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An Increased Osteoprotegerin Serum Release Characterizes the Early Onset of Diabetes Mellitus and May Contribute to Endothelial Cell Dysfunction

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Serum osteoprotegerin (OPG) is significantly increased in diabetic patients, prompting expanded investigation of the correlation between OPG production/release and glycemic levels. Serum levels of OPG, but not of its cognate ligand receptor activator of nuclear factor- κ B ligand (RANKL), were significantly increased in type 2 diabetes mellitus patients compared with healthy blood donors. Serum OPG was also significantly elevated in a subgroup of recently diagnosed diabetic patients (within 2 years). The relationship between serum OPG and diabetes mellitus onset was next investigated in apoE-null and littermate mice. Serum OPG increased early after diabetes induction in both mouse strains and showed a positive correlation with blood glucose levels and an inverse correlation with the levels of free (OPG-unbound) RANKL. The *in vitro* addition of tumor necrosis factor- α to human vascular endothelial cells, but not human peripheral blood mononuclear cells, markedly enhanced OPG release in culture. In contrast, high glucose concentrations did not modulate OPG release when used alone or in association with tumor necrosis factor- α . Moreover, the ability of soluble RANKL to activate the extracellular signal-regulated kinase/mitogen-activated protein kinase and endothelial nitric-oxide synthase pathways in endothelial cells was neutralized by preincubation with recombinant OPG. Altogether, these findings suggest that increased OPG production represents an early

event in the natural history of diabetes mellitus, possibly contributing to disease-associated endothelial cell dysfunction. (Am J Pathol 2006, 169:2236–2244; DOI: 10.2353/ajpath.2006.060398)

Receptor activator of nuclear factor (NF)- κ B ligand (RANKL) is a member of the tumor necrosis factor (TNF) family of cytokines, which exists either as type II membrane or as soluble protein.¹ RANKL was originally described as being expressed by activated T lymphocytes and osteoblasts, and it has been involved in the interaction between T lymphocytes and dendritic cells, osteoclast differentiation from monocytic precursor cells, and activation of mature osteoclasts.^{1–6} Two receptors for RANKL have been identified: transmembrane RANK and soluble osteoprotegerin (OPG).^{2,3,5} RANK mRNA is ubiquitously expressed in human tissues, but RANK protein expression has been characterized only in normal dendritic cells, CD4 and CD8 T lymphocytes, osteoclast monocytic precursors, and endothelial cells, suggesting that expression of this protein is posttranscriptionally regulated.⁶ For the purpose of this study, it is noteworthy that, by interacting with RANK, RANKL induces a variety of biological effects on endothelial cells, such as promotion of cell survival and angiogenesis.^{7–9} Although the affinity of RANKL for OPG is weaker than that for RANK,⁵ when present at high concentrations soluble OPG prevents RANKL interaction with transmembrane RANK, thus acting as a decoy receptor.^{3,5}

It has been shown that OPG is produced by a wide range of tissues, including the cardiovascular system, and that OPG levels are particularly high in aortic and renal arteries.^{10–12} Interestingly, different groups of investigators have reported that serum OPG is significantly increased in both type 1 and type 2 diabetic pa-

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Table 1. Characteristics of the Diabetic Study Group

Number of cases	88
Gender (M/F)	40/48
Age* (years)	65 (58 to 75)
BMI*	30 (22.4 to 41.5)
HbA1c* (%)	7.6 (5.3 to 11.5)
Pharmacological therapy [†] /diet	60/28
Patients with vascular complications	24
Duration diabetes* (years from diagnosis)	10 (<1 to 32)

*Median and range.

[†]Metformin, Glliclazide, Rosiglitazone, Glibenclamide.

tients,^{13–18} as well as in both diabetic and nondiabetic patients affected by coronary artery disease.^{17–20} Moreover, it has been demonstrated that up-regulated serum OPG levels have a negative prognostic value in heart failure after acute myocardial infarction as well as in patients affected by abdominal aortic aneurysm.^{21–23} Interestingly, it has also been shown that the levels of free RANKL are significantly decreased in the sera of patients affected by coronary artery disease²⁴ as well as in the endomyocardium in transplant coronary artery disease.²⁵ The aim of this study was to investigate whether serum OPG elevation represents an early or a late event in the natural history of diabetes mellitus and to investigate the correlation between OPG production/release and glycaemic levels both *in vivo* and *in vitro*.

Materials and Methods

Patients

Serum samples were obtained from 88 patients with type 2 diabetes mellitus and 41 control patients who had no metabolic disease. The study was approved by the “G. D’Annunzio” University Ethical Committee, and the consent was obtained from patients after full explanation of the procedure and its purpose, in accordance with Declaration of Helsinki of 1975. Characteristics of the patients are summarized in Table 1. In particular, 20 of 88 diabetic patients had microvascular complications: 12 had background diabetic retinopathy, four had preproliferative diabetic retinopathy, and four had undergone argon laser treatment for proliferative diabetic retinopathy. Two of the patients with background retinopathy also exhibited diabetic nephropathy (proteinuria >300 mg/24 hours). Only four patients had clinically manifest diabetic macroangiopathy (two had experienced a myocardial infarction, one had undergone coronary angioplasty, and one had undergone coronary artery bypass graft).

Animals

Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community, Council Directive 86/609, OJL 358, December 12, 1987). Sixteen apoE-null (ApoE^{tm1Unc}) mice, 6 weeks old, and eight littermates (C57Black/6J

strain) were rendered diabetic by five daily intraperitoneal injections of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO) at a dose of 55 mg/kg. Control apoE-null mice ($n = 10$) and littermates ($n = 8$) received citrate buffer alone and were processed in parallel to the diabetic mice. The animals had unrestricted access to water and were maintained on a 12-hour light-dark cycle in a nonpathogen-free environment on standard mouse chow (Harlan Nossan Correzzana, Milan, Italy). Serum glucose, total cholesterol, high-density lipoprotein, and triglyceride concentrations were determined by an autoanalyzer technique (Hitachi 717; Tokyo, Japan).

For the histological examination, after 3 months, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt; Boehringer, Ingelheim, Germany). The distribution and extent of atherosclerotic lesions in apoE-null mice were evaluated by the *en face* analysis, after staining with Sudan IV-Herxheimer’s solution (Sigma Chemical Co.), as previously described.²⁶ Aortic segments were then embedded in paraffin, and 4- μ m-thick cross-sectional serial sections were stained with hematoxylin and eosin to evaluate the atherosclerotic lesion complexity.

Reagents

Human OPG and RANKL levels were measured in serum samples as well as in cell culture supernatants using sandwich-type enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers’ instructions. The human OPG ELISA kit was purchased from Alexis Biochemicals (Lausen, Switzerland), and human RANKL kits were purchased from Apotech (Epalinges, Switzerland) and Biomedica (Vienna, Austria). Mouse RANKL and OPG serum levels were measured in sera from apoE-null and C57Black littermate mice using ELISA kits purchased from R&D Systems (Minneapolis, MN). The results were read at an optical density of 450 nm using an Anthos 2010 ELISA reader (Anthos Labtec Instruments Ges.m.b.H, Wals/Salzburg, Austria). Measurements were done in duplicates.

Of note, the ELISA assay for human RANKL from Apotech uses the two-site sandwich technique with two selected antibodies that bind to human sRANKL and OPG, allowing the determination of total (both free and OPG-bound) RANKL. On the other hand, the ELISA assay for human RANKL from Biomedica, as well as the assay for mouse RANKL (R&D Systems), detects only uncomplexed free RANKL. TNF- α , glucose, and insulin were purchased from Sigma; interleukin (IL)-1 β was from Roche Diagnostics (Mannheim, Germany); recombinant OPG was from R&D Systems; recombinant RANKL was from Alexis. For Western blot analyses, the following antibodies (Abs) were used: anti-extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-ERK1/2 (both from New England Biolabs, Beverly, MA), anti-phospho-endothelial nitric-oxide synthase (eNOS) (P-Ser1177; Cell Signaling Technology, Beverly, MA), anti-eNOS/NOS type III (BD Transduction Laboratories, Lexington, KY), and anti-tubulin (Sigma).

Cell Cultures

Primary human umbilical vein endothelial cells (HUVECs), obtained from BioWhittaker (Walkersville, MD), were used between passages 3 and 6 *in vitro*. Cells were grown on gelatin-coated tissue culture plates in M199 endothelial growth medium (BioWhittaker) supplemented with 20% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), 10 $\mu\text{g/ml}$ heparin, and 50 $\mu\text{g/ml}$ endothelial cell growth factor (Sigma), as previously described.²⁷ Human peripheral blood mononuclear cells (PBMCs) from healthy normal donors were separated by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories, Hornby, ON, Canada) and seeded at a density of 1 to 5×10^6 cells/well. For macrophage cultures, after incubation for 18 hours, non-adherent PBMCs were removed, and remaining adherent cells were maintained in RPMI medium containing 10% fetal bovine serum and 50 ng/ml human macrophage-colony-stimulating factor (PeproTech, London, UK). Expression of macrophagic markers was documented by flow cytometry using phycoerythrin-conjugated anti-CD14 (Immunotech, Marseille, France) and anti-CD36 antibodies (BD Pharmingen, San Diego, CA), and fluorescein isothiocyanate-conjugated anti-CD64 antibody (Immunotech). Cells were treated with glucose (30 mmol/L), insulin (1 $\mu\text{mol/L}$), or scalar concentration of inflammatory cytokines (TNF- α or IL-1 β ; 1 to 100 pg/ml) or of STZ (0.2 to 5 $\mu\text{mol/L}$). Supernatants were harvested at 24 and 72 hours after the treatments and analyzed for OPG and RANKL levels.

Immunoblot Analysis and Measurement of cGMP Formation

For immunoblot experiments, HUVECs were plated in 10-cm dishes and grown at subconfluence before treatments. To minimize activation by serum, HUVECs were subject to partial fetal bovine serum reduction (0.5%) and growth factor withdrawal for 18 hours before the addition of RANKL, used alone or in combination with OPG. The optimal concentrations for RANKL (10 ng/ml) and OPG (20 ng/ml) were determined in preliminary experiments in which HUVECs were exposed to serial dilutions (0.1 to 100 ng/ml) of the molecules. For protein preparation, cells were harvested in lysis buffer containing 1% Triton X-100, Pefablock (1 mmol/L), aprotinin (10 $\mu\text{g/ml}$), pepstatin (1 $\mu\text{g/ml}$), leupeptin (10 $\mu\text{g/ml}$), NaF (10 mmol/L), and Na_3VO_4 (1 mmol/L). Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of protein (50 μg) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were probed with antibodies for the phosphorylated ERK1/2 and eNOS. After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG, specific reactions were revealed with the enhanced chemiluminescence reagent detection system (DuPont-NEN, Boston, MA). Membranes were stripped by incubation in Re-Blot 1X antibody stripping solution (Chemicon Int., Temecula, CA) and re probed for the respective total

ERK1/2 and eNOS protein content and for tubulin levels, for verifying loading evenness. Densitometric values were expressed in arbitrary units and estimated by the ImageQuant software (Molecular Dynamics, Piscataway, NJ). Multiple film exposures were used to verify the linearity of the samples analyzed and avoid saturation of the film.

For NO-dependent guanosine 3',5'-cyclic monophosphate (cGMP) measurement, HUVECs were seeded in standard 96-well plates, incubated overnight at standard conditions, and subsequently treated, as indicated, for 30 minutes at 37°C in culture medium containing 0.6 mmol/L 3-isobutyl-1-methylxanthine. After cell lysis, cGMP levels were measured using an enzyme-immunoassay kit (cGMP EIA system; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

Statistical Analysis

The median, minimum, and maximum values were calculated for each group of data obtained from both human and mouse serum samples. Box plots were used to show the median, minimum, and maximum values and 25th to 75th percentiles. The results were evaluated by using Student's *t*-test and the Mann-Whitney rank-sum test. Correlation coefficients were calculated by the Spearman's method. Statistical significance was defined as $P < 0.05$.

Results

Serum Levels of OPG Are Significantly Elevated in Type 2 Diabetic Patients with Respect to Normal Healthy Blood Donors

In the first group of experiments, the serum levels of OPG and RANKL were examined in 88 type 2 diabetic patients in comparison to 41 healthy blood donors. The serum levels of OPG were significantly ($P < 0.05$) increased in diabetic patients (mean $\pm$ SD, 130 ± 41 pg/ml) with respect to sex- and aged-matched normal blood donors (mean $\pm$ SD, 80 ± 29 pg/ml) (Figure 1A). On the other hand, the serum concentration of total (free plus OPG-bound) RANKL did not show any significant variation between diabetic patients (mean $\pm$ SD, 79 ± 116 pg/ml) and normal controls (mean $\pm$ SD, 69 ± 75) (Figure 1B). Of note, in a limited group of diabetic patients ($n = 40$) and healthy controls ($n = 22$), we also examined the levels of free RANKL using an ELISA kit, which specifically recognizes free RANKL (ie, unbound to OPG). As shown in Figure 1C, the levels of free RANKL were significantly ($P < 0.05$) decreased in diabetic patients (mean $\pm$ SD, 5.2 ± 4 pg/ml) with respect to normal blood donors (mean $\pm$ SD, 10.5 ± 6 pg/ml), in line with the concomitant increase of serum OPG.

A significant correlation ($r = 0.29$, $P < 0.05$) was observed between serum OPG levels and the history of diabetes mellitus. In particular, because diabetic patients

Figure 1. Serum OPG and RANKL levels in diabetic patients and healthy individuals. Levels of OPG (**A**), total (**B**), and free (**C**) RANKL were determined by ELISA in sera from diabetic patients and from healthy patients. Horizontal bars are median, upper, and lower edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. * $P < 0.05$.

were clustered into two subgroups, ie, newly diagnosed diabetic patients (within 2 years from diagnosis) and patients with a longer history of diabetes (≥ 6 years from diagnosis), it is remarkable that recently diagnosed diabetic patients also showed significantly ($P < 0.05$) greater OPG serum levels with respect to normal controls (Figure 2A). Patients with a longer history of diabetes mellitus showed higher levels of OPG with respect to either recently diagnosed patients ($P < 0.05$) and normal controls (Figure 2A). Of note, patients with a long history of diabetes mellitus comprised most of the patients with documented microvascular and macrovascular complications (Table 1). The possibility that the increased serum OPG levels detected in patients with a long history of diabetes mellitus merely reflected an advanced age of these patients was excluded, because no significant cor-

Figure 2. Relation between serum OPG levels and years from diagnosis in diabetic patients. Serum levels of OPG were analyzed in patient subgroups, based on the indicated years from diagnosis (**A**) and in relation with patient age (**B**). Horizontal bars are median, upper, and lower edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. * $P < 0.05$. Coefficient of correlation is reported in the text.

relation was observed between serum OPG and age of the patients ($r = 0.05$, $P > 0.05$) (Figure 2B).

Correlation between OPG Serum Levels and Hyperglycemia in Diabetic ApoE-Null Mice and C57Black Littermates

Elevated levels of serum OPG have been reported in patients affected by either type 1 or type 2 diabetes mellitus.^{13–18} Therefore, to further analyze whether the serum levels of OPG were affected by hyperglycemia and/or by other aspect of the metabolic disorders associated to diabetes mellitus, such as hypercholesterolemia, the next experiments were performed in the apoE-null mice,²⁶ which are characterized by elevated levels of total serum cholesterol (mean $\pm$ SD, 14.27 ± 2.1 mmol/L) with respect to littermate mice (mean $\pm$ SD, 1.81 ± 0.42 mmol/L). In these animals, STZ-induced diabetes mellitus was associated to the development of widespread aortic atherosclerotic lesions, starting from 10 to 12 weeks from diabetes induction onwards (Figure 3A). Hyperglycemia started to become significantly ($P < 0.05$) increased from the 2nd week after the beginning of STZ treatment (Figure 3B).

Because we were interested in characterizing the OPG and RANKL levels in the early phases of diabetes mellitus, serum OPG and RANKL were measured in apoE-null mice starting from early time points from STZ treatment. A

Figure 3. Atherosclerotic aortic lesions and serum glucose levels in apoE-null mice after induction of diabetes. **A:** Representative H&E-stained histological cross-sectional sections from aorta of control and diabetic (3 months) apoE-null mice. **Arrowhead,** a wide atherosclerotic plaque. **B:** Levels of glucose were determined in serum samples from control and diabetic apoE-null mice at different weeks after diabetes induction by STZ treatment. Horizontal bars are median, upper, and lower edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. * $P < 0.05$. Original magnifications, $\times 10$.

significant ($P < 0.05$) increase in OPG levels was measured in diabetic mice with respect to nondiabetic apoE-null mice starting the 2nd week from diabetes induction (Figure 4A). At variance to the ELISA for human RANKL, which allowed us to measure either free or total (free plus OPG-bound) RANKL, the available ELISA for mouse RANKL only recognizes free, uncomplexed RANKL. As shown in Figure 4B, free RANKL was significantly ($P < 0.05$) decreased in apoE-null diabetic mice with respect to nondiabetic controls, in keeping with the concomitant elevation of serum OPG. Of note, OPG serum levels showed a significant inverse correlation ($r = -0.84$, $P < 0.05$) with the levels of free RANKL (Figure 5A) and a significant positive correlation ($r = 0.66$, $P < 0.05$) with the levels of glycemia (Figure 5B).

To investigate further the findings illustrated above, pointing to a role of hyperglycemia in the early induction of OPG elevation during diabetes mellitus, additional experiments were performed on C57Black littermates. As shown in Figure 6A, STZ treatment induced an increase of glycemia with kinetics similar to that previously observed in apoE-null mice. Concomitantly with the elevation of glycemia, OPG serum levels showed a significant ($P < 0.05$) increase also in C57Black whereas the levels

Figure 4. Serum OPG and RANKL levels in diabetic and control apoE-null mice. Levels of OPG (**A**) and free RANKL (**B**) were determined in sera from control and diabetic apoE-null mice at different weeks after induction of diabetes. Horizontal bars are median, upper, and lower edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. * $P < 0.05$.

of free RANKL showed a concomitant decrease (Figure 6, B and C).

OPG Is Released in Vitro by Endothelial Cells in Response to Inflammatory Cytokines but Not to High Glucose Levels

Next experiments were performed *in vitro* to investigate whether endothelial cells might contribute to the serum OPG elevation observed in diabetic environment. For this purpose, HUVECs were exposed to high glucose concentrations (30 mmol/L), insulin (1 μ mol/L), or inflammatory cytokines (1 to 100 pg/ml), which are known to be elevated in the sera of diabetic patients.^{28,29} As shown in Figure 7A, HUVECs spontaneously secreted measurable levels of OPG in culture. Neither high glucose levels nor insulin were able to significantly modulate basal OPG release. On the other hand, recombinant TNF- α dose dependently up-regulated the release of OPG in the culture medium after 24 hours of exposure. It is particularly noteworthy that TNF- α induced a twofold ($P < 0.05$) increase of OPG release at concentrations as low as 10 pg/ml (Figure 7A). On the other hand, a significant increase of OPG release in response to IL-1 β was observed only after stimulation with 100 pg/ml IL-1 β (Figure 7A), a concentration that might be beyond the physiological levels of this cytokine in the plasma of diabetic patients. The association between high concentrations of

Figure 5. Relation between serum levels of OPG and free RANKL or glucose. Relation between serum levels of OPG and free RANKL (**A**) and between serum levels of OPG and glucose (**B**) in diabetic apoE-null mice. Coefficients of correlation are indicated.

glucose and $\text{TNF-}\alpha$ did not result in a further increase of OPG release, even after 72 hours of exposure (Figure 7B), suggesting that inflammatory cytokines, rather than hyperglycemia per se, might mediate the up-regulation of OPG release observed in both humans and mice. Furthermore neither HUVECs nor primary human PBMCs produced OPG in response to increasing concentrations of STZ, excluding the possibility that STZ has a direct effect on OPG production, at least in these cell types (Figure 7B). In parallel, OPG release was also measured in the culture supernatants of primary human macrophages, taking into account that macrophage infiltration represents a major event in both microvascular and macrovascular complications associated to diabetes mellitus. OPG was not detected in the supernatant of cultured macrophages in any tested condition, even after stimulation with inflammatory cytokines (ie, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$) (data not shown). Finally, neither endothelial cells nor PBMCs released detectable amounts of soluble RANKL, either spontaneously or after addition of high glucose levels, insulin, $\text{TNF-}\alpha$, or $\text{IL-1}\beta$ (data not shown).

OPG Blocks the Intracellular Signaling Induced by RANKL in Endothelial Cells

It has been previously shown that soluble RANKL, by interacting with its cognate transmembrane receptor RANK, triggers a variety of intracellular signal transduction pathways in endothelial cells, which result in protec-

Figure 6. Serum OPG and RANKL levels in diabetic and control C57 littermate mice. Diabetes was induced by STZ injection in C57Black littermate mice. Levels of glucose (**A**), OPG (**B**), and free RANKL (**C**) were measured in sera from control and diabetic mice at different weeks after STZ treatment. Horizontal bars are median, upper, and lower edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. * $P < 0.05$.

tive effects, such as promotion of endothelial cell survival and angiogenesis.⁷⁻⁹ To ascertain whether the enhanced OPG production/release observed in sera of diabetic patients might interfere with the beneficial biological activity of RANKL on endothelial cells, we investigated the activation of ERK/mitogen-activated protein kinase and eNOS intracellular pathways in endothelial cells after treatment with RANKL in the presence or absence of recombinant OPG. As shown in Figure 8, RANKL induced

Figure 7. OPG release in endothelial cells and PBMC cultures. **A:** HUVECs were either left untreated or stimulated with glucose, insulin, TNF- α , or IL-1 β . After 24 hours, the levels of OPG released in culture supernatant were measured by ELISA. **B:** HUVECs and PBMCs were either left untreated or stimulated with TNF- α $\pm$ glucose or STZ. After 72 hours, the levels of OPG released in culture supernatant were measured by ELISA. Results are expressed as means $\pm$ SD of three to four independent experiments, each performed in triplicate. * P < 0.05.

phosphorylation of ERK1/2 and of eNOS. The activation of both pathways was completely abrogated by preincubation of RANKL with OPG, clearly indicating that OPG was effective in inhibiting the biological activity of RANKL in endothelial cells. As expected on the basis of the Western blot data illustrated above, we next investigated whether eNOS-expressing cells were able to generate bioactive NO. For this purpose, we measured the formation of cGMP, a good proxy for NO, because soluble guanylate cyclase is activated by nM concentrations of the gas.³⁰ Exposure to RANKL resulted in a significant (P < 0.05) increase in cGMP over controls (652 ± 40 and 263 ± 30 fmol/ 10^6 cells, respectively; n = 3), which was inhibited by the preincubation with OPG (302 ± 39 fmol/ 10^6 cells, n = 3). The presence of high glucose concen-

Figure 8. Effect of OPG on RANKL-induced intracellular signaling in endothelial cells. Quiescent HUVEC cultures were stimulated with RANKL or RANKL + OPG for the indicated time intervals (0 to 60 minutes). **A:** Cell lysates were analyzed for ERK1/ERK2 and eNOS activation by Western blot of total and phosphorylated (P) proteins using specific antibodies. Equal loading of protein in each lane was confirmed by staining with the antibody to tubulin. **B:** Protein bands were quantified by densitometry, and levels of P-ERK1/2 and P-eNOS were calculated for each time point, after normalization to ERK1/2, and eNOS, respectively. Unstimulated basal expression was set as unity. One of three experiments with similar results is shown.

trations in culture medium did not determine any significant modulation of these results (data not shown).

Discussion

We have here demonstrated that serum OPG levels are increased in the early onset of diabetes mellitus and progressively increase with the duration of the disease.

Diabetic vasculopathy has an underlying low-grade inflammatory component, manifesting itself in the up-regulation of genes responsive to inflammatory processes.^{27–31} In this respect, it should be emphasized that OPG is an NF- κ B-inducible gene,³² whose release in endothelial cell culture is significantly increased by inflammatory cytokines as also shown in this study. Because of the enormous surface area of the endothelium throughout the body as well as the relatively substantial levels of constitutive and regulated OPG produced not only by human endothelial cells but also by vascular smooth muscle cells,^{33,34} vascular cells likely significantly contribute to circulating OPG levels measured in serum. On the other hand, our *in vitro* data do not support for a significant role of endothelial cells, PBMCs, or macrophages in releasing significant amounts of RANKL. Other authors have reported a local expression of OPG and RANKL in the tunica media of big arteries during the process of vascular calcification,¹⁰ but the relative contribution of these cytokines to the vascular calcification is still incompletely understood.

Although we have analyzed a cohort of type 2 diabetic patients, it is noteworthy that a couple of recent studies have demonstrated that serum OPG is also elevated in patients affected by type 1 diabetes,^{13,14} suggesting that hyperinsulinemia and insulin-resistance are unlikely to play a key role in OPG induction. Consistently, *in vitro* data have demonstrated that insulin rather down-regulates OPG expression in vascular smooth muscle cells.¹² In line with the hypothesis that insulin is not involved in the induction of OPG expression and secretion, we have also demonstrated that OPG release is significantly up-regulated in the sera of diabetic apoE-null mice early after the induction of diabetes mellitus by STZ treatment. Of note, OPG serum levels in diabetic apoE-null mice positively correlated with the glycemic levels whereas they were inversely correlated to the levels of free RANKL. Elevated levels of OPG were also observed in C57Black littermates concomitantly with the induction of diabetes mellitus, suggesting that hypercholesterolemia, characterizing apoE-null mice, did not play a major role in the up-regulation of serum OPG associated to diabetes mellitus. Although we cannot exclude the possibility that autoimmune responses associated to STZ treatment are involved in the increase of OPG expression/release, the fact that OPG serum levels started to increase in both apoE-null mice and control littermates not during STZ treatment but only subsequent to the rise of glycemia renders this possibility unlikely.

Despite the *in vivo* data obtained in the mouse models of STZ-induced diabetes, in which we have demonstrated the existence of a positive correlation between OPG and glycemic serum levels, high glucose levels per se were insufficient to modulate OPG release in endothelial cells, PBMCs, and macrophages. On the other hand, the proinflammatory cytokine TNF- α , which is known to be elevated in the sera of diabetes mellitus,^{28–30} dose dependently up-regulated OPG secretion by endothelial cells. Importantly, the concentrations of TNF- α (10 pg/ml) required to induce a significant (approximately twofold) increase in OPG, a situation mimicking the OPG rise

observed in the serum of diabetic patients, were in the range of plasma concentrations reported in diabetic patients.^{28–30} These *in vitro* findings, coupled to the data obtained in the diabetic mouse models, clearly suggest that the inflammation-driven hyperglycemia, rather than the high glucose levels per se, is involved in the increase of OPG observed in both diabetic patients and diabetic mice.

It is possible that the imbalance of OPG versus RANKL serum levels in both diabetic patients and diabetic apoE-null mice might contribute to endothelial cell dysfunction by blocking RANKL signaling, which is able to activate protective intracellular pathways in endothelial cells, such as the eNOS pathway. In this respect, it should be emphasized that diabetic vascular dysfunction is a major clinical problem that predisposes patients to a variety of cardiovascular diseases. In fact, diabetic patients frequently suffer from macroscopic and microscopic vasculopathy and accelerated atherosclerosis. The early impairment of nitric oxide release is a key feature of endothelial dysfunction, which invariably precedes permanent vascular alterations.³⁵ Our results also suggest a mechanism to explain why altered serum OPG levels have been shown to reflect the development or status of vascular disease in both diabetic and nondiabetic patients,^{13–24} indicating that therapeutic strategies aimed to decrease the OPG serum levels may be suitable for improving the vascular function in diabetes mellitus and possibly in other vascular pathologies characterized by a chronic inflammatory state.

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