Endocr (2007) 32:52–58
DOI 10.1007/s12020-007-9007-0

ORIGINAL PAPER

Bone morphogenetic proteins regulate osteoprotegerin and its ligands in human vascular smooth muscle cells

Kirsten Q. T. Nguyen · Ping Olesen · Thomas Ledet · Lars Melholt Rasmussen

Received: 19 June 2007 / Accepted: 11 September 2007 / Published online: 29 September 2007

Abstract The bone-related protein osteoprotegerin (OPG) may be involved in the development of vascular calcifications, especially in diabetes, where it has been found in increased amounts in the arterial wall. Experimental studies suggest that members of the TGF-superfamily are involved in the transformation of human vascular smooth muscle cells (HVSMC) to osteoblast-like cells. In this study, we evaluated the effect of BMP-2, BMP-7 and transforming growth factor beta (TGF-β1) on the secretion and mRNA expression of OPG and its ligands receptor activator of nuclear factor-$\kappa\beta$ ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL) in HVSMC. All three growth factors decreased OPG protein production significantly; these results were paralleled by reduced OPG mRNA expression. TRAIL mRNA levels were also decreased. RANKL mRNA expression declined when treated with TGF-β1 but were increased by both BMPs. Members of the TGF-superfamily, i.e. TGF-β1, BMP-2 and BMP-7 exert effects on OPG and its ligands, indicating that these peptides may be involved in the development of vascular calcifications. The downregulation of OPG by these peptides does, however, not suggest that these factors are directly involved in OPG accumulation in diabetes.

Keywords Vascular calcification · Atherosclerosis · Diabetes

K. Q. T. Nguyen · P. Olesen · T. Ledet · L. M. Rasmussen
Research Laboratory for Biochemical Pathology, Aarhus University Hospital, Aarhus, Denmark

L. M. Rasmussen (✉)
Clinical Biochemistry and Clinical Genetics, Institute of Clinical Research, Odense University Hospital, 5000 Odense C, Denmark
e-mail: lmr@dadlnet.dk

Introduction

Linear media calcifications are often seen in diabetes and are known to predict future events of coronary heart disease and total cardiovascular mortality independently [1]. Analyses of vessels with linear and plaque calcification have revealed the presence of osteoblast-like cells, changes in the expression of bone-regulatory proteins as well as osteo- and chondrocytic transcriptional factors, suggesting that these factors could play an active role in the calcification process [2, 3]. However, at present the mechanisms leading to vascular calcifications, for example, in diabetes, are unknown.

Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor family. It is secreted as a decoy protein neutralizing the receptor activator of nuclear factor-$\kappa\beta$ ligand (RANKL), which leads to uncoupling of osteoblast activation and inhibited osteoclast function. In some cells, OPG also prevents cell death induced by tumor necrosis factor receptor-apoptosis inducing ligand (TRAIL) [4]. A study on osteoporosis in OPG-knock-out mice first implicated its role in the pathogenesis of vascular calcification, when mice deficient of OPG developed calcification in the tunica media of the large arteries, a condition that could be prevented by re-establishing OPG transgenically [5].

Several clinical studies support a role for OPG in cardiovascular disease. Elevated serum OPG is related to the presence and severity of both coronary and peripheral arterial disease [6, 7] and a high risk of cardiovascular mortality [8, 9]. More recently increased concentration of plasma OPG has been associated with cardiovascular disease in both type 1 and type 2 diabetic patients [10–12] and Olesen et al. who found OPG accumulation in the aortic tunica media from diabetic individuals, also showed

that it synthesis rate in vascular smooth muscle cells is enhanced by cytokines such as TNF-α and IL-1β, but decreased by insulin [13]. Others have observed increased levels with platelet derived growth factor and angiotensin II [13, 14]. The increase of OPG seen in these studies suggests that the upregulation could be an anti-calcification response to mediators of vascular calcification. The information on such OPG regulators in diabetes, however, is still scant.

Transforming growth factor-beta and the bone morphogenetic proteins (BMPs) belong to the TGF-β-superfamily. TGF-β may mediate development of diabetic nephropathy [15], whereas the BMP family regulate a number of processes in many different tissues [16]. In relation to the vasculature, it has been proposed that both BMP-2 and BMP-7 could be involved in the formation of calcification seen here. More recently increased vascular mRNA expression of BMP-2 and -7 was recently found in murine model of type 1 diabetes mellitus [17].

The mechanisms behind the increased levels of OPG in diabetes are unknown but could be related to development of arterial calcification. We propose that members of the TGF-β-superfamily may be important contributing factors involved in medial calcification possibly by altering the synthesis of OPG and its ligands RANKL and TRAIL in arterial smooth muscle cells.

Materials and methods

Materials

Cell culture reagents: Dulbecco's modified Eagles' medium (DMEM, D5523), endothelial cell growth factor (ECGS, E2759), new born calf serum and L-glutamine were purchased from Sigma Aldrich, Minimum Essential Medium (MEM, 21430) from Invitrogen, ampicillin and fungizone from Bristol–Myers and gentamycin sulphate from Bie & Berntsen, Denmark.

The following recombinant human proteins were supplied from R&D systems: TNF-α (210-TA-10), IL-1β (201-LB), TGF-β_1 (240-B), BMP-2 (355-BM) and BMP-7 (354-BP). TGF-β_1 and the BMPs were reconstituted in sterile 4 mM HCl containing 1% human serum albumin (HSA). Human insulin (Actrapid) was acquired from Novo Nordisk.

Cell culture

Human vascular smooth muscle cells (HVSMC) were established from explants of normal aortic tissue obtained from excess donor vasculature at kidney transplantations [18]. Cells were maintained at 37°C in 5% CO_2 and 95% atmospheric air and at confluence washed with PBS before detachment with 0.125%–PBS diluted trypsin solution (Gibco, 25090-028). Prior to all experiments, the cells were counted in a Bürker Türk chamber before replating, passages from 2 to 5 were used. One day before the experiments, cells were changed to DMEM + 1% HSA. At all time, medias were supplemented with 2 mM L-glutamine, 50 µg/l gentamycin sulphate, 2 µg/l fungizone and 100 µg/l ampicillin.

To establish whether growth factors from the TGF-β-superfamily influence OPG production, HVSMC were treated DMEM + 1% HSA with TGF-β_1 (0.001–10 ng/ml), BMP-2 (1–500 ng/ml) and BMP-7 (1–500 ng/ml). For time-dependent experiments, the following concentrations of TGF-β_1 (1 ng/ml), BMP-2 (10 ng/ml), BMP-7 (100 ng/ml) and TNF-α (5 ng/ml) were used.

OPG secretion per hour was determined by the treating the cells with test substances for 5, 12 or 20 h, washing them gently three times before a final 1 h incubation in DMEM containing only 1% HSA.

Osteoprotegerin protein measurements

Total OPG concentration in the conditioned medium was estimated by a sandwich ELISA (DuoSet, R&D systems, DY805); 96-well plates were coated with mouse anti-human OPG capture antibody (2.0 µg/ml) and detected with 100 ng/ml biotinylated goat anti-human OPG. Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP) and substrate solution (R&D systems, DY999) were applied for detection and visualization, respectively. The colour reaction was stopped with 2 mol/l H_2SO_4, the optical density read at 450 nm on a microplate reader (Bio-Rad) and compared to a duplicate seven point standard curve ranging from 62.5 to 4,000 pg/ml made with recombinant human OPG.

RANKL and TRAIL: estimation of free proteins and proteins in complex with OPG

Free TRAIL was detected with an ELISA (DuoSet, R&D systems, DY375) using 2.0 µg/ml mouse anti-human TRAIL for coating and detection with 50 ng/ml biotinylated goat anti-human TRAIL antibody. The procedures were carried out as indicated previously and values were related to a duplicate standard curve ranging from 24.3 to 1,500 pg/ml.

For the free RANKL ELISA, soluble RANKL in the conditioned medium was captured with 1 ng/ml monoclonal anti-human RANKL antibody, incubated for 2 h with

secondary monoclonal anti-human RANKL (100 ng/ml) (R&D systems, MAB626 and BAF626) and compared to a standard curve (24.3–1,500 pg/ml) made with human recombinant RANKL.

Measurements of RANKL and TRAIL in complex with OPG (RANKL:OPG and TRAIL:OPG) where done by coating plates with the previously mentioned primary antibodies against either RANKL or TRAIL. The samples were incubated for 2 h in room temperature or 37°C, the latter to see if a physiologic temperature would favour complex formation. When this did not seem to be the case, the subsequent sample incubations were carried out in room temperature (data not shown). Polyclonal anti-OPG antibody was used for detection and concentrations of RANKL:OPG and TRAIL:OPG were compared to a normal OPG standard curve.

RNA isolation and RT-PCR analysis

Three millilitres medium containing 35,000 cells/ml HVSMC were seeded in six-well plates. After 5 days the cells were serum-starved and treated test substance for 2, 12 and 24 h. Total RNA was extracted with 500 µl Trizol reagent pr. well and quantified by spectrophotometry at 260 nm. No differences between any of the different experimental situations were observed concerning total RNA amounts.

Reverse transcription was made from 1 µg RNA by incubation with 200 U Superscript III-reverse transcriptase (Invitrogen) in 30 µl reaction cocktail containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.4 mM of each dNTP, 50 pM random hexamers, 1 mM DTT and incubated in a 37°C warm oven for 75 min, followed by 5 min at 99°C.

Real-time PCR was performed using the iCycler iQ system (Bio-Rad). For amplification 2 µl cDNA was mixed with 23 µl reaction solution consisting of 12.5 µl SYBR Green Supermix (Bio-Rad, CA) and 10 pmol human specific primer sets (DNA Technology, Aarhus, DK): OPG forward 5′ GGA ACC CCA GAG CGA AAT AC 3′, reverse 5′ CCT GAA GAA TGC CTC AC 3′; β-actin forward 5′ GTC CAG ACG CAG GAT GGC ATG 3′, reverse 5′ CTA CAA TGA GCT GCG GTG GGC 3′; RANKL forward 5′ TCC CAT CTG GTT CCC ATA AA 3′, reverse 5′ CTT GGG ATT TTG ATG CTG GT 3′; TRAIL forward 5′ GAG AAC CTC TGA GGA AAC CAT T 3′, reverse 5′ TTT CAT GGA TGA CCA GTT CAC C 3′ to a total volume of 25 µl. cDNA from all samples were pooled and diluted to make a relative standard curve (1:1, 1:5, 1:25 and 1:125). Duplicate standards and negative controls (–superscript III) were included in all PCR reactions. The samples were heated to 95°C for 4 min and the cDNA amplified for 35 cycles, each cycle in three steps of 95°C for 30 s, 57°C for 1 min and 72°C for 45 s, respectively. Sample threshold values were calculated with iCycle iQ software and transformed to arbitrary units using the linear standard curve.

Statistics

Statistical analyses were performed in Excel. Differences between groups were tested by one-way ANOVA and Student's *t*-test and a two-tailed *P*-value <0.05 was considered statistically significant. All results are presented as means ± SEM.

Results

Synthesis of osteoprotegerin and its ligands in HVSMC

HVSMC produce high levels of OPG while no free soluble RANKL or TRAIL in the conditioned medium was detected with the specified ELISAs. However, both RANKL:OPG and TRAIL:OPG were present each complex type constituting approximately 10% of total OPG content (data not shown).

Modification of OPG synthesis by growth factors

TGF-β_1 and BMP-2 decrease OPG secretion in a dose-dependent manner (Fig. 1A, B). The influence of TGF-β_1 and BMP-2 was seen with doses as low as 0.1 and 5 ng/ml, respectively, whereas an effect by BMP-7 required a concentration of 100 ng/ml (Fig. 1C) The growth factors also affected the production of OPG time-dependently with a reduction starting 5 h after incubation with BMP-2 ($P < 0.03$) and 12 h for BMP-7 ($P < 0.01$) and TGF-β_1 ($P < 0.004$) that still persisted after 20 hours. TNF-α quickly induced OPG secretion (Fig. 2).

RANKL and TRAIL protein

Although soluble RANKL and TRAIL proteins could not be detected in the conditioned medias, decreased levels of RANKL:OPG were seen with the addition of TGF-β_1. The BMPs also reduced RANKL:OPG (Fig. 3A). Decreased TRAIL:OPG was observed after incubation with TGF-β_1 and BMP-2 but not BMP-7 (Fig. 3B).

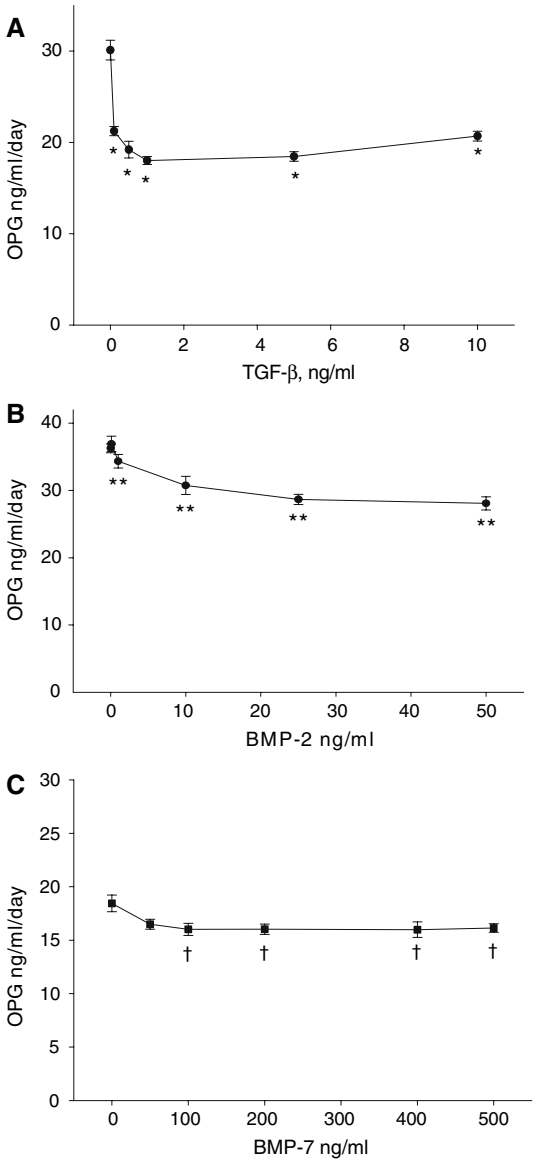

Fig. 1 Dose–response effects of TGF-β_1, BMP-2 and BMP-7 on OPG production in HVSMC. OPG was estimated in conditioned media after treatment of HVSMC with (**A**) TGF-β_1 0, 0.1, 0.5, 1, 5 or 10 ng/ml or low doses (ii) 0, 1, 5, 10, 50 or 100 pg/ml, (**B**) BMP-2 0, 1, 5, 10, 25 or 50 ng/ml or (**C**) BMP-7 0, 50, 100, 200, 300, 400 or 500 ng/ml. Results are presented as means ± SEM (n = 6–8). All values obtained with high TGF-β_1 doses were significantly different from control cells (*$P < 3.2 \times 10^{-6}$). **$P < 0.007$, $^{\dagger}P < 0.04$ compared to untreated cells

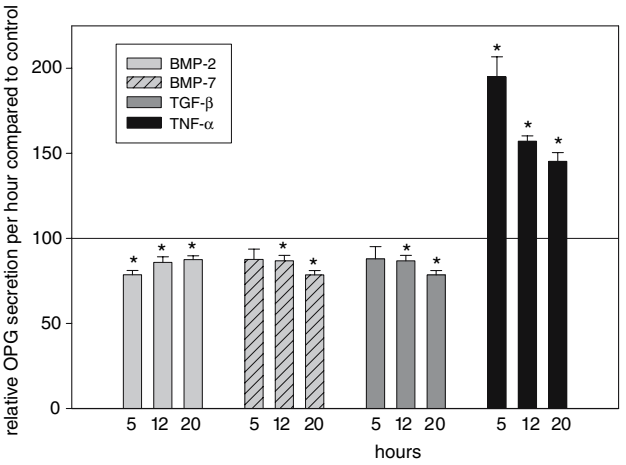

Fig. 2 Time-dependent effects of TGF-β_1, BMP-2 and -7 and TNF-α on OPG secretion in HVSMC. Cells were treated with TGF-β_1 (1 ng/ml), BMP-2 (10 ng/ml), BMP-7 (100 ng/ml) or TNF-α (5 ng/ml) for 5, 12 and 20 h, washed with serum-free medium and then incubated in serum-free medium for another hour. Results are expressed as means ± SEM per hour (n = 12) and are shown as percentage of the values of non-treated cultures at the same time-point indicated by the horizontal line at the 100 mark (*$P < 0.03$)

Regulation of OPG, RANKL, TRAIL and β-actin mRNA expression

As indicated in Fig. 4A, similar changes were observed in OPG mRNA expression. BMP-2 downregulated mRNA after 2 h while the effects of BMP-7 and TGF-β_1 showed a significantly decrease after 12 and 24 h. TNF-α stimulated OPG mRNA expression.

All three growth factors decreased TRAIL mRNA expression significantly starting at 12 h ($P < 0.04$) while mRNA levels were increased 8.2 times by TNF-α (Fig. 4B).

In the case of RANKL expression the properties of the BMPs and TGF-β_1 differed from protein data. Both BMP-2 and -7 elevated RANKL mRNA 2.8 and 2.2-fold, respectively, compared to untreated cells after 12 h incubation ($P < 0.004$). While RANKL mRNA had normalised in BMP-7 treated cells at 24 h, the effect of BMP-2, although on retreat, still persisted ($P = 0.01$). Contrary to the BMPs, TGF-β_1 significantly decreased RANKL mRNA ($P = 0.03$) while TNF-α also induced RANKL expression (Fig. 4C).

The three factors, in particular TGF-β_1, time-dependently upregulated β-actin mRNA as previously seen [19, 20], whereas no effect from TNF-α was observed (Fig. 4D).

Discussion

Osteoprotegerin may play an important role in development of medial vascular calcification in diabetes. Although its role in the vasculature is still unclear, the arterial calcification seen in OPG–/– mice [5] as well as OPG's ability to inhibit warfarin and vitamin-D induced arterial calcification [21], indicate that this bone-related protein might prevent vascular calcification. As the main source for the high amounts of OPG found in the arterial tunica media is likely to be the smooth muscle cells, the upregulated

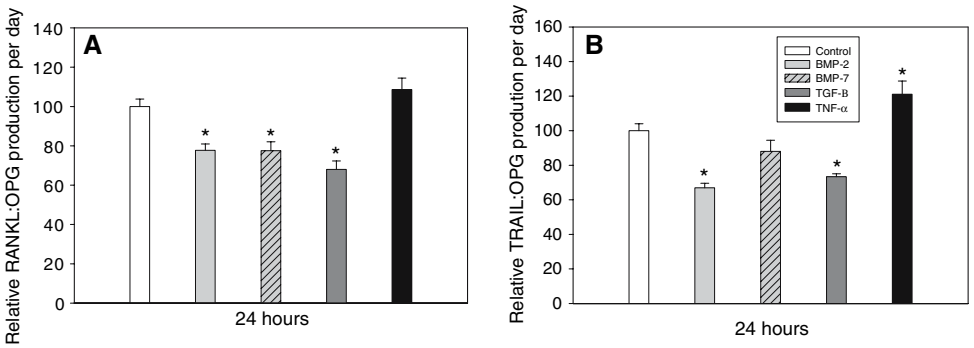

Fig. 3 Effects of the TGF-β-superfamily and TNF-α on (**A**) RANKL:OPG and (**B**) TRAIL:OPG in conditioned HVSMC medium. Complexes of OPG-bound RANKL and TRAIL were estimated by ELISA after treatment with TGF-β_1 (1 ng/ml), BMP-2 (10 ng/ml), BMP-7 (100 ng/ml) and TNF-α (5 ng/ml) for 24 h. Data are presented as means ± SEM (n = 6–19). *$P < 0.02$ and are shown as percentage of the values compared to control

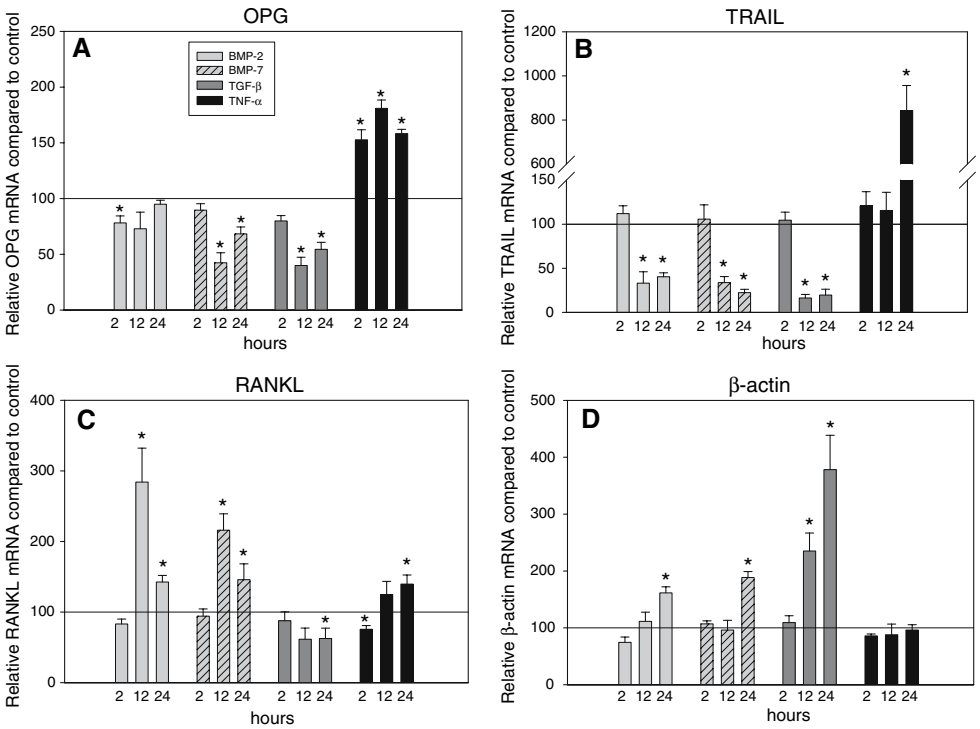

Fig. 4 mRNA expression of (**A**) OPG, (**B**) TRAIL, (**C**) RANKL and (**D**) β-actin after incubation with growth factors and TNF-α for 2, 12 and 24 h. mRNA for the different genes are quantified and standardised by qRT-PCR as stated in Method section. Results are presented as means ± SEM (n = 4–6) and are shown as percentage of the values of non-treated cultures at the same time-point, *$P < 0.04$. No differences in the amounts of harvested total RNA in any situation

synthesis seen in diabetics could be a compensatory response to calcification-inducing factors in the diabetic metabolism such as cytokines, hormones and growth factors.

Although BMP-2 has been found in human atherosclerotic plaques [22, 23] and ossified cardiac valves [24] and TGF-β and BMP-7 have been implicated as respective promoters and inhibitors of diabetic nephropathy, it is only recently that a role for these growth factors has arisen in relation to diabetes-induced large vessel disease. Towler et al. has demonstrated that LDLR–/– mice fed with a high fat diet acquire diabetes as well as concomitant stimulation of aortic BMP-2 and Msx2, a BMP-2 regulated transcription factor that promotes osseous bone formation [23, 25]. Treatment of aortic myofibroblasts with BMP-2 induces Msx2 expression and increased ALP activity just as transduction of Msx2 into the cells does [26]. The authors also have shown that CMV-Msx2 transgenic mice on the same high fat diet develop calcification of the tunica media independently of intimal atheroma formation [27] and propose that the process of vascular calcification in diabetes is associated with a BMP-2 transformation of arterial cells to cells with osteoblasts-like properties. Others have found elevated BMP-2 expression in the aortas of mice with type 1 diabetes [17] while cell culture studies show that BMP-2 expressed in both adventitial and endothelial cells is upregulated by TNF-α, oxidised LDL, hypoxia and vascular endothelial growth factor [28–30]. Based upon

this, we hypothesised that members of the TGF-β-superfamily might contribute to the augmented amounts of OPG by increasing OPG production in vascular smooth muscle cells. Contrary to this expectation, however, what we found that TGF-β_1, BMP-2 as well as BMP-7 decrease OPG gene expression and protein secretion in a both a dose-dependent and time-dependent manner.

To our knowledge, our study is the first study to indicate that the BMPs, a subgroup of the TGF-β-superfamily, regulate OPG in HVSMC. Our finding of a TGF-β_1 effect on OPG production is consistent with prior experiments [31] and furthermore similar to a study of TGF-β in endothelial cells [32] which also showed decreasing OPG mRNA in a dose-dependent manner. BMP-2 and TGF-β_1 have been reported to increase OPG expression and protein levels in murine bone marrow stromal cells and mouse calvarial osteoblasts [33–35]. The discrepancy could be due to differences in cell confluence, differentiation stage and incubation with or without serum. To our knowledge no one has looked at the effect of the BMPs on OPG in other vascular cells.

The role of RANKL in vascular cells is even more unclear than that of OPG. In the study of OPG–/– mice the authors noticed that expression of RANKL and its receptor RANK were only present in calcified arteries. Immunohistochemical staining of non-diseased human arteries show weak RANKL expression confined to the medial smooth muscle cells while higher immonureactivity is found in the extracellular matrix surrounding the calcium deposits of fibrocalcificated plaques [23].

We found expression of RANKL mRNA in normal HVSMC and that RANKL secreted from the cells exists in complex with OPG. Free soluble RANKL is also likely to be present in the conditioned medium but in too small amounts making it undetectable by ELISA. RANKL mRNA was induced by BMP-2, -7 and TNF-α while TGF-β_1 caused a decrease. Again, this is opposite from what has been observed not only in bone-related cells but also in vascular endothelial cells [32, 35]. In contrast to our RANKL mRNA data, we observed a fall in RANKL:OPG, which may seem conflicting at first glance. However, the majority of soluble RANKL exists as the OPG-bound form in equilibrium with small amounts of free RANKL, since we could not detect this form. Thus, the decrease in RANKL:OPG may reflect decreased OPG levels and therefore a lower binding capacity. The amounts of free, active RANKL could thus be increased but merely not assessable with the particular assay that we have employed. Alternatively, RANK (receptor activator of nuclear factor κB) might be concomitantly upregulated by the cytokines which would favour a RANKL:RANK interaction reducing levels of soluble RANKL. Finally, it should be kept in mind that increased amounts of mRNA do not necessarily lead to increased accumulation of the protein. Hence, the absence of increased protein should therefore be interpreted with care.

Osteoprotegerin's other ligand, TRAIL has also been found concurrently with OPG adjacent to apoptotic cells in medial and atherosclerotic calcifications suggesting that it might play a role in arterial calcification [36]. Similarly, treatment of smooth muscle cells with the three growth factors and TNF-α led to TRAIL changes that were associated with changes in OPG expression. Hence, TGF-β_1 and the BMPs downregulated TRAIL mRNA, while levels were increased by TNF-α., TRAIL:OPG concentrations were reduced in parallel with the mRNA changes.

While it is generally accepted that TRAIL induces apoptosis in malignant cells, the role of TRAIL in normal vascular cells is still uncertain. Exogenous TRAIL has been shown to have a cytotoxic effect in both HVSMC and HUVEC [37, 38], an outcome that could be reversed by OPG. On the other hand, in the same cells TRAIL could also promote survival and proliferation [39, 40]. A possible role for the changes in TRAIL by the members of the TGF-β-superfamily and TNF-α therefore remains unclear.

Contrary to our initial hypothesis increased BMPs or TGF-β_1 do not augment concentrations of arterial OPG in HVSMC and are therefore not likely to be directly responsible for the increased amounts seen in diabetics. However, quite interestingly these factors inhibit transcriptional activation and secretion of OPG. Could the mechanism whereby the TGF-β-superfamily members induce vascular calcification be through a decrease of OPG, a putative calcification inhibitor? Further investigation of OPG and BMPs in relation to the arterial wall is required to analyse their role in the development of diabetic macroangiopathy.

Acknowledgements The technical assistance of Anne Lone Larsen is greatly appreciated. The study was supported by the University of Aarhus Research Foundation, the Danish Heart Foundation, NOVO Nordisk Foundation and the Danish Medical Research Council.

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