that affects multiple aspects of the vascular tree, and particularly the cardiac valves and the intimal and medial aspects of the arterial wall [1]. VC exerts numerous detrimental effects on the vasculature, contributing to increased arterial stiffness, widened pulse pressure, increased rates of left ventricular hypertrophy, and an increased risk of coronary artery dissection after angioplas-

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ty [2]. However, despite the recognition of a clear association between VC burden and rates of cardiovascular morbidity and mortality [3], the cellular mechanisms that promote and inhibit VC remain unclear, and clinical interventions aimed at inhibiting or reversing VC have produced, for the most part, disappointing results [4].

Receptor activator of nuclear factor- κ B ligand (RANKL) is a member of the tumor necrosis factor (TNF) receptor superfamily which binds to the RANK receptor on cell membranes [5]. RANKL colocalizes with areas of calcification within the vasculature and has been demonstrated by our own group and others to promote the calcification process by inducing the emergence of pro-osteoblastic cells within the vascular wall [6–8]. Osteoprotegerin (OPG), also present within the vascular wall, is a decoy receptor for RANKL. OPG binds to RANKL and blocks its biological activities, with OPG knockout mice developing early and widespread VC [9, 10]. Interestingly, there have been comparatively few basic assessments of the factors controlling the production and secretion of RANKL and OPG within the vascular wall – and, particularly, what effects stimuli associated with VC *in vivo* (inflammation, hyperglycemia, etc.) have on these two antagonistic proteins. Those limited studies that have been conducted in this area have typically examined murine rather than human cells, have often employed cells from regions of the vasculature that are not traditionally prone to VC, and/or have employed just one vascular cell type, ignoring the potential paracrine contribution of other cells inherent within the vessel wall [11–15]. Thus, the translational applicability of the results of these studies to human VC is questionable. Moreover, whilst the effects of OPG on RANKL are clear (OPG binds to and blocks RANKL from exerting any biological function), the “opposing” effects of RANKL on OPG production and secretion by vascular cells have yet to be assessed. Without this information, it is difficult to determine (a) whether the RANKL/OPG system is involved in the increased rates of VC that typically accompany inflammatory and hyperglycemic conditions *in vivo* and (b) whether RANKL itself potentially feeds back upon the production and secretion of OPG under specific physiological and pathological circumstances.

In view of this knowledge deficit, the goals of the present study, therefore, were to separately characterize the production and secretion of RANKL and OPG within two pivotal vascular cell populations (human aortic endothelial cells [HAECs] and human aortic smooth muscle cells [HASMCs]). Moreover, for comparative purposes, it was decided to perform these studies under pathophysiologi-

cal conditions that typically promote VC (inflammation and hyperglycemia), and under hemodynamic conditions (e.g., cyclic strain) associated with a more physiological vascular cell phenotype [16, 17]. Finally, we aimed to assess whether RANKL could exert any effect on OPG production or secretion under the aforementioned treatment conditions. Based on the previously demonstrated ability of SMCs to adopt osteoblast-like behavior [6, 7], we hypothesized that these particular vascular cells would produce significant concentrations of OPG, and, furthermore, that this OPG production would be influenced by stimuli relevant to the development of VC *in vivo*.

Materials and Methods

Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Dublin, Ireland). Recombinant human RANKL was purchased from R&D Systems (Minneapolis, MN, USA). TNF- α was purchased from Merck Millipore (Danvers, MA, USA). Primers were purchased from Eurofins Genomics (Ebersburg, Germany). BioFlex[®] plates were purchased from Dunn Labortechnik GmbH (Asbach, Germany). Monoclonal antibody to RANK (category No. 374360) was purchased from Santa Cruz (Dallas, TX, USA), and the secondary antibody (category No. 7076s) was purchased from Cell Signaling Technology (Danvers, MA, USA). Commercial enzyme-linked immunosorbent assay (ELISA) kits included the OPG and RANKL DuoSet kits (R&D Systems), the Ampli-sRANKL assay kit (Biomedica, Vienna, Austria), and the sRANKL assay kit (BioVendor, Heidelberg, Germany).

Cell Culture

HAECs and HASMCs were purchased from Promocell GmbH (Heidelberg, Germany), as were the culture media for both cell types. The HAECs (positive for von Willebrand factor and CD31) were received from two donors, a 28-year-old Caucasian male (utilized in the majority of experiments) and a second batch from a 23-year-old Caucasian male. The HASMCs (positive for α -actin) were received from two donors, one batch from a 23-year-old Caucasian male and a second batch from a 19-year-old Caucasian male. Expression of ACTA2 (smooth muscle alpha-actin 2) and TAGLN (transgelin, SM22) was used to confirm persistence of the smooth muscle cell phenotype throughout the series of experiments (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000486787). HAECs were cultured in endothelial cell growth medium with the following supplements: fetal calf serum (0.05 mL/mL), endothelial cell growth supplement (0.004 mL/mL), epidermal growth factor (10 ng/mL), heparin (90 μ g/mL), and hydrocortisone (1 μ g/mL). This medium was also supplemented with penicillin (100 IU/mL) and streptomycin (100 μ g/mL). HASMCs were cultured in smooth muscle cell growth medium supplemented with antibiotics (as above), in addition to fetal calf serum (0.05 mL/mL), epidermal growth factor (0.5 ng/mL), basic fibroblast growth factor (2 ng/mL), and insulin at 5 μ g/mL. Basic fibroblast growth factor, which has been report-

ed as promoting OPG production (albeit at higher concentrations), was retained in the media to maintain acceptable levels of growth and viability for experimental purposes [18].

During culture, cells were maintained in a humidified incubator at 37°C and 5% CO₂. Passages 5–9 were routinely used for experimental purposes. The cells were cultured in standard 6-well dishes. HAECs were seeded at a density of 250,000 cells per well, whereas HASMCs were seeded at a density of 150,000 cells per well. At confluency, the cultures were washed with sterile PBS, fresh medium was applied, and experiments were commenced. Once the growth medium had been added to the cells, soluble factors (TNF- α , glucose, etc.) could be added as required to produce experimental conditions. Cell number and viability were routinely measured using the Advanced Detection and Accurate Measurement (ADAM™) cell counter (Digital Bio, Seoul, Korea), both for seeding density purposes and to allow normalization of results where necessary. This process involved the digital analysis of cells on custom AccuChip slides following the addition of propidium iodide, with or without a lysis solution, providing total numbers of both viable and nonviable cells.

For the experiments involving the application of cyclic circumferential strain, a potent hemodynamic stimulus experienced in arteries, HAECs, and HASMCs was seeded to each well of a BioFlex® plate at densities of 350,000 and 250,000 cells, respectively. The BioFlex® plates utilized were 6-well culture dishes with flexible, ProNectin®-coated silicon membrane bottoms, whose shape was altered via the use of a microprocessor-controlled Flexcell FX-4000™ Tension Plus vacuum system (Flexcell International, Hillborough, NC, USA). Stretching of the flexible membrane allowed us to apply equibiaxial strain (0–10% strain, 60 cycles/min or 1 Hz, cardiac waveform) to the cells [19].

Our experimental approach was subsequently divided into two main areas: (1) assessment of RANKL and OPG production and secretion from HAECs/HASMCs under physiological and pathophysiological stimulation and (2) assessment of the effects of RANKL on OPG production and secretion by HASMCs.

Assessment of RANKL and OPG Production and Secretion (HAECs and HASMCs)

As OPG and RANKL are both active in the soluble, secreted form, and it was unclear from previous research whether the production and secretion of these proteins are affected in different ways by stimuli, we chose to measure both production and secretion in our experiments. Accordingly, the production and secretion of RANKL and OPG from HAECs and HASMCs under basal (untreated) conditions were investigated. HAECs and HASMCs were grown to confluency in standard 6-well dishes, and at the commencement of the experiments fresh growth medium was added to the cells. To measure the intracellular levels of RANKL and OPG protein in HAECs and HASMCs, whole-cell lysates were harvested at 24–72 h for ELISA. To measure the secretion of RANKL and OPG from the two cell types, supernatant medium was harvested from each culture well at 24, 48, and 72 h and tested by ELISA as well.

After our initial assessment of HAECs and HASMCs under basal conditions, we assessed the effects of TNF- α (0 and 100 ng/mL), glucose (15 and 30 mmol/L; concentrations reflecting moderate and severe hyperglycemia, respectively), and cyclic strain (5% for HAECs and 10% for HASMCs, reflecting the differing strain amplitudes likely encountered by each cell type in vivo) [20, 21] on

the production and secretion of RANKL and OPG from HAECs and HASMCs. All treatments lasted 72 h, following which the supernatant media and whole-cell lysates/mRNA were harvested for analysis of RANKL and OPG secretion and production, respectively.

Assessment of Effects of RANKL on OPG Production and Secretion (HASMCs)

HASMCs were exposed for 72 h to TNF- α (0 and 100 ng/mL), glucose (15 and 30 mmol/L), or cyclic strain at 10% in the absence and presence of exogenous RANKL (50 ng/mL). This concentration of RANKL ensured an excess of exogenous RANKL relative to the endogenous RANKL-blocking OPG released by HASMCs (see Results), thereby avoiding confounding RANKL results due to decoy receptor release and also avoiding the need for the utilization of anti-OPG neutralizing antibodies in our experiments. After a 72-h exposure, the supernatant media and whole-cell lysates/mRNA were harvested for analysis of OPG secretion and production, respectively.

ELISA

In order to measure the secretion of OPG and RANKL from HAECs and HASMCs, supernatant medium was harvested and then centrifuged at 1,000 rpm (4°C) for 10 min to remove any cellular debris. With regard to the harvesting of cell lysates, this was accomplished on ice (<4°C) using a radioimmunoprecipitation assay lysis buffer. The lysates were then transferred to fresh tubes and stored. All samples were stored at –80°C, with all relevant analyses being performed within 1 month following a single freeze-thaw cycle. OPG levels were measured with the DuoSet ELISA kit as per the manufacturer's instructions. A 10 $\times$ dilution of all harvested supernatant medium samples from HASMCs proved necessary, as OPG concentrations from this cell population were significantly above the upper limit of the standard curve. RANKL levels were monitored via three different ELISA approaches. These included the RANKL DuoSet kit, the Ampli-sRANKL assay kit, and the sRANKL assay kit, and all were performed as per the manufacturer's instructions. The concentrations of OPG and RANKL in the supernatant medium samples were routinely normalized for viable HAEC and HASMC numbers, whilst the concentrations of OPG and RANKL in whole-cell lysates were routinely normalized for total protein concentrations measured with a bicinchoninic acid assay.

Quantitative Real-Time RT-PCR and Western Blot

For quantitative real-time RT-PCR (RT-qPCR), posttreatment HAECs and HASMCs were harvested for extraction of total RNA and analysis of gene expression, as previously described by Rochfort et al. [22], using an ABI 7900 HT Fast RT-PCR System (Applied Biosystems/Life Technologies, Paisley, UK). Each cDNA sample was assayed in triplicate, and results were analyzed by the comparative CT method. S18 was routinely used for normalization purposes. Primer pairs were screened for their correct product size by 1% agarose gel electrophoresis and underwent melting curve analysis for primer dimers – S18 (250 bp): forward 5'-cagccaccgagattgagca-3', reverse 5'-tagtagcgacggcggtgtg-3'; OPG (241 bp): forward 5'-ggcaacacagctcacaaga-3', reverse 5'-ctgggttgatgcctt-3'; RANK (182 bp): forward 5'-caccatgaacccatgtttac-3', reverse 5'-cgactcctatctcacttaggc-3'; RANKL (180 bp): forward 5'-agagcgcagatggatccta-3', reverse 5'-ttcctttgcacagctcctt-3';

Fig. 1. The secretion of osteoprotegerin (OPG) from human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) under basal conditions. HAECs (**a**) and HASMCs (**b**) were cultured for 24, 48, and 72 h. At each time point, the supernatant medium surrounding the cells was harvested for quantification of OPG protein. The histograms represent concentrations of OPG in the supernatant media corrected for viable cell numbers relative to untreated control HAECs or HASMCs. Data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

ACTA2 (122 bp): forward 5'-ctatgaggctatgccttgcc-3', reverse 5'-gctcagcagtagtaacgaagga-3'; TAGLN (104 bp): forward 5'-ccgtg-gagatccaactgg-3', reverse 5'-ccatctgaaggccaatgacat-3'. For RANK protein expression in HASMCs, lysates were assessed by Western immunoblotting as described by Rochfort et al. [22] with minor modifications. Primary monoclonal RANK antibody was prepared in TBS-T (Tris-buffered saline plus 0.1% Tween 20) containing 1% BSA at 1/1,000 dilution. Secondary anti-mouse IgG was employed at 1/1,000 dilution in TBS-T (+ 1% BSA).

Statistical Analysis

Data were analyzed using GraphPad Prism version 4.03 (GraphPad Software, La Jolla, CA, USA), and are expressed as mean $\pm$ SEM (normality was tested for via the Kolmogorov-Smirnov test). Unless otherwise stated, all experiments were conducted a minimum of 3 times. Statistical comparisons between controls and treatment groups were made by ANOVA in conjunction with a Dunnett post hoc test for multiple comparisons. A Student unpaired t test was also employed for pair-wise comparisons. A value of $p \leq 0.05$ was considered significant.

Results

OPG, but Not RANKL, Is Produced and Secreted by HAECs and HASMCs under Basal Conditions

Upon examination of the production and release of OPG under basal conditions, the concentration of OPG within HAEC lysates was measured at 598 ± 30 pg/mg cell protein ($n = 9$). HAECs also secreted small amounts

of OPG into the supernatant media, with concentrations of 24.5 ± 1.4 pg/10,000 cells measured after 72 h. With regard to HASMCs, the concentration of OPG within HASMC lysates was considerably higher, measuring $26,945 \pm 983$ pg/mg protein ($n = 3$) after 72 h. HASMCs were also observed to secrete very high amounts of OPG into the supernatant media, with concentrations of $2,096 \pm 186$ pg/10,000 cells measured after 72 h (Fig. 1). Interestingly, while we did find evidence of RANKL mRNA expression in HASMCs (but not HAECs), we found no evidence of significant concentrations of RANKL protein within either HAEC or HASMC whole-cell lysates, nor did we detect significant concentrations of RANKL within the supernatant media of either cell type after 72 h of culture, despite a lower limit of detection in the pg/mL range (see online suppl. Fig. S2). In view of this negative result, and to rule out any potential flaws with our ELISA procedure, we utilized three different ELISA assay kits for the detection of both bound and free RANKL. To ensure there was no assay interference, we mixed a known quantity of RANKL standard with (a) the culture media utilized and (b) a high concentration of OPG. Neither of these control conditions affected the ability of the ELISA kits to identify and quantify the known level of RANKL, further indicating that RANKL protein was not present in detectable quantities.

Fig. 2. Levels of osteoprotegerin (OPG) mRNA in human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) under basal and stimulated conditions. HAECs (**a**) and HASMCs (**b**) were treated for 72 h with 5 or 10% cyclic strain, hyperglycemia (15 and 30 mmol/L), and TNF- α (100 ng/mL), after which they were harvested and monitored for changes in mRNA levels of OPG. Representative agarose gel insets verify HAEC or HASMC OPG expression and correct fragment sizes, with lanes labelled O1 and O2, respectively. The histograms represent fold change in mRNA levels relative to untreated controls normalized for expression against S18. Data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

Inflammation and Hyperglycemia, but Not Cyclic Strain, Modulate OPG Production and Secretion from HAECs

Following exposure of HAECs to 5% cyclic strain (72 h), a very slight reduction in levels of OPG mRNA was noted, but this was not accompanied by any significant change in either intracellular or released OPG protein levels. Exposure of HAECs to hyperglycemia (either 15 or 30 mmol/L glucose, 72 h) resulted in a trend towards

higher intracellular concentrations of OPG protein, although mRNA and secreted OPG levels remained virtually unchanged. Finally, whilst a significant reduction in levels of OPG mRNA was noted following exposure to 100 ng/mL TNF- α (72 h), both intracellular and secreted concentrations of OPG protein were increased (Fig. 2a, 3a, 4a). There were no significant levels of RANKL detected in HAECs in response to any of the conditions tested.

Fig. 3. Intracellular concentrations of osteoprotegerin (OPG) in human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) under basal and stimulated conditions. HAECs (**a**) and HASMCs (**b**) were treated for 72 h with 5 or 10% cyclic strain, hyperglycemia (15 and 30 mmol/L), and TNF- α (100 ng/mL), after which whole-cell lysates were harvested for quantification of OPG protein. The histograms represent concentrations of OPG in the cell lysates corrected for total protein concentrations relative to untreated controls. Data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

Inflammation, Hyperglycemia, and Cyclic Strain Increase OPG Production and Secretion from HASMCs

In contrast to HAECs, exposure to 10% cyclic strain (72 h) induced significant increases in levels of OPG mRNA and protein (intracellular and secreted). Exposure to 100 ng/mL TNF- α had similar effects. Finally, whilst exposure to hyperglycemia (either 15 or 30 mmol/L glucose, 72 h) was not associated with a significant increase in levels of OPG mRNA, significantly increased levels of intracellular and secreted OPG protein were observed (Fig. 2b, 3b, 4b). As before, there were no significant levels of RANKL detected in response to any of the conditions tested.

RANKL Causes a Temporary Increase in OPG Production and Secretion from HASMCs at 24 h That Is Followed by a Significant Decrease by 72 h

As our initial series of experiments demonstrated that HASMCs produce much greater amounts of OPG than do HAECs, and we did not identify any endogenous production of RANKL, we chose to focus our subsequent experiments on HASMCs and the effects of exogenous RANKL on OPG production and secretion. We note that the expression of RANK mRNA and protein was demonstrated in untreated HASMCs, indicating the presence of the RANKL receptor in significant quantities in this cell population (see online suppl. Fig. S2). When HASMCs

Fig. 4. Concentrations of osteoprotegerin (OPG) in the supernatant media of human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) under basal and stimulated conditions. HAECs (**a**) and HASMCs (**b**) were treated for 72 h with 5 or 10% cyclic strain, hyperglycemia (15 and 30 mmol/L), and TNF- α (100 ng/mL), after which the supernatant media surrounding the cells were harvested for quantification of OPG protein. The histograms represent concentrations of OPG in the supernatant media corrected for viable cell numbers relative to untreated control HAECs or HASMCs. Data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

were exposed to 50 ng/mL RANKL, a divergent response in OPG production and secretion was seen over the 72 h. Initially, we noted that at 24 h, levels of OPG mRNA, intracellular OPG, and secreted OPG had all increased significantly. Following this response, however, all of these parameters decreased, and by 72 h the net effect of RANKL on HASMCs was to induce a significant decrease in levels of OPG mRNA, intracellular OPG, and secreted OPG protein (Fig. 5). Finally, a dose-response assessment of 50 and 500 pg/mL as well as 5 and 25 ng/mL concentrations

of RANKL on OPG production and secretion was performed, which indicated that at 72 h, a downregulation of OPG mRNA and intracellular OPG concentrations was seen with 5 ng/mL RANKL, but that decreased secretion was not apparent until 25 ng/mL RANKL was utilized.

RANKL Prevents the Increased Production and Secretion of OPG in HASMCs Exposed to Cyclic Strain

When HASMCs were exposed to 50 ng/mL RANKL in the presence of 10% cyclic strain, a complete abrogation

Fig. 5. Effects of RANKL on osteoprotegerin (OPG) production and secretion at 24, 48, and 72 h. Human aortic smooth muscle cells were treated with 50 ng/mL RANKL for 24, 48, and 72 h. After treatment, cells were harvested and monitored for changes in OPG mRNA expression (**a**), intracellular OPG protein (**b**), and

released OPG protein (**c**). The histogram in **a** displays fold changes to untreated controls. All data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

of the cyclic-strain-induced upregulation of OPG was observed, with levels of OPG mRNA and protein (intracellular and released) all below control levels in the presence of RANKL (Fig. 6).

RANKL Strongly Attenuates the Increased Production of OPG Protein (but Not OPG mRNA or Release) in HASMCs Exposed to Hyperglycemia

When HASMCs were exposed to 50 ng/mL RANKL for 72 h under hyperglycemic conditions (15 and 30 mmol/L glucose), the level of OPG mRNA was unchanged relative to glucose treatment alone (Fig. 7a, d). By contrast, the glucose-induced production of intracellular OPG protein (but not OPG release) was almost completely abrogated under hyperglycemic (15 mmol/L [Fig. 7b, c] and 30 mmol/L [Fig. 7e, f]) conditions.

RANKL Weakly Attenuates the Increased Production of OPG Protein (but Not OPG mRNA or Release) in HASMCs Exposed to TNF- α

When HASMCs were exposed to 50 ng/mL RANKL for 72 h in the presence of 100 ng/mL TNF- α , a slight increase in the level of OPG mRNA was observed relative to TNF- α treatment alone (Fig. 7g). By contrast, the cytokine-induced production of intracellular OPG protein (but not OPG release) was moderately decreased (Fig. 7h, i).

Discussion

The biological process of VC has become a subject of increasing interest in recent years as a both a precipitant and accelerant of cardiovascular disease, especially in the diabetes and renal failure populations. The promoters and inhibitors of VC remain poorly understood, but a

Fig. 6. Effects of RANKL $\pm$ cyclic strain on osteoprotegerin (OPG) production and secretion by human aortic smooth muscle cells (HASMCs) at 72 h. HASMCs were treated with 50 ng/mL RANKL for 72 h in the absence or presence of cyclic strain (10%). After treatment, cells were harvested and monitored for changes in OPG

mRNA expression (**a**), intracellular OPG protein (**b**), and released OPG protein (**c**). The histogram in **a** displays fold changes to untreated controls. All data are presented as mean $\pm$ SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

mounting body of evidence suggests that RANKL promotes VC and that OPG inhibits VC by blocking RANKL. The present study demonstrates a number of important findings regarding the production and secretion of OPG and RANKL from two human vascular cell populations believed to be intimately linked to the emergence of VC in vivo. A brief summary of our findings indicates that (1) in keeping with our initial hypothesis, OPG is produced in very significant amounts by HASMCs, and OPG production by both HAECs and HASMCs alters significantly in response to inflammatory and hemodynamic stimuli; (2) the source of RANKL within the vascular wall in vivo is unlikely to originate from either the endothelial or the smooth muscle cell layer; and (3) RANKL can down-regulate the production of its own decoy ligand under basal and stimulated (e.g., cyclic strain) conditions. It is also noteworthy that a bimodal OPG response to RANKL in HASMCs was seen over time, with a temporary up-regulation by 24 h that was then followed by a longer-term downregulation. To the best of our knowledge, this is the first time the inhibitory influence of RANKL upon OPG production in this manner has been reported – indeed, these are the first data on the effects of cyclic strain on the OPG/RANKL system within the vasculature to be reported.

Our initial experiments assessed the production and secretion of OPG and RANKL from HAECs and HASMCs. Few groups have previously examined the production of these proteins from clinically relevant human vascular cell populations, in particular under stimuli known to be associated with VC in vivo, and there is virtually no research on the effects of cyclic strain, a potent hemodynamic stimulus within arteries, upon the production of OPG and RANKL from vascular cells. With this in mind, we report that HASMCs produce and release OPG in concentrations at least two orders of magnitude greater than HAECs under basal conditions. Furthermore, although we did not formally compare levels of OPG mRNA expression between HASMCs and HAECs, we did compare the threshold at which OPG mRNA amplification occurred during RT-qPCR for the two cell populations, with amplification of OPG mRNA occurring in a much earlier cycle in HASMCs. These data strongly suggest that HASMCs may be the primary source of OPG within the vascular wall. We also report that the production and secretion of OPG by either cell population altered in response to both injurious and healthy stimuli. We speculate that the upregulation of OPG in both cell types following exposure to pathologic stimuli such as inflammatory cytokines or high glucose [23, 24] may be an adaptive response designed to inhibit

Fig. 7. Effects of RANKL ± hyperglycemia/TNF-α on osteoprotegerin (OPG) production and secretion by human aortic smooth muscle cells (HASMCs) at 72 h. HASMCs were treated with 50 ng/mL RANKL for 72 h in the absence or presence of hyperglycemia (15 and 30 mmol/L glucose) or TNF-α (100 ng/mL). After treatment, cells were harvested and monitored for changes in OPG

mRNA expression (**a, d, g**), intracellular OPG protein (**b, e, h**), and released OPG protein (**c, f, i**). The histograms in **a, d**, and **g** display fold changes to untreated controls. All data are presented as mean ± SEM ($n = 18$ for the total number of wells analyzed after the experiments had been performed in triplicate).

it the early emergence of VC in areas of vessel wall damage. With regard to cyclic strain, this hemodynamic stimulus, which reproduces the outward stretching experienced by arterial walls *in vivo*, typically causes endothelial and smooth muscle cells to adopt a “healthy” phenotype [19]. In terms of OPG production, the responses to cyclic strain that we observed were somewhat nuanced, with limited effects on OPG production and secretion seen in HAECs, but quite significant upregulation seen for both in HASMCs. These findings are quite novel, with the only other studies on OPG and strain in the scientific literature at present involving research into osteoblast cells in dentistry [25]. Ultimately, our results suggest that cyclic strain is not a potent stimulus for OPG production by HAECs, but that it is a very significant stimulus for OPG production and secretion by HASMCs. We hypothesize that it is likely that the increased production and secretion of OPG by HASMCs under cyclic strain reflects a closer picture of the true levels of OPG production by smooth muscle cells under healthy conditions *in vivo*, with a large production and secretion of OPG conferring a basal level of protection from the induction of VC by RANKL. The natural corollary to this, however, is that once VC becomes established within a vessel wall, the compliance and flexibility of the vessel will be reduced, and gradually its responsiveness to cyclic strain. This may contribute to an injurious cycle of increasing calcification, decreasing strain, decreasing OPG production, and further increased calcification, a hypothesis supported in part by the observation that abnormal areas of hemodynamic stress often colocalize with atherosclerosis and VC within the vascular tree *in vivo* [26].

It is noteworthy that we did not identify any significant levels of RANKL within either the whole-cell lysates or the supernatant media surrounding the HAECs and HASMCs. Moreover, this did not change when the cells were exposed to either TNF- α , hyperglycemia, or cyclic strain. With regard to previous research in this area, there is considerable inconsistency in findings. In endothelial cells, for example, Collin-Osdoby et al. [11] reported the presence of RANKL protein via immunostaining. By contrast, when heart and umbilical vein endothelial cells were assessed in a similar fashion by Zannettino et al. [27], no evidence of RANKL mRNA or protein was detected. Finally, while Osako et al. [7] did report RANKL protein expression via Western blotting in HAECs, this expression was very faint and they did not describe absolute concentrations. With regard to vascular smooth muscle cells, Nguyen et al. [28] measured

concentrations of RANKL and TRAIL (TNF-related apoptosis-inducing ligand) in the supernatant media of HASMCs by ELISA. While this group reported findings similar to ours in that there was an absence of free RANKL, they did report its presence in relatively low concentrations bound to endogenous OPG. It may be noted that a novel ELISA protocol generated *de novo* by Nguyen and co-workers for this particular study was utilized, although no data on the validity of this ELISA protocol was provided. This is particularly relevant as both OPG and RANKL, due to their interlinked nature and propensity for dimer and trimer formation, can lead to false positives on ELISA. As such, it is therefore possible that methodological differences may account for the discrepancies between that study and our own. Ultimately, we submit that the lack of significant quantities of RANKL identified by our approach – which utilized multiple ELISAs with lower limits in the picogram and picomole range, and which captured both free and bound forms of RANKL – indicates that infinitesimally little or no RANKL is produced by cultured HAECs or HASMCs, and certainly nothing sufficient to lead to any biological activity given the significant basal release of OPG decoy receptor from these cells. This in turn suggests that alternate cell populations are responsible for the presence of RANKL in areas of VC identified *in vivo*, with circulating T lymphocytes identified as one possible source [5]. It should, however, be noted that these results are only applicable to the endothelial and smooth muscle cells from the human aortic vascular bed under the conditions assessed, and that endothelial and smooth muscle cells harvested from different vascular beds and/or cultured under different conditions (including altered hemodynamic flow) may produce different results.

For our final series of experiments, we focused on the effects of RANKL on HASMCs. We chose this approach due to the significantly greater production of OPG by HASMCs in our initial experiments, the known involvement of HASMCs in the emergence of VC, and previous preliminary work by our group on osteoblastic activity in vascular cell populations suggesting that HAECs respond minimally in terms of OPG following exposure to RANKL [6, 7]. We note that while there has been some controversy in the literature regarding the presence of RANK in smooth muscle cell populations, we demonstrated that this receptor for RANKL was present at the mRNA and protein level in our own HASMCs. Upon adding RANKL to HASMCs, we demonstrated that exogenous RANKL suppressed OPG production and secretion by these cells under basal conditions. We do note

that while the concentrations of RANKL utilized in our experiments was significantly in excess of reported circulating concentrations of this protein, the endogenous production of RANKL within the vascular wall, as reported by other groups, suggests that vascular concentrations *in vivo* may well be higher than circulating levels [29]. Interestingly, the effect of RANKL on OPG was time dependent, with an initial upregulation of OPG production and release seen at 24 h that was then followed by a longer-term downregulation at 48 and 72 h. This bimodal response is also a novel finding by our group and may explain some of the inconsistencies in previous studies on OPG production and release, with the OPG system evidently able to demonstrate diametrically opposing short- and long-term responses to stimuli. As our own interest in OPG and RANKL stemmed from its potential role in the chronic process of VC, we retained our focus on the 72-h results in the subsequent experiments, but clearly the bimodal response seen here should be taken into account in future studies of this system. Furthermore, these findings highlight the possibility that different signaling pathways may be involved in OPG production versus secretion in the cell populations, a phenomenon which will require additional study, potentially including RANKL receptor blockade, to characterize fully. RANKL was also shown to potentially block the upregulation of OPG production and release normally induced by physiological levels of cyclic circumferential strain. These data suggest that once RANKL is present in sufficient concentrations, it not only promotes VC via mechanisms such as upregulation of bone morphogenetic protein, as previously shown [6], but also decreases OPG production, likely neutralizing an important protective influence of cyclic strain upon smooth muscle cells and the vascular wall. In this context, one can hypothesize that a “tipping point” towards VC may exist within the vascular wall. Once RANKL concentrations exceed those of OPG, unopposed RANKL activity may induce VC, directly decrease the production of its own neutralizing decoy receptor (OPG), and (as noted previously) facilitate a cycle of VC leading to decreased vessel flexibility, decreased cyclic strain responsiveness in smooth muscle cells, and ultimately decreased OPG production and further unopposed RANKL activity. If true, this hypothesis suggests that primary prevention of VC may be more successful than inhibiting or reversing the process once established. Finally, whilst RANKL could abrogate the strain-induced production of OPG by HASMCs, it did not affect the upregulation of OPG production by these cells following exposure to elevated lev-

els of TNF- α and hyperglycemia, stimuli that are integral to diabetes, atherosclerosis, and, of course, the emergence of VC. Whilst the reason for this is unknown at present, these divergent results may be attributable to a specific influence of RANKL upon strain-mediated mechanosignaling pathways [30].

In conclusion, this paper characterizes the production of OPG by HAECs and HASMCs under both physiological and pathological conditions, noting a considerably greater production of OPG by the latter cell type under all conditions tested. Interestingly, neither cell population expressed or produced RANKL under any of the conditions tested, suggesting that RANKL reported within the vessel wall derives from nonvascular cells. We also present novel data describing how RANKL treatment of HASMCs downregulates the production of its own decoy receptor under basal conditions, and completely abrogates the upregulation of OPG production induced by physiological levels of cyclic strain. These findings may have direct relevance to the establishment of VC at biomechanically compromised points within arteries. Ultimately, these findings are of particular importance to research into VC because (1) they establish a baseline characterization of the OPG/RANKL system under a variety of relevant conditions in two major vascular cell populations and (2) they suggest a “tipping-point” hypothesis that would alter the focus of current efforts aimed at reversing or inhibiting already established VC in favor of primary prevention of the emergence VC in the first place.

Acknowledgements

This work was supported by a research training fellowship grant supplied by the Health Research Board of Ireland to C.D. (grant reference HPF/2010/30). Additional financial support was provided by a DCU O'Hare Scholarship and a Government of Ireland – Irish Research Council Postgraduate Scholarship to E.H. (grant reference GOIPG/2015/3758).

Disclosure Statement

The authors have no conflicts of interest or additional financial ties to report.

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