The RANKL/RANK/OPG Signaling Pathway Mediates Medial Arterial Calcification in Diabetic Charcot Neuroarthropathy

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OBJECTIVE—The receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) signaling pathway (RANKL/RANK/OPG signaling) is implicated in the osteolysis associated with diabetic Charcot neuroarthropathy (CN); however, the links with medial arterial calcification (MAC) seen in people with CN are unclear. This study aimed to investigate the role of RANKL/OPG in MAC in patients with CN.

RESEARCH DESIGN AND METHODS—Enzyme-linked immunosorbent assay and Bio-plex multiarray technology were used to quantify a range of cytokines, including RANKL and OPG in sera from 10 patients with diabetes, 12 patients with CN, and 5 healthy volunteers. Human tibial artery segments were immunohistochemically stained with Alizarin red and human RANKL antibody. Human vascular smooth muscle cells (VSMCs) were also explanted from arterial segments for in vitro studies.

RESULTS—We demonstrate colocalization and upregulation of RANKL expression in areas displaying MAC. Systemic levels of RANKL, OPG, and inflammatory cytokines (interleukin-8, granulocyte colony–stimulating factor) were elevated in those with CN compared with diabetic patients and healthy control subjects. Human VSMCs cultured in CN serum showed accelerated osteoblastic differentiation (alkaline phosphatase activity) and mineralization (alizarin red staining) compared with cells treated with diabetic or control serum ($P < 0.05$). Coincubation with OPG, the decoy receptor for RANKL, attenuated osteogenic differentiation of VSMCs and was independent of a high calcium-phosphate milieu. The accelerated mineralization induced by RANKL and CN serum correlated with nuclear translocation of nuclear factor-κB, a process abrogated by OPG.

CONCLUSIONS—Our data provide direct evidence that RANKL/RANK/OPG signaling is modulated in patients with CN and plays a role in vascular calcification. This study highlights this pathway as a potential target for intervention. *Diabetes* 60:2187–2196, 2011

Vascular calcification is a strong independent predictor of cardiovascular mortality (1). In people with diabetes, medial arterial calcification (MAC) has emerged as a strong predictor of lower limb amputation and cardiovascular mortality (2,3). This may be to the result of an increase in arterial stiffness, pulse wave velocity, and systolic blood pressure, ultimately leading to reduced coronary perfusion and ventricular hypertrophy (4). MAC in people with diabetes is more common in those with peripheral neuropathy, who also display increased bone resorption (osteolysis) (5–7), typically seen in Charcot neuroarthropathy (CN). The signaling pathway of the receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and its decoy receptor osteoprotegerin (OPG) has been suggested as the link between vascular and bone metabolism (8,9). In fact, RANKL has been shown to mediate osteolysis in CN by stimulating osteoclastic differentiation of monocytes/macrophages, an effect that is attenuated by OPG, the decoy receptor (10). This has led to nascent theories implicating RANKL/OPG signaling as the potential pathogenetic basis for CN.

RANKL exists in two biologically active soluble forms secreted by T cells, endothelial cells, or osteoblasts or proteolytically cleaved from cell surfaces. RANKL binds to its target receptor RANK on cell surfaces (including vascular smooth muscle cells [VSMCs]) to generate multiple intracellular signals that regulate cell differentiation, function, and survival (8,11,12). In the vasculature, RANKL is expressed and upregulated in calcifying vascular cells (13) and enhances the recruitment and infiltration of cells that have been shown to stimulate VSMC mineralization (14).

Most of the evidence for a direct role of RANKL/OPG signaling in vascular calcification is derived from animal studies with limited human data. For instance, with the use of VSMCs from rat aorta, RANKL has been shown to increase VSMC calcification via activation of the alternate nuclear factor-κB (NF-κB) pathway (15). However, to enhance translational applications, we extend these data to human VSMCs and use of patient serum.

In diabetic CN, there is osteolysis and simultaneous vascular calcification, potentially leading to amputation (16,17). Therefore, the aim of this study was to determine the role of RANKL/OPG signaling in MAC in diabetic CN by using an in vitro model of vascular calcification.

RESEARCH DESIGN AND METHODS

Approval from the local research ethics committee was granted for the use of human tissue, and procedures were in accordance with institutional guidelines and the Declaration of Helsinki.

Patient serum. CN was confirmed clinically, supported by radiologic features as described previously (18). Serum was obtained via the antecubital fossa from 12 patients with CN at the time of diagnosis of acute stage disease, 10 patients with diabetes, and 5 nondiabetic volunteers, all matched for age, sex, and renal function. Ten milliliters of venous blood was withdrawn aseptically into sterile, heparinized tubes and centrifuged at 3,200 rpm for 15 min at 4°C. The resultant sera were measured in aliquots and stored at −80°C until future use. A recent analysis of patient serum, the Rancho Bernardo study, reported that storage at −70°C for up to 11 years did not affect RANKL or OPG concentrations (19).

RANKL and OPG enzyme-linked immunosorbent assay. Serum RANKL and OPG levels were measured using a sandwich ELISA technique according to the manufacturer's protocol. According to the manufacturer, the intra-assay and interassay coefficients of variation for the soluble RANKL (ampli-sRANKL, BI-20452, lot no. P83C) ELISA assay are 8–9 and 3–6%, respectively, whereas the same values for the OPG assay (OPG, BI-20402 lot no. 873aC) are 4–40 and 7–8%, respectively (Biomedica Gruppe, Vienna, Austria). Absorbance was measured at 450 and 560 nm for the OPG and RANKL, respectively.

Serum cytokine analysis. A Bio-plex angiogenesis panel ("Pro-Human Angiogenic 9-plex plate" catalog number 171-304060) obtained from Bio-Rad Laboratories Inc. (Hercules, CA) was used according to the manufacturer's protocol for analysis of angiopoietin, vascular endothelial growth factor, hepatocyte growth factor, leptin, platelet endothelial cell adhesion molecule or CD-31, and follistatin and two inflammatory cytokines, granulocyte colony–stimulating factor (GCSF) and interleukin-8 (IL-8). The intra-assay and interassay coefficients of variation of the Bioplex angiogenesis assay were ≤15 and ≤25%, respectively, with an accuracy of 70–130%. Data were analyzed using Bio-Plex Manager software version 5.0.

Tissue collection and immunohistochemistry for RANKL expression. Segments of tibial artery were obtained from patients undergoing surgery (amputation) and used for imunohistochemistry and explantation of VSMCs as described previously (20). A panel of internal mammary artery (IMA) (deemed surplus to requirements from patients undergoing cardiac artery bypass graft surgery) was used as noncalcified control artery. Arterial sections were stained using rabbit anti-human RANKL antibody (dilution 1:200 catalog number ab9957; AbCAM, Cambridge, U.K.) and a nonimmune serum (IgG) as negative control as described previously (20).

Explanting and culture of human VSMCs. Human VSMCs were obtained by tissue explantation (21) using smooth muscle cell growth medium obtained from PromoCell (PromoCell GmbH, Heidelberg, Germany) and subsequently maintained in regular growth media (Dulbecco's modified Eagle's medium, high glucose, 4.5 g/L) containing 5% FBS, 10 mmol/L sodium pyruvate, 1.8 mmol/L $CaCl_2$, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were used between passages three and eight.

Induction and determination of calcification. VSMC mineralization was induced using a modification of the method described by Petrova et al. (22). Briefly, VSMCs were seeded in a 24-well plate at densities of 5×10^4 cells/cm^2 and cultured in regular growth media until they reached 80% confluence. Cells were then switched to osteogenic media, i.e., regular growth medium containing 2.6 mmol/L $CaCl_2$, and 5 mmol/L β-glycerophosphate for up to 7 days. Cells were treated with 5% serum from *1*) healthy volunteers, *2*) diabetic patients, or *3*) patients with CN. Recombinant OPG (20 pmol/L) (catalog number GF120; Millipore Corporation, Hayward, CA) was added in a parallel set of experiments when cells were changed to osteogenic media and subsequently added each time the medium was replaced, as were vehicle control subjects. All experiments were performed in triplicate using three populations of VSMC, each from different patients. Alkaline phosphatase (ALP) activity assays and Alizarin red staining were carried out as described previously (20,21).

Immunocytochemical localization of NF-κB. Cells were seeded at a density of 5×10^4 cells/cm^2 into four-well chamber slides (Laboratory-Tek Chamber Slides, Fisher Scientific UK LTD, Leicestershire, U.K.). At confluence, they were subcultured for 1 h in osteogenic medium (OM) containing 5% Charcot serum, 20 pmol/L human recombinant soluble RANKL, and 20 pmol/L human recombinant soluble RANKL plus 20 pmol/L human recombinant OPG. Cells were fixed in 4% paraformaldehyde, blocked in 5% goat serum/PBS and followed by incubation with primary rabbit anti–NF-κB antibody (1:500 dilution in 5% goat serum/PBS) and an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Invitrogen, Paisley, U.K.). Rabbit IgG was used as intra-assay negative control. Cells were counterstained with 4 µg/mL DAPI and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Statistical analysis. Each experiment was performed in triplicate with three different populations of human VSMCs explanted from different patients. Data were expressed as mean ± SD for the ELISA, Bio-plex, and cell culture assays (optical density [OD] and ALP activity). Differences between groups were analyzed using an unpaired Student *t* test (two groups) or a one-way ANOVA (three or more groups) as appropriate. Where multiple samples were compared using ANOVA, a post hoc Bonferroni analysis was performed to enable comparison between groups. Data that were not normally distributed were log-transformed before performing statistical tests. A *P* value < 0.05 was considered statistically significant.

RESULTS

The general clinical characteristics of the different patient groups are presented in Table 1.

TABLE 1
General characteristics of study patients

	Charcot	Diabetes	Healthy control subjects
Age (years, mean ± SD)	56 ± 8	58 ± 12	52 ± 14
Sex (male:female)	7:5	6:4	3:2
Type 1: type 2 diabetes	2:10	1:9	—
Time since onset of diabetes (years)	19 ± 10	21 ± 12	—
eGFR (mL/min)	53 ± 20	48 ± 29	52 ± 12

eGFR, estimated glomerular filtration rate.

Localized RANKL expression and medial arterial calcification. To determine whether local RANKL expression was upregulated in calcified versus noncalcified arteries, sections of calcified tibial artery and noncalcified IMA were stained immunohistochemically with anti-human RANKL antibody and Alizarin red. Figure 1 shows a representative section of calcified human tibial artery and a noncalcified IMA. The tibial artery displayed both medial and intimal calcification with positive RANKL staining detected within the vicinity of the calcified areas (B1, B2, and B3; C1 and C2). There was a distinct lack of positive RANKL staining in noncalcified areas (D1 and D2) and control IMA segments (A1, A2, and A3).

Serum OPG and RANKL levels in patients with CN, diabetic patients, and healthy control subjects. ELISA was performed to determine serum levels of RANKL and OPG in the two patient groups and control subjects. Serum OPG levels were higher in patients with CN (8.2 ± 2.7 pmol/L, ANOVA: *P* = 0.031) compared with diabetic patients (7.7 ± 3.3 pmol/L) and healthy control subjects (4.2 ± 0.7 pmol/L) (Fig. 2*A*). The Bonferroni post hoc test showed that OPG levels were higher in patients with CN versus control subjects (*P* = 0.01) and in diabetic patients versus control subjects (*P* = 0.029), but not significantly different between patients with CN and patients with diabetes (*P* = 0.638). In a similar trend, RANKL levels in patients with CN were 0.3 ± 0.042 pmol/L, approximately 8- and 15-fold higher than in patients with diabetes (0.04 ± 0.05 pmol/L) and healthy control subjects (0.02 ± 0.01 pmol/L), respectively (ANOVA test, *P* = 0.055). The Bonferroni test showed that patients with CN had higher serum levels of RANKL than diabetic patients (*P* = 0.033) or healthy control subjects (*P* = 0.063), but there were no significant differences between diabetic patients and control subjects (*P* = 0.895) (Fig. 2*B*). Most important, the RANKL/OPG ratio was significantly higher in patients with CN ([36.8 ± 43.1] × 10^{-3}, ANOVA *P* = 0.033) compared with those with diabetes ([5.2 ± 4.9] × 10^{-3}) or healthy control subjects ([4.6 ± 3.1] × 10^{-3}) (Fig. 2*C*). The Bonferroni test showed that the RANKL/OPG ratio was higher in patients with CN than in diabetic patients (*P* = 0.019) and in patients with CN than in control subjects (*P* = 0.051), but differences between diabetic patients and control subjects were not significant (*P* = 0.974).

Serum protein analysis of inflammatory and angiogenic cytokines. To determine whether an inflammatory milieu/stimulus may be modulated in patients with CN and thus driving elevated RANKL expression and subsequent secretion into systemic circulation in this group, we analyzed a panel of nine cytokines using a Bio-plex multiarray suspension assay (Fig. 3). We demonstrated that two
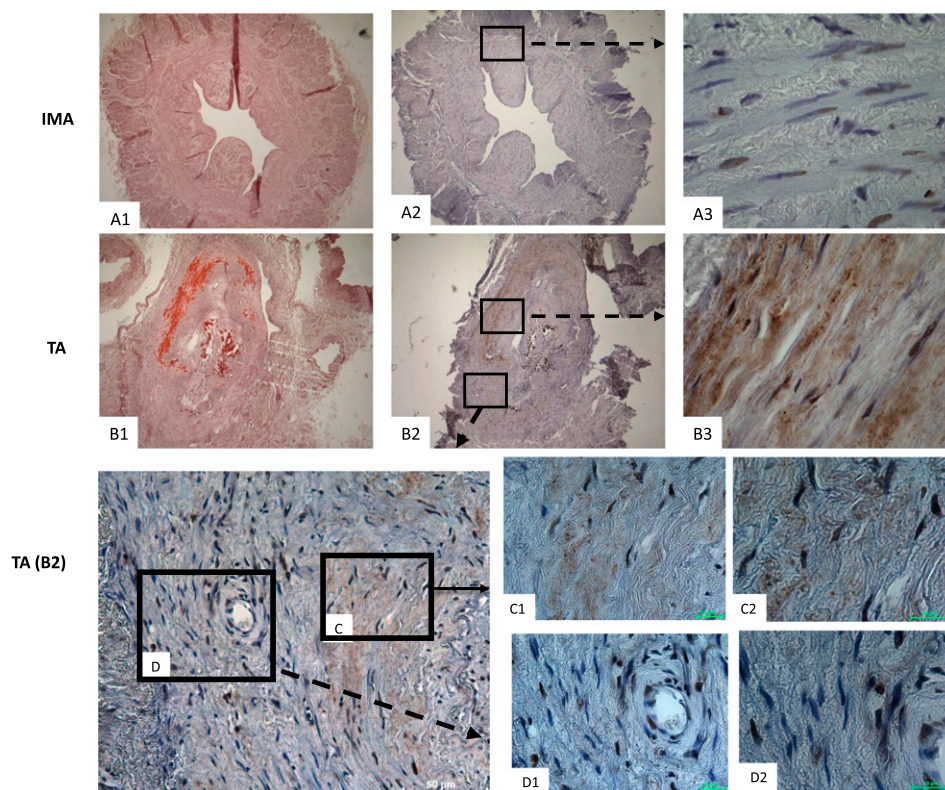
FIG. 1. Representative phase-contrast photomicrographs. Paraformaldehyde-fixed paraffin-embedded sections (7 μm) from control IMA (A1) and diseased tibial artery (TA) (*B1*) were stained with Alizarin red. *A1, A2, B1*, and *B2* were viewed at magnification ×5; *A3* and *B3* were viewed at magnification 63; white scale bar in image FA (*B2*) = 50 μm; green scale bars in *C1, C2, D1*, and *D2* = 10 μm. Calcified areas of FA are colored red, including both medial and intimal calcifications, whereas the IMA control shows a negative stain. Immunohistochemical stain of a contiguous section of the same artery shows upregulated RANKL expression (brown stain) in the vicinity of calcified areas of the tibial artery (*B2* and *B3*) but negative staining of the control IMA (*A2* and *A3*). Within the same TA, calcified areas (*C, C1*, and *C2*) showing upregulated RANKL expression are found adjacent to noncalcified areas (*D, D1*, and *D2*). (A high-quality digital representation of this figure is available in the online issue.)

proinflammatory cytokines (GCSF and IL-8) were significantly higher in patients with CN compared with diabetic patients and control subjects. This may well reflect an elevated inflammatory environment in patients with CN acting as the stimulus for elevated RANKL secretion. The serum levels of noninflammatory cytokines were not significantly different among the patients with CN, compared with patients with diabetes, and control patients.

Charcot serum accelerates osteogenic differentiation and mineralization of VSMCs. Figure 4*A* shows results from the culture of human VSMCs at day 7 under the different conditions. Cells treated with Charcot serum displayed increased calcification as evidenced by Alizarin red staining. After dye elution and quantification, there were overall differences (ANOVA: $P < 0.0001$) in the extent of mineralization between the different culture conditions (Fig. 4*C*). Mineralization was higher in human VSMCs coincubated in Charcot serum (OD = 0.16 ± 0.01 vs. OM control subjects: 0.09 ± 0.003, $P = 0.0003$) compared with diabetic serum (OD = 0.15 ± 0.01 vs. OM, $P = 0.0004$) or healthy serum (OD = 0.14 ± 0.03 vs. OM, $P = 0.037$) (Fig. 4*C*).

Likewise, one-way ANOVA test showed that the early osteogenic differentiation marker, ALP, was significantly different ($P < 0.0001$) in human VSMCs subcultured in any of the three patient sera compared with OM that contained FBS (same concentration) in place of patient serum. The effect was most marked in Charcot than in diabetes serum.

At day 7, the ALP activity of human VSMCs cultured in Charcot serum (49.9 ± 7.3) was approximately twofold higher than that induced by diabetes serum (29.8 ± 3.7 μmol/L phosphate/mg protein/min, $P = 0.013$) and threefold higher than either healthy serum (17.6 ± 10.1, $P = 0.011$) or OM control subjects (15.3 ± 5.4 μmol/L phosphate/mg protein/min, $P = 0.003$) (Fig. 4*B*).

Charcot serum and RANKL induce human VSMC mineralization in the absence of a high calcium phosphate milieu. To establish whether factors in Charcot serum or RANKL triggered the mineralization process per se or needed the presence of the elevated calcium and phosphate in the osteogenic conditions, cells were grown in ordinary growth medium to which the Charcot serum or RANKL was added. The VSMCs were shown to have a greater capacity to mineralize when cultured in the presence of Charcot serum (OD: 0.07 ± 0.003, $P = 0.0009$) or recombinant RANKL (OD 0.072 ± 0.003, $P = 0.0002$) compared with ordinary growth medium only (OD = 0.059 ± 0.002) (data not shown). The high levels of mineralization detected in the VSMCs cultured in the presence of Charcot serum or recombinant RANKL were similar to those induced by OM (OD = 0.075 ± 0.002).

OPG attenuates Charcot serum-induced osteoblastic differentiation of VSMCs. The anti-RANKL effect of OPG on VSMC osteoblastic differentiation and mineralization induced by Charcot and diabetes sera is shown in Fig. 5*A–C*. Compared with VSMCs subcultured in Charcot
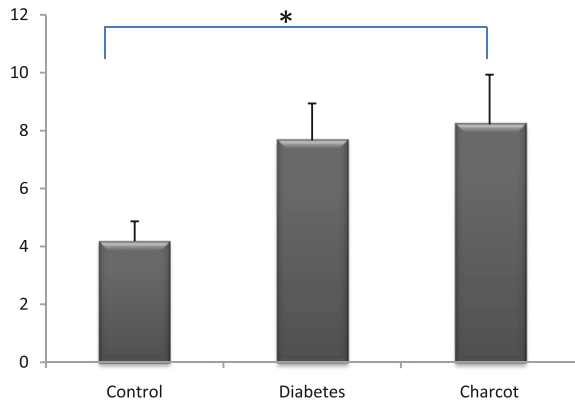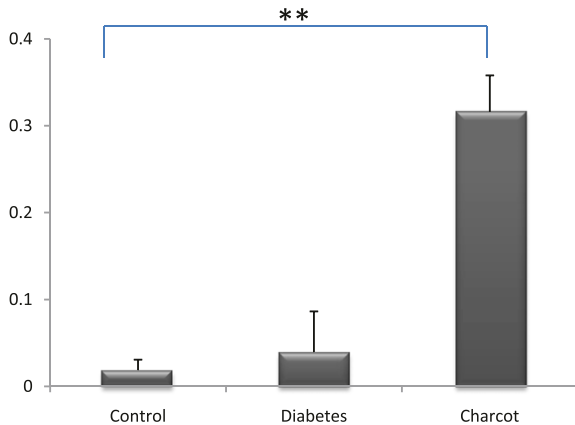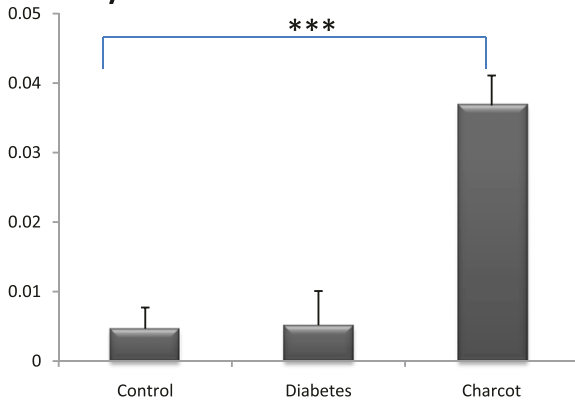
A OPG

B RANKL

C RANKL/OPG ratio

FIG. 2. ELISA kits (Oxford Biosystems, Oxford, U.K.) were used to determine levels of OPG and RANKL in the serum of patients with CN ($n = 12$), diabetic patients ($n = 10$), and nondiabetic control subjects ($n = 5$). OPG and RANKL levels are expressed in picomoles per liter. As shown, OPG and RANKL levels and the RANKL/OPG ratio were elevated in patients with CN compared with diabetic patients and nondiabetic control subjects (*$P = 0.031$, **$P = 0.055$, and ***$P = 0.033$, respectively). (A high-quality color representation of this figure is available in the online issue.)

serum alone, coincubation of VSMCs with Charcot serum plus OPG resulted in an attenuation of osteoblastic differentiation (ALP activity: 49.9 ± 7.3 vs. 12.2 ± 8.6, $P = 0.004$) and mineralization (OD: 0.16 ± 0.01 vs. 0.13 ± 0.003, $P = 0.018$) of these cells (Fig. 5B and C).

However, VSMCs coincubated with diabetes serum and OPG showed only a trend toward less mineralization

(OD = 0.14 ± 0.004) and reduced osteoblastic differentiation (ALP activity, 20.9 ± 10.9) compared with those cultured in diabetes serum alone (Fig. 5B and C); the difference failed to achieve statistical significance.

Charcot serum and RANKL induce mineralization by NF-κB nuclear translocation in vitro. To establish the downstream effects of Charcot serum or RANKL on transcription factors that may be involved in the mineralization of human VSMCs, we investigated the activation of the NF-κB pathway. VSMCs were subcultured for 1 h in Charcot serum (5% v/v) or recombinant RANKL (20 pmol/L) and stained immunocytochemically for the p65 component of NF-κB to detect its intracellular localization. Representative photomicrographs obtained after immunofluorescent staining for NF-κB are shown in Fig. 6A and B. After 1 h of culture of VSMCs in osteogenic media to which Charcot serum was added, NF-κB translocated from its cytoplasmic location to a predominantly nuclear and perinuclear location (Fig. 6A, images D and G). When VSMCs were incubated with recombinant RANKL in lieu of Charcot serum, nuclear translocation of p65 component of NF-κB was equally observed (Fig. 6B, images D and G).

OPG blocks Charcot and RANKL-induced translocation of NF-κB. To validate that the RANKL/OPG signaling involved translocation of NF-κB during mineralization of VSMCs, immunostaining was performed on subcultures of VSMCs (Charcot or RANKL) coincubated with OPG. The presence of OPG abrogated the NF-κB nuclear translocation induced by Charcot serum (Fig. 6A, images E and H) or recombinant RANKL (Fig. 6B, images E and H), thus confirming that NF-κB activation induced by Charcot serum directly involved RANKL/RANK/OPG signaling.

DISCUSSION

Our results have demonstrated that RANKL/RANK/OPG signaling is implicated in human MAC in people with diabetes and CN. The RANKL immunolocalization data suggest a direct association between localized tissue expression of RANKL and MAC. Patients with CN also displayed high levels of inflammatory cytokines (IL-8, GCSF), OPG, RANKL, and particularly elevated RANKL/OPG ratio compared with diabetic patients or healthy volunteers. The high systemic level of RANKL enables it to bind to its receptor, RANK, and drive vascular smooth muscles cells into an osteoblastic differentiation pathway resulting in accelerated deposition of a mineralized matrix leading to MAC. This differentiation process is mediated by RANKL-induced translocation of NF-κB from the cytoplasm into the nucleus (Fig. 6A and B), leading to downstream nuclear mechanisms culminating in upregulated osteoblastic genes and increased ALP activity (Fig. 4B). The specific decoy receptor for RANKL, OPG, abrogates the osteoblastic differentiation of human VSMCs.

It should be emphasized that this was an in vitro study, and we did not seek to correlate the clinical stage of CN to the extent of MAC or the serum level of RANKL/OPG. This is an obvious limitation that will be addressed through future correlative clinical studies.

High serum levels of inflammatory cytokines and RANKL/OPG ratio expressed in patients with CN. The bone changes seen in CN are customarily thought to be triggered by events that enhance an inflammatory milieu, inducing monocytes into an osteoclastic differentiation program (10,22,23). We show that IL-8 and GCSF are two proinflammatory cytokines that are elevated in the
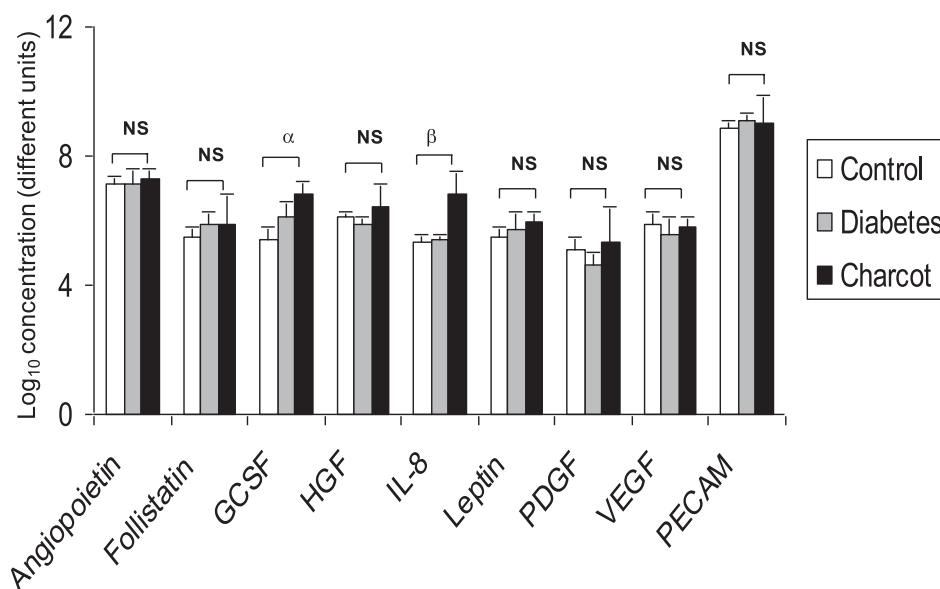
FIG. 3. Bio-plex angiogenesis panel (catalog number171–304060), obtained from Bio-Rad Laboratories Inc. (Hercules, CA), was used to assay various cytokines in the serum of patients with CN, diabetic patients, and nondiabetic control subjects, using Luminex suspension array technology. Results were log-transformed before ANOVA test was performed. Patients with CN displayed higher levels of the inflammatory cytokines (GCSF and IL-8) compared with diabetic patients and nondiabetic control subjects. ANOVA tests represented by α: GCSF $P = 0.002$, β: IL-8 $P < 0.0001$. HGF, hepatocyte growth factor; NS, not significant; PDGF, platelet-derived growth factor; PECAM, platelet endothelial cell adhesion molecule.

serum of patients with CN. Inflammatory cytokines induce activated T cells to secrete RANKL (24,25), and attendant to this, OPG levels increase to counteract the effects of RANKL (26). Figure 2 illustrates that both RANKL and OPG levels are higher in patients with CN. The current study is the first to provide data on systemic levels of RANKL and OPG in patients with CN. There is evidence in the literature reporting elevated levels of RANKL and OPG in other disease conditions associated with high bone turnover and vascular calcification, notably rheumatoid arthritis (27,28), Paget's disease (29,30), and osteoporosis (31–33). In particular, most studies reveal that the RANKL/OPG ratio is the more significant predictor of these disease conditions. Our data showing that the ratio of RANKL/OPG is significantly higher in patients with CN than in diabetic patients or nondiabetic control subjects (Fig. 2C) support these findings. Whether there is a cutoff ratio that may be diagnostic of CN or the ratio can be used to monitor disease activity remains to be elucidated. However, the high levels of the RANKL/OPG ratio can be anticipated to orchestrate deleterious effects on the vasculature as discussed below.

Local expression of RANKL in the vicinity of MAC. Local RANKL expression within the medial layer of the vasculature coincided with areas of MAC. Intense RANKL staining was observed in the vicinity of calcified areas confirmed by alizarin staining and a lack of staining in the negative tissue control (shown here as IMA) and the nonimmune IgG intra-assay control (results not shown). These findings agree with and support results from previous studies demonstrating increased RANKL protein and mRNA expression patterns in MAC (Monckeberg's sclerosis) (34,35). Schoppet et al. (35) also found that OPG was predominantly adjacent to areas of calcification and areas of apoptosis throughout the whole circumference of their arterial specimens. However, the data for OPG immunostaining are less consistent in the literature. A

study by Kaden et al. (13) on calcific aortic stenosis confirmed the positive correlation between RANKL immunostaining and calcification, but showed an inverse relationship between OPG immunostaining and calcified aortic stenosis. However, in the study by Kaden et al., decalcification was performed by placing tissue sections for 48 h in 270 mmol/L EDTA, a protocol that has not been replicated elsewhere and that may affect epitope exposure.

Charcot serum and RANKL accelerate mineralization and osteoblastic differentiation of VSMCs via NF-κB nuclear translocation. Our data demonstrate that explanted resident VSMCs undergo an osteogenic differentiation when cultured in media containing Charcot serum (Fig. 4B), a milieu that we show contains a high RANKL/OPG ratio (Fig. 2C). Our data suggest that systemic OPG is increased in patients with CN probably in response to and to mitigate the effects of high levels of RANKL. It is worth noting that although both OPG and RANKL levels were higher in diabetic patients compared with healthy control subjects, the RANKL/OPG ratio was not different between these two groups. It is therefore likely that elevated OPG levels in diabetic patients do compensate for elevated RANKL levels such that the ratio remains comparable to that of nondiabetic control subjects.

To further support the hypothesis that RANKL mediates MAC in patients with CN, immunocytochemical studies reveal that both RANKL- and Charcot-induced differentiation of human VSMCs are associated with the translocation of NF-κB from the cytoplasm into the nucleus (Fig. 6).

Of note, RANKL and Charcot serum enhanced mineralization of human VSMCs even in nonosteogenic conditions, i.e., normal growth medium without any added β-glycerophosphate or calcium. This finding refutes commonly held suggestions that MAC is merely a direct precipitation of inorganic (calcium and phosphate) ions when their levels are high in systemic circulation. Our data provide
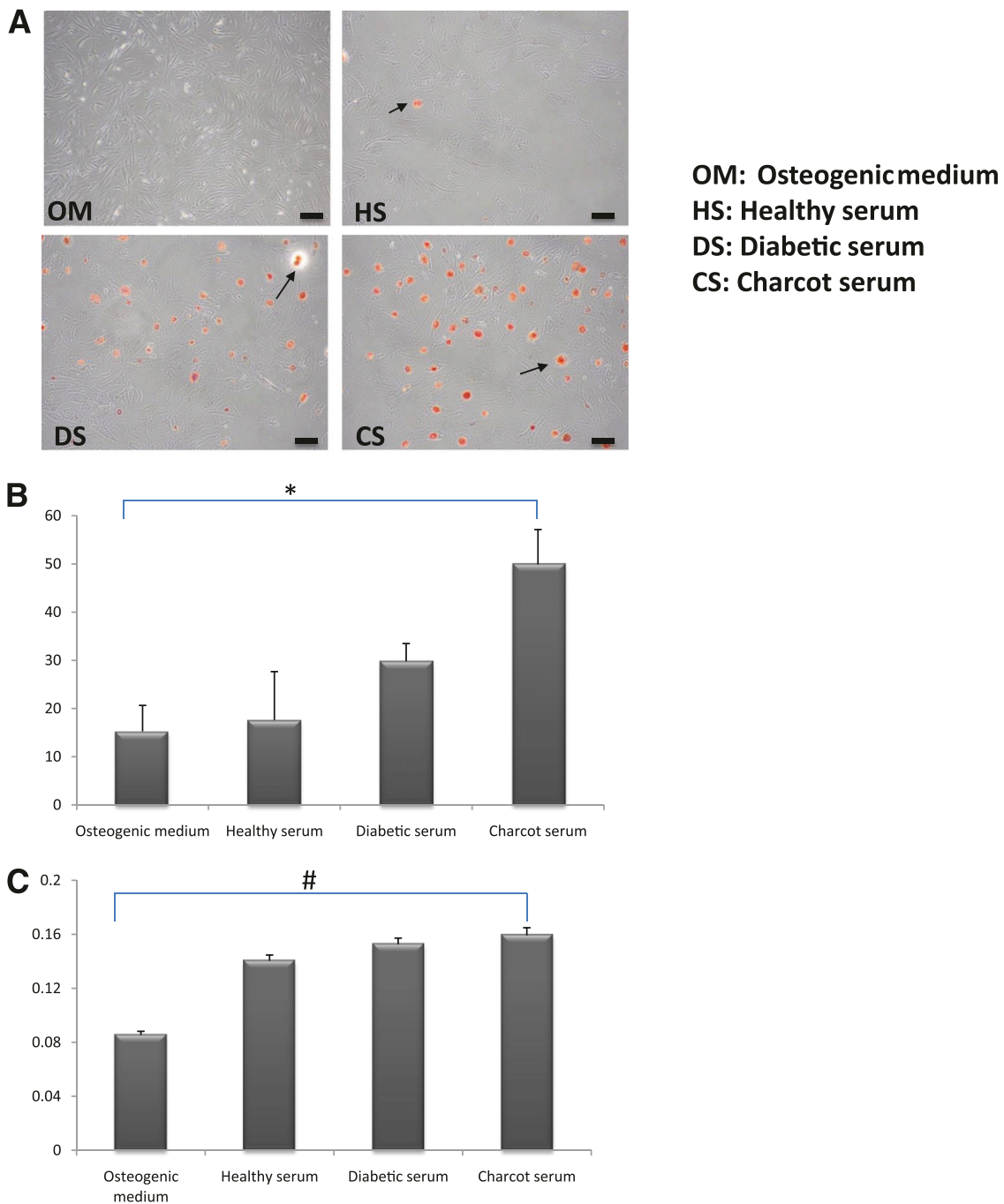
FIG. 4. *A*: Representative phase-contrast photomicrographs of human VSMCs cultured for 7 days in OM, OM plus healthy serum, OM plus diabetic serum, and OM plus Charcot serum. Mineralization was assessed by the Alizarin red method, which stains mineralized nodules orange-red. Black scale bar = 100 μm. After day 7, the number and density of mineralized cells (*black arrows*) were greater in Charcot serum compared with diabetic and healthy serum or OM control subjects. *B*: ALP (μmol/L phosphate/mg protein/min) was used as a marker of early osteoblastic differentiation. Total protein was determined by bicinchoninic acid assay, and the para-nitrophenyl phosphate method was used to quantitate ALP activity. ALP activity was markedly elevated in human VSMCs cultured with Charcot serum compared with diabetic serum, healthy serum, or osteogenic control medium. *ANOVA test for overall differences, *P* < 0.0001. *C*: Alizarin dye was eluted from human VSMCs, and the amount of dye uptake was used to quantify mineralization by measuring optical densities at 414 nm. Human VSMCs cultured in Charcot serum displayed an accentuated mineralization response compared with diabetic serum, healthy serum, or osteogenic control medium. #ANOVA test for overall differences *P* < 0.0001. CS, Charcot serum; DS, diabetic serum; HS, healthy serum. (A high-quality digital representation of this figure is available in the online issue.)

direct evidence of mineralization of human VSMCs in vitro by using nonosteogenic culture conditions. In fact, vascular calcification is an intricate and highly organized process involving cell differentiation, matrix deposition, and secondary ossification resulting in the formation of hydroxyapatite. In this study, we show that the cells are not only depositing a mineralized matrix but also actually switching their phenotype into osteoblastic cells as suggested by the elevated ALP activity, which is a marker of early osteogenic differentiation.

There is some controversy regarding the origin of cells that undergo osteogenic differentiation in MAC. Although some authors have suggested that circulating osteogenic precursor cells migrate from the blood through the vessel
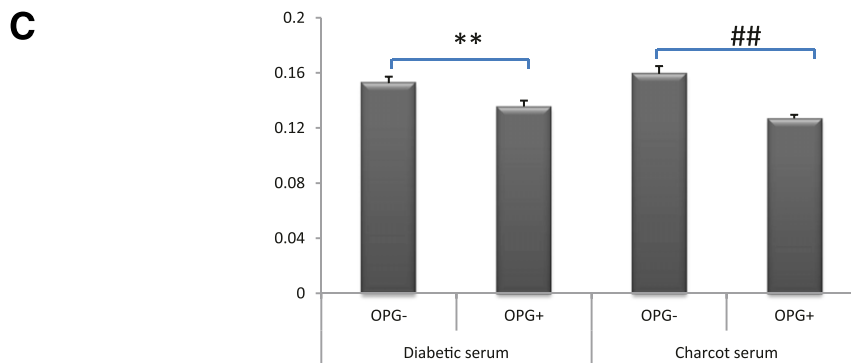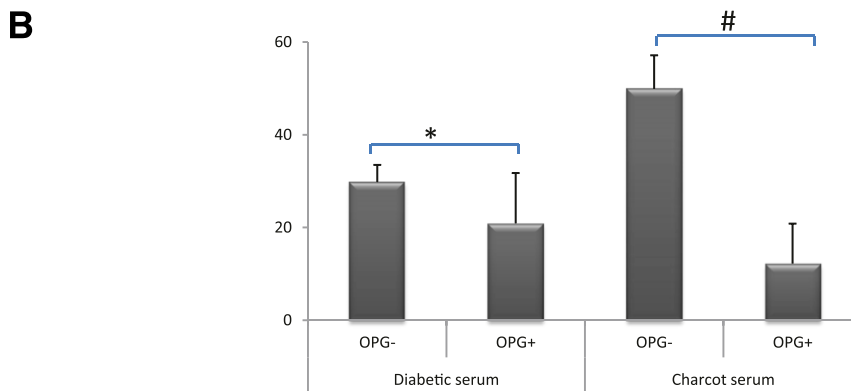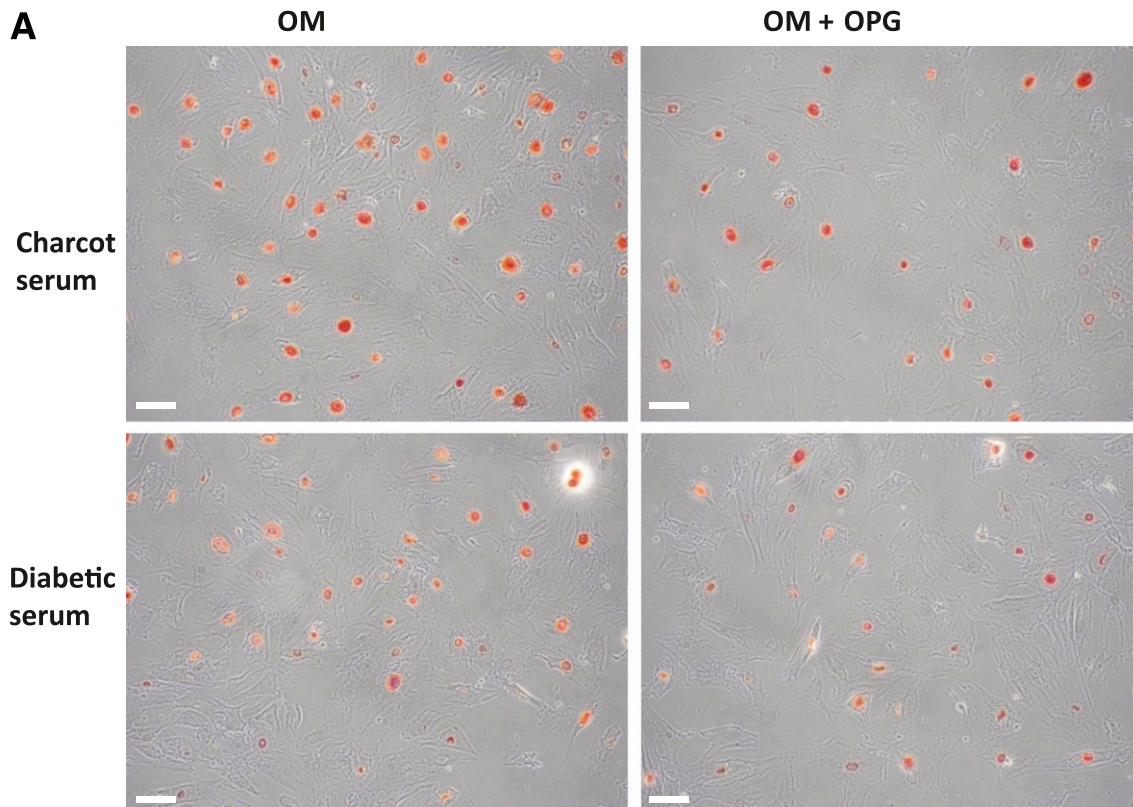
FIG. 5. *A*: Representative phase-contrast photomicrographs of human VSMCs at day 7 in culture for 7 days. Cells were cultured in OM and Charcot (5%) or diabetic (5%) sera (*left*) or 20 pmol/L recombinant OPG in addition to the same sera (*right*). The orange-red spots indicate mineralized nodule formation. White scale bar = 100 µm. *B*: ALP activity (µmol/L phosphate/mg protein/min) of cells at day 7. The inhibitory effect of OPG on the osteoblastic differentiation of human VSMCs is more marked in Charcot serum (#*P* = 0.011) compared with diabetic serum (*P = 0.120). *C*: Extent of mineralization of human VSMCs at day 7. The inhibitory effect of OPG on human VSMC mineralization is markedly significant in Charcot serum (##*P* = 0.004) but not significant in diabetic serum (**P = 0.249). (A high-quality digital representation of this figure is available in the online issue.)
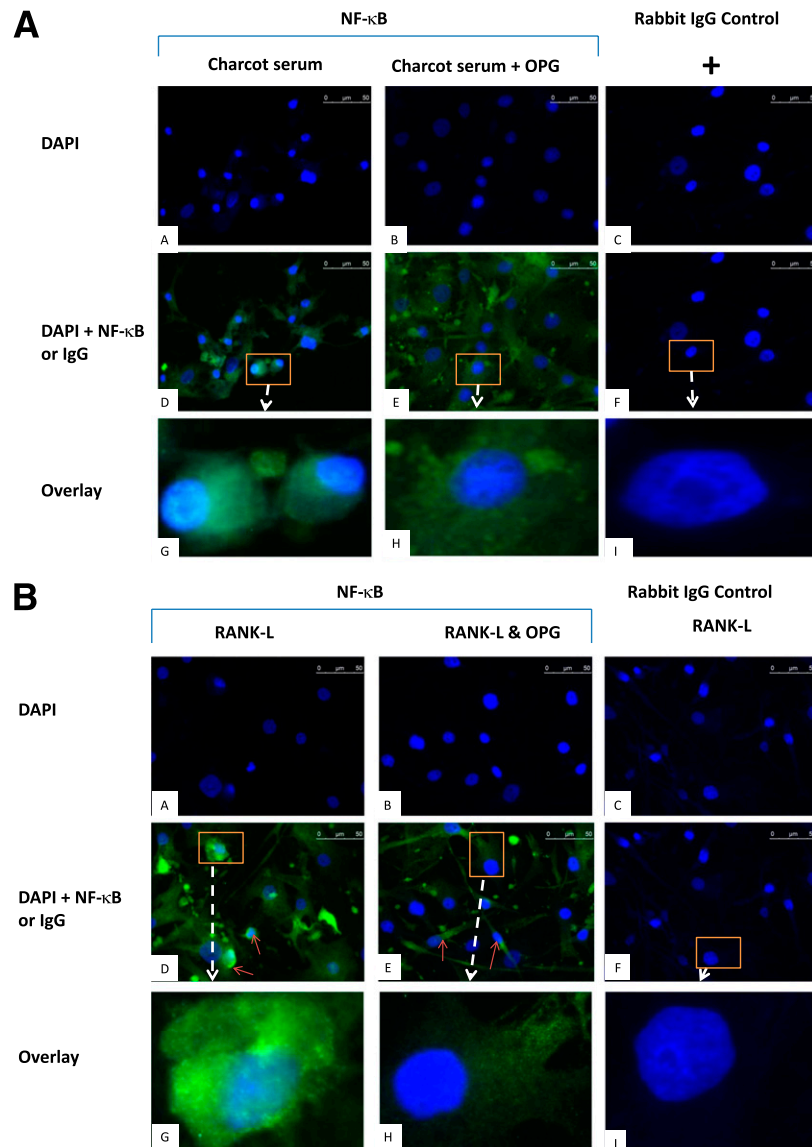
FIG. 6. Fluorescent photomicrographs from immunocytochemical experiments. Human VSMCs were cultured to confluence in growth media and subsequently incubated for 1 h in (*A*) OM with Charcot serum (5% v/v) or coincubated with Charcot (5% v/v) serum plus 20 pmol/L recombinant OPG and (*B*) OM, including 20 pmol/L recombinant human RANKL with or without plus 20 pmol/L recombinant human OPG. After 1 h, immuno-histochemical staining to localize NF-κB was performed using rabbit anti-human p65 antibody. Goat anti-rabbit secondary antibody conjugated to a fluorescent dye (Alexa-Flour 488) was used to locate NF-κB staining (green) (*D* and *E*), whereas DAPI was used to stain the cell nucleus (blue) (*A–C*). Nonimmune rabbit IgG was used as negative antibody control (*C*, *F*, and *I*). Charcot serum induced nuclear translocation of NF-κB as seen by the intense perinuclear green stain (*A*, image *G*). Coincubation with OPG prevented the translocation of NF-κB, which remained predominantly cytoplasmic (*A*, image *H*). Likewise, RANKL induced nuclear translocation of NF-κB as seen by the intense perinuclear green stain (*B*, image *G*). Coincubation with OPG prevented the translocation of NF-κB, which remained predominantly cytoplasmic (*B*, image *H*). (A high-quality digital representation of this figure is available in the online issue.)

wall to differentiate locally into osteoblastic cells, others have maintained that these osteoblastic cells originate from residential human VSMCs displaying marked phenotypic plasticity under specific stimuli. Our study supports the latter hypothesis because it demonstrates using an in vitro model that explanted human VSMCs of medial origin directly differentiate into an osteoblastic phenotype. Our data support those of Schoppet et al. (35), who found that the medial tissue surrounding calcified lesions in MAC contained only smooth muscle cells, whereas macrophages and lymphocytes were absent.

By using VSMCs from rat aortas, Panizo et al. (15) demonstrated that RANKL induced mineralization in a dose-dependent manner, an effect abrogated by OPG. Data reported in this study support and extend those of Panizo et al., implicating RANKL/OPG signaling in MAC by using smooth muscle cells from human origin.

OPG blocks RANKL- and Charcot-induced NF-κB nuclear translocation, and mineralization of human VSMCs. This study showed that OPG, a specific decoy receptor for RANKL, attenuated mineralization and osteoblastic differentiation of VSMCs induced by Charcot serum or RANKL. Our data also suggest a more pronounced effect of OPG on Charcot-mediated differentiation and mineralization of VSMCs compared with that induced by diabetes serum. Because OPG directly and specifically blocks the action of RANKL, these data provide additional evidence of RANKL/RANK signal modulation in CN-induced MAC. The
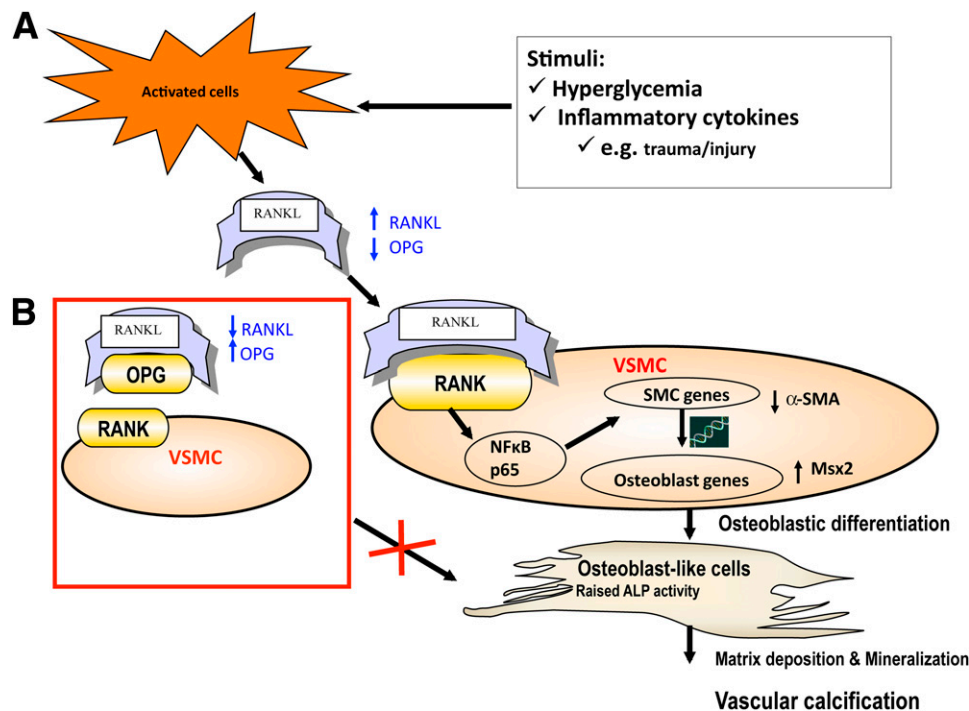
FIG. 7. External stimuli (hyperglycemia, inflammation resulting from trauma, infection, or other injury) activate certain cells (e.g., endothelial cells, osteoblasts) to release RANKL. When the RANKL/OPG ratio is high as in CN (*A*), RANKL binds to its receptors (RANK) on VSMCs to activate NF-κB and cause downstream cell signaling cascades, resulting in osteoblastic differentiation and deposition of mineralized matrix leading to MAC. However, when the OPG levels are low or the RANKL/OPG ratio is not elevated (*B*), OPG acts as a decoy receptor mopping excess RANKL and preventing human VSMCs from undergoing osteoblastic differentiation and mineralization. SMC, smooth muscle cell.

nuclear translocation of NF-κB was also abrogated by coincubation with OPG, thus lending support to the key mechanistic role played by NF-kB in RANKL-induced mineralization. In fact, binding of RANKL to its receptor RANK may activate the canonical or the alternative pathway of NF-κB signaling (36,37).

Tseng et al. (38) recently used a murine model of calcification to show that atherosclerotic calcification involves bone remodeling with simultaneous activation of both osteoclastic and osteoblastic differentiation. Although OPG attenuated Forskolin-induced osteoclastic differentiation, thus confirming similar reports by Mabilleau et al. (10), Tseng et al. reported that calcification was not abrogated by OPG and concluded that pathways different from RANKL/OPG signaling were implicated. This is not surprising because murine models of calcification, and likewise, atherosclerotic calcification, may involve different pathways than in humans. However, we provide evidence for the direct involvement of RANKL/OPG in human MAC as typified in CN.

In summary, patients with CN have high levels of inflammatory cytokines potentially modulating the RANKL/OPG signaling pathway. They further display elevated systemic (serum) RANKL levels and high RANKL/OPG ratio, driving VSMCs into an osteoblastic differentiation pathway, resulting in deposition of a mineralized matrix in vitro. Differentiation of VSMCs occurs via the RANKL/RANK signaling cascade, setting intracellular mechanisms involving the nuclear translocation of NF-κB. This study provides additional data demonstrating that MAC in CN is independent of the high calcium-phosphate microenvironment. Furthermore, OPG is shown to prevent nuclear translocation of NF-κB and abrogate differentiation and

mineralization of VSMCs induced by RANKL or Charcot serum. On the basis of the findings from this study, a working model of MAC as occurs in patients with CN is proposed (Fig. 7).

When interpreted in the light of other studies confirming the crucial role of RANKL/OPG signaling in osteoclastic bone resorption (10), our data provide a potential unifying hypothesis for the underlying pathogenetic mechanisms resulting in diabetic CN. Abnormal RANKL/OPG signaling, therefore, may be proposed as the mechanism underpinning the paradoxical osteolysis and MAC seen in CN and similar disease conditions. This holds promise for the treatment of CN, especially because new drugs specifically targeting RANKL are already marketed for use in osteoporosis. Clinical trials are warranted to assess the efficacy of anti-RANKL therapy in the treatment or prevention of CN and vascular calcification.

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