Osteoprotegerin upregulates endothelial cell adhesion molecule response to tumor necrosis factor-α associated with induction of angiopoietin-2

Simone H. Mangan, Ann Van Campenhout, Catherine Rush, Jonathan Golledge*

Vascular Biology Unit, Department of Surgery, School of Medicine, James Cook University, Townsville, 4811 Australia

Received 10 October 2006; received in revised form 18 July 2007; accepted 26 July 2007
Available online 2 August 2007
Time for primary review 29 days

Abstract

Objective: Osteoprotegerin (OPG) and osteopontin (OPN) have been identified within unstable atherosclerosis and circulating concentrates have been linked to cardiovascular events. We studied the influence of OPG and OPN on endothelial adhesion molecule expression and monocyte binding.

Methods: Resting or tumor necrosis factor (TNF-α) activated human endothelial cells were incubated with OPG (0, 0.5, 5, and 10 ng/mL) or OPN (0, 2.5, 10 and 50 nmol/L). The expression of endothelial genes and proteins was investigated with the Oligo GEArray microarray series, multiplexed gene expression analysis, flow cytometry, ELISA and immunohistochemistry. Monocyte-binding studies were carried out using fluorescently labeled THP-1 cells and analysed by flow cytometry.

Results: OPG but not OPN stimulated a dose-dependent increase in the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin by endothelial cells in the presence of TNF-α (p ≤ 0.05) which was reflected by enhanced binding of THP-1 monocytes. In the absence of TNF-α, OPG had no significant effect on adhesion molecule expression but upregulated angiopoietin-2. When the induction of angiopoietin-2 was inhibited using interfering RNA the ability of OPG to upregulate adhesion molecules in the presence of TNF-α was abolished. OPN did not effect adhesion molecule expression by resting or activated endothelial cells.

Conclusion: OPG upregulates angiopoietin-2 in human endothelial cells sensitizing them to the effects of TNF-α. These findings suggest a mechanism by which OPG may stimulate inflammation in atheroma and thereby promote the progression and complications of atherosclerosis.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Arteries; Atherosclerosis; Gene array analysis; Gene expression; Inflammation

1. Introduction

Calcification is a frequent finding in advanced atherosclerosis with the process having similarities to bone remodeling [1–3]. A number of bone remodeling cytokines, in particular osteoprotegerin (OPG) and osteopontin (OPN), have been identified adjacent to areas of atheroma calcification [4–6]. The presence of OPG and OPN within atheroma suggests that these cytokines may actively modulate the progression and complications of atherosclerosis. In support of a role for OPG and OPN in atherosclerosis, local atheroma and circulating concentrations of these cytokines have been associated with cardiovascular events in a variety of different patient populations, such as those with hypertension, diabetes and recent cardiac events [6–12].

Evidence supporting a role for OPN in atherosclerosis is also available from experimental studies. In animal models deficiency of OPN inhibits atherosclerosis development...
while upregulation of OPN promotes atheroma progression [13,14]. In addition, in vitro studies have demonstrated an important role for OPN in inflammatory cell chemotaxis [15,16]. Data from experimental studies examining the importance of OPG in atherosclerosis are more controversial than that for OPN [17,18]. Studies in vitro suggest that OPG stimulates changes in vascular smooth muscle cells typically demonstrated in atherosclerosis by promoting apoptosis and matrix metalloproteinase release [17]. In a pro-atherosclerotic mouse model however, deficiency of OPG was associated with enhanced development of atherosclerosis [18].

Accumulation of monocyte-macrophages has been linked to both atherosclerosis progression and plaque rupture and therefore is believed to be an important target for the medical treatment of cardiovascular disease [19–21]. Control of inflammatory cell accumulation is primarily determined by endothelial expression of adhesion molecules [22,23]. At the time of this study the role of OPN in endothelial–monocyte interactions had not been previously examined. We hypothesized that OPG and/or OPN accumulated locally within atheroma or the circulation favoured monocyte-macrophage entry by altering endothelial expression of adhesion molecules. In this study we investigated this hypothesis in vitro.

2. Methods

2.1. Patients

Umbilical cords were collected from consenting mothers (n=30). The study was approved by the ethics committees of the Townsville Hospital and James Cook University and protocol conformed to ethical guidelines of Declaration of Helsinki.

2.2. Materials

Recombinant human OPG was purchased from ImmunoKontact. Endotoxin concentrates were demonstrated to be below detectable limits by the LAL method (<0.1 ng/μg) [24]. We did not exclude LPS contamination by heat-inactivation experiments in addition since mild heat treatment has recently been demonstrated to inactivate 90% of the TNFα inducing ability of LPS and therefore for our experiment would be a poor guide to LPS contamination [25]. OPN, tumor necrosis factor (TNF)-α and interleukin (IL)-1β were purchased from R&D Systems. Specific monoclonal antibodies to vascular cell adhesion molecule (VCAM)-1 (1.4C3), E-selectin (1.2B6), CD31 (JC70A), Von Willebrand Factor (F8/86) and smooth muscle α-actin (1A4), as well as a goat–anti-mouse FITC (F0479) and HRP (P0447) and rabbit anti-goat-HRP (P0160)-conjugated secondary antibody were from DakoCytomation. The monoclonal antibody against intercellular adhesion molecule (ICAM)-1 (R6.5) was kindly donated by Boehringer-Ingelheim and a polyclonal antibody against angiopoietin-2 (N-18) was from SantaCruz Biotechnology.

2.3. Cell culture

We elected to utilize human umbilical vein endothelial cells (HUVECs) rather than commercial cells. This allowed experiments to be carried out on a range of different endothelial cells rather than limiting studies to those of a single donor. HUVECs were isolated from freshly collected human umbilical cords by collagenase (Worthington Biochemical Corporation) digestion and cultured according to the method of Jaffe et al. [26]. Briefly, umbilical cords were rinsed in DMEM (TropBio) and incubated at 4 °C overnight in fungizone (Invitrogen) (10 μg/mL). Umbilical veins were rinsed with hanks balanced salt solution (JRH Biosciences) and perfused with 0.1% pre-warmed collagenase (type I) in PBS and incubated for 16 min at 37 °C. Endothelial cells were collected and cultured at 37 °C/5% CO₂ in EGM-2 culture media (Cambrex Bio Science Australia). Media were changed within 24 h of plating then every 3 days. For experiments, cells were passaged with trypsin–versene (Cambrex BioScience Australia) to 24-well plates. HUVEC purity was ascertained by the typical ‘cobblestone’ morphology of endothelial cells and by immunohistochemistry showing positive staining for Von Willebrand Factor and CD31 and negative staining for smooth muscle α-actin. Cells were used between passages 3 and 5. The monocyte THP-1 cell line was kindly provided by Dr. Rajiv Khanna (Tumor Immunology Laboratory, Queensland Institute of Medical Research) and maintained at 0.5×10⁶ cells/mL in RPMI, supplemented with 25 mM HEPES (JRH Biosciences), antibiotics and 10% fetal bovine serum (FBS) (Invitrogen).

2.4. Cell based ELISA

Preliminary studies to investigate the pattern of TNF-α-induced adhesion molecule expression by HUVECs were carried out by a cell based ELISA as previously described [27]. Briefly, after cytokine treatment HUVEC monolayers were washed with PBS and fixed with freshly prepared 0.5% periodate–lysine–paraformaldehyde buffer for 10 min at room temperature. For antigen detection, 40 μL of monoclonal antibody in PBS/0.05% tween/1% BSA was added to each monolayer and incubated with shaking at room temperature for 1 h, followed by 3 washes with PBS. Monolayers were then incubated with a peroxidase conjugated goat–anti-mouse antibody and washed. The peroxidase substrate 3,3′,5,5′-Tetramethylbenzidine solution (Sigma Australia) was added (100 μL/well) and incubated with shaking in the dark at room temperature for 15 min. The colour reaction was stopped by the addition of 100 μL 2N HCL and the optical density at 450 nm was read in a Sunrise TECAN microplate reader (Q-Lab). To adjust for cell number, wells were washed with tap water and stained with 50 μL 0.08% crystal violet in PBS for 5 min. Cells were washed and the nuclear stain was solubilised with 100 μL 33% acetic acid and the optical density was measured at 595 nm. Time course studies where monolayers were stimulated with TNF-α (300 U/mL) for 0,
6, 8, 12 and 24 h showed that maximal inductions of ICAM-1, VCAM-1 and E-selectin expression were 12, 8 and 6 h respectively (results not shown). Dose–response curves for TNF-α and IL-1β (0–1000 U/mL) were also carried out. Based on these curves, a concentration of TNF-α or IL-1β of 50 U/mL was chosen to activate the monolayers for latter experiments. This concentration was sub-maximal and allowed for the detection of both an up- or downregulation of expression due to other cytokine treatments.

2.5. Flow cytometry

Analysis of adhesion molecule expression was also performed by flow cytometry. After incubation with various

![Graphs showing the effect of incubation of OPG (A) and OPN (B) on unstimulated HUVEC ICAM-1 (i), VCAM-1 (ii) and E-selectin (iii) surface expression. Adhesion molecule levels were measured using cell based ELISA at 6, 8 and 12 h for VCAM-1, E-selectin and ICAM-1, respectively. These incubation periods were based on preliminary time–response curves (see Methods). Results are expressed as OD 450/595 (n = 3). Adhesion molecule expression was not significantly altered. Statistical analysis was by ANOVA.](https://academic.oup.com/cardiovascres/article-abstract/76/3/494/487932)
cytokines cell monolayers were rinsed with cold wash buffer (0.1% BSA/0.1% NaN₃/PBS). Due to reported trypsin sensitivity cells were labeled directly before trypsinisation for the detection of E-selectin (4 μg/mL) and VCAM-1 (4 μg/mL) [28]. For ICAM-1 (10 μg/mL) cells were stained following trypsinisation. Cells were washed twice with wash buffer and stained with a FITC-conjugated secondary antibody. Labeled cells were re-suspended in PBS and stained with propidium iodide (0.5 μg/mL) to detect dead cells. The cell-associated fluorescence of 10000 events per sample was analysed in a FACScan flow cytometer (Becton Dickinson) using the Cell Quest software and measured as mean fluorescence intensity (MFI) in the FL-1 channel. Propidium iodide positive cells were excluded from analysis.

Fig. 2. The effect of co-incubation of OPG and TNF-α on HUVEC ICAM-1 (A), VCAM-1 (B) and E-selectin (C) surface expression. Adhesion molecule levels were measured using flow cytometry at 6, 8 and 12 h for VCAM-1, E-selectin and ICAM-1, respectively. Results are expressed as % of control (±standard error) of 3 independent experiments. Statistical analysis was by ANOVA.

Fig. 3. The effect of co-incubation of OPN and TNF-α on HUVEC ICAM-1 (A), VCAM-1 (B) and E-selectin (C) surface expression. Adhesion molecule levels were measured using flow cytometry at 6, 8 and 12 h for VCAM-1, E-selectin and ICAM-1, respectively. Results are expressed as % of control (±standard error) of 3 independent experiments. Adhesion molecule expression was not significantly altered. Statistical analysis was by ANOVA.
2.6. Monocyte-binding study

a) Monocyte labeling: THP-1 cells were collected and washed three times in serum-free RPMI. Cells (1–5 × 10^6) were re-suspended in serum-free RPMI and stained with calcein-AM (Molecular probes) (10 μM) for 30 min at 37 °C/5% CO2. The staining process was stopped by 3 washes with cold RPMI/10% FBS. b) Monocyte binding: THP-1 cells were re-suspended in RPMI/10% FBS and 0.5 mL of cells were added to rinsed cytokine treated HUVEC monolayers in fresh EGM-2 (10⁶ cells per monolayer). Plates were incubated for 30 min at 37 °C with shaking (75 rpm). Plates were rotated 90° 3 times during the binding step. Wells were washed 4 times with RPMI to remove unbound THP-1s. The remaining cells were removed with 1 mM EDTA/PBS, collected and re-suspended in wash buffer. c) Flow cytometry: Re-suspended cells were analysed as described above with 20,000 events collected. To determine the ratio of THP-1 to endothelial cells, calcein-positive and -negative cells were gated and expressed as a ratio. Controls included labeled and unlabeled THP-1 cells, unlabeled HUVECS and HUVECS incubated with the supernatant of stained THP-1 cells.

Fig. 4. The effect of incubation of TNF-α activated HUVEC monolayers with OPG on the binding of THP-1 moncytic cells. HUVEC monolayers were pre-incubated with TNF-α (50 U/mL) and OPG (0–10 ng/mL) prior to exposure to monocytes for 30 min. The ratio of firmly adherent THP-1 cells to HUVECs was measured by flow cytometry. Results are representative of 1 of 3 independent experiments carried out in triplicate. Monocyte binding is expressed as the ratio of THP-1 cells bound per 1000 HUVECs (±standard error) (A). Parallel wells were fixed and a haematoxylin and eosin stain performed (B) 0 treatment (i), TNF-α (50 U/mL) (ii), OPG (5 ng/mL)/TNF-α (50 U/mL) (iii), OPG (10 ng/mL)/TNF-α (50 U/mL) (iv). Statistical analysis was by ANOVA.
2.7. Immunohistochemistry and haematoxylin and eosin staining

For haematoxylin and eosin staining, coverslips with HUVECs/THP-1 cells attached were fixed in 75% methanol/PBS for 10 min at 4 °C and dried overnight. The coverslips were incubated with haematoxylin (4 min) followed by eosin (2 min) and dehydrated in graded alcohol and mounted with DEPEX (ProSciTech). For angiopoietin-2 staining, cells fixed to coverslips were stained with a polyclonal antibody (1/50) followed by a secondary rabbit anti-goat-HRP (1/100). Staining was developed using DAB (DakoCytomation).

2.8. Preparation of total RNA

Total RNA from endothelial cells stored in RNAlater (Ambion) was extracted using the RNeasy Mini Kit (Qiagen) using QiaShredder (Qiagen) for homogenisation, according to the manufacturer’s instruction. The RNA was eluted in RNAse-free water and the integrity was verified by electrophoresis on a 1.2% agarose gel and was visualized with ethidium bromide staining. The concentration was quantified by UV absorption with a nanodrop spectrophotometer (BioLab Ltd).

2.9. cDNA SuperArray analysis

The Oligo GEArray microarray series (SuperArray Inc.) were used to quantify the expression of 113 genes associated with endothelial cell biology. This technique is highly specific (90% homology) and has an inter-assay coefficient of variation <10%. Briefly 3 μg total RNA was reverse transcribed into Biotin-16-dUTP-labeled cDNA probes with the TrueLabeling-AMP method according to the manufacturer’s instructions. The SuperArray membranes (OHS-015) were pre-hybridized at 60 °C for at least 2 h. Hybridization of the Biotin-labeled cDNA probes to the membranes was carried out at 60 °C overnight with slow agitation in a hybridization oven. The hybridized membranes were washed in saline sodium citrate buffer once in solution I (one in 2× SSC, 1% SCS) and once in solution II (0.1× SSC, 0.5% SDS). For detection, membranes were incubated with alkaline phosphatase-conjugated streptavidin, washed and

Fig. 5. (A) The effect of OPG on HUVEC gene expression detected using SuperArray membranes. Results are representative of 1 of 3 independent experiments carried out. Endothelial cells were incubated with 0 treatment (i), TNF-α (50 U/mL) (ii), OPG (10 ng/mL) (iii), OPG (10 ng/mL)/TNF-α (50 U/mL) (iv). Differences in the expression of genes such as 1, angiopoietin-2; 2, VCAM-1; 3 E-selectin are noted by an arrowhead. (B) Bars and error bars represent mean± standard error of intensities for specific genes from three independent membranes. OPG significantly increased expression of E-selectin and ICAM-2 on activated HUVECs. *p ≤ 0.05.
incubated with the chemiluminescent substrate CDP-Star. Images of the membranes were acquired using the Chemidoc XRS system (Biorad Laboratories) and analysed with the web-based GEArray Expression Analysis Suite software. The relative expression level of each gene was determined by comparing the signal intensity of each gene in the array after correction for background and normalisation.

2.10. Quantitative gene expression (QGE)

A novel gene expression analysis method that combined competitive PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to confirm the results of the membrane arrays. This technique has excellent reproducibility (inter-assay coefficient of variation < 3%) and high sensitivity of 5 copies of a gene in a sample prior to PCR [29]. Total RNA was sent to the Australian Genome Research Facility (AGRF) and the assay was carried out as per the developers protocol which has been previously validated with comparison to other quantitative gene expression analysis methods [30].

2.11. Small interfering RNA (siRNA) studies

Human angiopoietin-2 (RefSeq NM_001147) sense (CCA-GACGGCUGUGAUGAUAtt), antisense (UAUCAUCA-CAGCCGUCUGGtt) and control (Silencer™ control #1) siRNA oligonucleotides were obtained from Ambion. HUVECs were prepared and cultured as described above and transfected in 6-well plates using siPORT Amine transfection reagent (Ambion) according to the manufacturer’s reverse transfection protocol. Trypsinised HUVECs were added at a concentration of 3 × 10^5 cells in 2.3 mL EGM-2 medium (without antibiotics) and transfected using 20 nM siRNA and 10 μl siPORT Amine per well. Growth medium was replaced 8 h after transfection to minimise cytotoxicity. Angiopoietin-2 knockdown was assessed by real-time RT-PCR using total RNA extracted from cells 48 h post-transfection. Cells were used 48 h post-transfection with siRNAs in experiments to

**Table 1**

<table>
<thead>
<tr>
<th>Adhesion molecule (pM)</th>
<th>IL-1β (50 U/mL)</th>
<th>OPG (10 ng/mL) and IL-1β (50 U/mL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>3.38±0.43</td>
<td>3.53±0.19</td>
<td>0.77</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>0.13±0.03</td>
<td>0.19±0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>E-selectin</td>
<td>16.57±2.08</td>
<td>15.90±0.55</td>
<td>1.00</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>1.19±0.54</td>
<td>0.94±0.03</td>
<td>0.08</td>
</tr>
</tbody>
</table>

HUVECs were incubated with IL-1β (50 U/mL) alone or in addition to 10 ng/mL of OPG. Gene expression was assessed by MALDI-TOF MS. Data is presented as mean±standard error and compared by Mann–Whitney U test.
examine the role of angiopoietin-2 in changes induced by OPG in TNF-α activated HUVECs.

2.12. Real-time RT-PCR

Real-time PCR primers for human angiopoietin-2 and GAPDH were designed using PCR Express® primer design software and were as follows: angiopoietin-2 sense (TTCC-TCCTGAGAGATGGGA), angiopoietin-2 antisense (TGCAACAGCATTGGACAGTA); GAPDH sense (GAC-CACCTTGTCAAGCTCATTTCC); GAPDH antisense (GTGAGGTTCTCCTCTTTTCTTGT). One-step SYBR green I-based RT-PCR was performed on the Rotor-Gene 3000 (Corbett Research) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. Briefly, 25 μl reactions contained total RNA (40–400 ng), 500 μM each primer, 0.25 μl QuantiTect RT mix and 1× QuantiTect SYBR Green RT-PCR Master Mix. Cycling conditions were: reverse transcription for 30 min at 50 °C; RT inactivation and polymerase activation step 15 min at 95 °C; quantitative real-time PCR for 45 cycles: 94 °C for 15 s (denaturation), 57 °C for 30 s (annealing), 72 °C for 30 s (elongation). Fluorescence was measured during the elongation phase. Negative controls were included in which template was absent and RNA was tested for DNA-contamination in reactions in which QuantiTect RT mix was omitted. The comparative Ct method was used to detect relative gene expression ratios normalised to the housekeeping gene, GAPDH. A template dilution series using angiopoietin-2 and GAPDH PCR primers, proved that the rate of Ct change versus the rate of target copy change (i.e. reaction efficiency) was identical for both angiopoietin-2 and GAPDH, hence the DDCt method was used to calculate relative gene expression [31]. Amplicons were run as duplicates in separate tubes to allow for quantification of angiopoietin-2 cDNA normalised to GAPDH cDNA. Each reaction contained 40 ng total RNA. The relative angiopoietin-2 expression in angiopoietin-2 siRNA-transfected versus negative control siRNA-transfected HUVECS was estimated using the following formula:

\[2^{-\Delta\Delta Ct}}\text{, where }\Delta\Delta Ct=\left[\text{Ct angiopoietin-2 (angiopoietin-2 siRNA)} - \text{Ct GAPDH (angiopoietin-2 siRNA)} \right] - \left[\text{Ct angiopoietin-2 (control siRNA)} - \text{Ct GAPDH (control siRNA)} \right].\]

Each Ct represents the mean Ct value of sample duplicates, and relative expression was calculated using samples from 2 independent experiments. The percent knockdown by angiopoietin-2 siRNA was calculated as \((1 - 2^{-\Delta\Delta Ct}})\times100.

2.13. Statistical analysis

Each experiment was carried out on HUVECS isolated from at least 3 different cords. Results were expressed as a mean±standard error of percentage of the control for flow cytometry. For dose–response experiments a one-way ANOVA was used to test for statistical significance. For the membrane arrays and QGE, results were expressed as mean and standard error and statistical significance was tested using the Mann–Whitney U test. \(p\leq0.05\) was considered statistically significant. Analyses were performed using SPSS software 13.0 for Windows.

3. Results

3.1. The effect of OPG and OPN on resting endothelial adhesion molecule expression

Incubation of OPN or OPG with non-activated HUVEC monolayers did not influence adhesion molecule expression (Fig. 1).

3.2. Effect of OPG and OPN on endothelial adhesion molecule expression in the presence of TNF-α

HUVEC monolayers were incubated with both TNF-α (50 U/mL) and increasing concentrations of OPG (0.5, 5, 10 ng/mL) or OPN (2.5, 10, 50 nmol/L). Based on preliminary time–response experiments, VCAM-1, E-selectin and ICAM-1 surface expression were measured at 6, 8 and 12 h, respectively. Incubation with OPG stimulated a dose-
dependent upregulation of all three adhesion molecules on TNF-α activated HUVECs (Fig. 2). OPN had no significant effect on adhesion molecule expression (Fig. 3).

3.3. The effect of OPN and OPG on monocyte binding

To further investigate the functional and pathological significance of OPG, monocyte-binding studies were carried out using the THP-1 cell line. Confluent activated HUVEC (TNF-α 50 U/mL) monolayers in 24-welled plates were treated with increasing concentrations of OPG (0, 0.5, 5, 10 ng/mL) for 12 h, prior to incubation with THP-1 monocyte cells for 30 min. The number of THP-1 cells firmly adhered per 1000 endothelial cells was measured by flow cytometry. OPG stimulated a dose-dependent upregulation of THP-1 binding (Fig. 4A) with a 47% increase in bound THP-1 cells in the presence of 10 ng/mL OPG. Parallel experiments of HUVECS grown on cover slips and stained

Fig. 8. Effect of OPG on adhesion molecule expression in TNF-α activated HUVECs transfected with control (i) or angiopoietin-2 targeted (ii) siRNA. HUVECs were transfected with siRNA 48 h prior to incubation with TNF-α (50 U/mL) and OPG (10 ng/mL) for 4 h. Gene expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C), ICAM-2 (D) and angiopoietin-2 (E) was measured by MALDI-TOF MS. In control siRNA-transfected HUVECs OPG induced an upregulation in ICAM-1, VCAM-1, E-selectin, ICAM-2 and angiopoietin-2 expression (*p ≤ .05). However, in endothelial cells transfected with angiopoietin-2 targeted siRNA OPG had no effect on adhesion molecule or angiopoietin-2 expression (p=0.4–0.9).
with haematoxylin and eosin supported this finding (Fig. 4B). OPN had no effect on monocyte binding (data not shown).

### 3.4. The effect of OPG on resting and activated endothelial gene expression

Resting and activated (TNF-α 50 U/mL) HUVECs monolayers were incubated with OPG (10 ng/mL) for 4 h to investigate the effect of OPG on the expression of genes associated with endothelial cell biology. The original images captured for one complete set of membrane arrays are shown in Fig. 5A. This experiment was carried out on 3 separate occasions and the genes that had significant differences between treatments were selected after they were corrected for background and normalised using the interquartile range for the entire set of genes. OPG stimulated significant upregulation of endothelial E-selectin and ICAM-2 expression in the presence of TNF-α but not alone (Fig. 5B).

Further gene expression analysis using MALDI-TOF MS supported a role for OPG in upregulating endothelial cell adhesion molecule expression. OPG significantly upregulated the expression of ICAM-1, VCAM-1, E-selectin and ICAM-2 in TNF-α activated HUVECs (Fig. 6). OPG did not influence the expression of adhesion molecules in IL-1β activated HUVECs (Table 1).

### 3.5. OPG upregulates endothelial angiopoietin-2

We carried out a number of further experiments in order to investigate the mechanisms that might be responsible for the OPG induced upregulation of adhesion molecules in TNF-α activated HUVECs. Firstly we assessed whether OPG had no effect on the expression of TNF receptor subfamily, member 1a (mean expression 35.6±1.9 in OPG treated compared to 41.4±1.4 FM in control cells, n=4, p=0.049) or TNF receptor subfamily, member 1b (mean expression 21.4±1.7 in OPG treated compared to 27.8±3.8 FM in control cells, n=4, p=0.34). Analysis of the SuperArray membranes showed that OPG induced the expression of angiopoietin-2 by resting HUVECs (Fig. 5A iii). The adjusted angiopoietin-2 spot intensity for resting HUVECs was 2.8±0.752 compared to 5.6±0.756 for OPG treated cells, p=0.04. These results were supported by analysis using the MALDI-TOF MS, and similarly indicated a significant 2-fold increase in angiopoietin-2 levels (mean expression 162.3±26.9 in OPG treated HUVECS compared to 85.2±8.9 FM in control cells, n=4, p=0.03). HUVEC angiopoietin-2 protein expression was assessed by immunohistochemistry analysis of HUVECs grown on coverslips and treated with OPG (10 ng/mL). Initial time course studies (3, 6, 8, 10, 12, 24 and 36 h) of OPG treatment were carried out to identify if and when OPG had an effect on angiopoietin-2 protein expression. OPG induced expression at 8 h. Representative images (Fig. 7) show the effect of OPG on HUVEC angiopoietin-2 expression.

Positive staining is seen in a granular pattern in cells exposed to OPG for 10 h (Fig. 7C). This granular pattern has been previously identified to be angiopoietin-2 expressed within Weibel–Palade bodies [32].

### 3.6. Interfering with angiopoietin-2 upregulation by OPG inhibits increased expression of adhesion molecules

We used targeted siRNA to investigate whether angiopoietin-2 was important in the effect of OPG on TNF-α activated HUVECs. Angiopoietin-2 expression was measured by real-time PCR in angiopoietin-2 siRNA- and control siRNA-treated cells. Expression was normalised to GAPDH and reported relative to control siRNA. The relative expression of angiopoietin-2 was 0.3±0.05 and 1.0±0.5 in angiopoietin-2 siRNA- and control siRNA-treated cells, respectively, n=3, p<0.05, which represents a 70% inhibition in expression. Having established that angiopoietin-2 siRNA treatment knocked down angiopoietin-2 expression in HUVECs, we then compared the effect of OPG in angiopoietin-2 siRNA- and control siRNA-treated cells exposed to TNF using quantitative gene expression (Fig. 8A–D). OPG had no influence on the expression of angiopoietin-2 in cells transfected with siRNA targeted at angiopoietin-2 (Fig. 8). Similarly, OPG did not effect ICAM-1, VCAM-1, E-selectin or ICAM-2 expression in TNF-α activated HUVECs transfected with angiopoietin-2 siRNA (Fig. 8). The ability of OPG to upregulate adhesion molecules and angiopoietin-2 in the presence of TNF-α was preserved in HUVECs transfected with control siRNA (Fig. 8).

### 4. Discussion

Both OPG and OPN were originally identified in bone [33,34]. OPN is component of the non-collagenous matrix of bone and has been shown to have important effects on cell migration through its ability to bind a variety of integrins [35]. OPG is critical in the control of bone remodeling through inhibition of osteoclast function [33]. Both proteins have been associated with vascular calcification, atherosclerosis and cardiovascular events [4–12]. Since inflammation plays an important role in the progression and complications of atherosclerosis the effect of OPG and OPN on endothelial adhesion molecules is of particular interest.

In this study we demonstrated that OPG upregulates the expression of a number of adhesion molecules in TNF-α activated HUVECs (Fig. 2). In contrast OPN failed to stimulate such changes. The functional importance of this effect is supported by the ability of OPG to promote monocyte adhesion to TNF-α activated endothelial cells. The effects stimulated by OPG were dose-dependent being evident at 5 ng/mL (Figs. 2 and 4). We have previously demonstrated important effects of OPG at these concentrations on vascular smooth muscle cells, with estimates of tissue concentrations within atherosclerosis and aortic aneuwysm being within this range [17]. The circulating concentrations of OPG measured in patients with atherosclerosis have
varied between 1 and 6 ng/mL, suggesting the concentrations we used in vitro are relevant to the in vivo situation [36–38]. Of note the actual concentrations measured are dependent on the assay utilized which makes it difficult to relate in vivo concentrations accurately to the in vitro situation [38]. Interestingly OPG has previously been demonstrated to promote endothelial survival in vitro, however, this effect was demonstrated at 500–2000 ng/mL, a concentration unlikely to be relevant to the in vivo situation [39].

The effects of OPG on adhesion molecule expression were only evident when endothelial cells were co-activated with the cytokine TNF-α. OPG had no influence on endothelial adhesion molecule expression alone or in the presence of the cytokine IL-1β (Fig. 1 and Table 1). Initially we postulated that OPG might act to upregulate TNF receptors in endothelial cells thereby explaining this co-stimulation effect. We demonstrated that OPG had no effect on the expression of TNF receptor subfamily members 1a and 1b. Instead it appears that OPG acts to sensitize endothelial cells to TNF-α by upregulating angiopoietin-2. Angiopoietin-2 has been previously demonstrated to markedly enhance the endothelial adhesion of monocytes in response to TNF-α [40]. These effects were demonstrated both in vitro using HUVECs and in vivo within a mice model [40]. In the present study we demonstrate that OPG induces a 2-fold upregulation of angiopoietin-2 expression using gene expression analysis. Furthermore transfection of HUVECs with siRNA targeted at angiopoietin-2 abolished the ability of OPG to upregulate adhesion molecules in the presence of TNF-α (Fig. 8). Our findings therefore suggest that OPG facilitates monocyte adhesion by indirectly sensitizing endothelial cells to the influence of TNF-α which then promotes increased transcription of relevant adhesion molecule genes.

Thus our study supports a role of OPG in promoting inflammation, recognized as an important mechanism in the progression and complications of atherosclerosis [19–21]. Both OPG and TNF-α are present within unstable atherosclerosis and therefore would be able to act together on the endothelial cells of vasa vasorum or the arterial lumen [4,6,41]. It should be noted however that the exact role of OPG in atherosclerosis remains controversial [18]. A large body of epidemiological evidence supports an association between OPG and cardiovascular disease and events [4,5,7–10]. In one study carried out in the pro-atherosclerotic apolipoprotein knock-out mouse, deficiency of OPG was associated with increased development of atherosclerosis [18]. It is unclear how this finding in an animal model relates to atheroma progression and complications in humans. The evidence supporting a role for OPN in atherosclerosis is even stronger than that for OPG. In addition to numerous studies demonstrating an association between circulating concentrations of OPN and atherosclerosis presence and subsequent events, animal studies support the ability of OPN to promote atheroma development [6,11–14]. Our study demonstrates that OPN has no influence on the expression of endothelial adhesion molecules and thus OPN likely promotes atherosclerosis via its well demonstrated ability to promote inflammatory cell migration [15,16,42,43].

Our study has a number of limitations which should be noted. Firstly all our studies we carried out in HUVECs in vitro. We utilized HUVECs in our studies as this enabled us to confirm findings in cells extracted from a number of different patients as compared to limiting studies to one commercial cell line. Further studies in other endothelial cells and animal models would support our findings, although information from mice models can be difficult to relate to humans. Secondly, the degree of change in each individual adhesion molecule was quite small with upregulation of between 1.2 and 1.8 fold (Fig. 2). The number of adhesion molecules altered though supports the importance of these findings, which translated to a very significant increase in monocyte adhesion (Fig. 4). Ideally monocyte adhesion experiments should be carried out under laminar flow conditions however we did not have access or experience with these techniques.

In conclusion the findings of this study demonstrate the ability of OPG to stimulate upregulation of adhesion molecules in TNF-α activated endothelial cells suggesting one potential mechanism for the association between OPG and atherosclerosis complications.

Acknowledgements

This project is supported in part by a bursary from the British Journal of Surgery and grant numbers RO1 HL080010-01 from The National Institute of Health and 379600 from the National Health and Medical Research Council Australia. JG is supported by Practitioner Fellowships from the NHMRC, Australia (431503). The authors would like to thank Dr. Rajiv Khanna from the Tumor Immunology Laboratory, Queensland Institute of Medical Research for kindly providing the THP-1 cell line as well as Boehringer-Ingleheim for supplying the monoclonal anti-ICAM-1 antibody. We also thank Drs Maria Nataatmadja and Allison Sutherland from the Department of Medicine, University of Queensland for their assistance with cell culture techniques.

References


