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Regular Article

Osteoprotegerin is higher in peripheral arterial disease regardless of glycaemic status ☆

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ABSTRACT

Introduction: Peripheral arterial disease (PAD) and type 2 diabetes mellitus (DM) are both associated with excessive vascular calcification and elevated levels of inflammatory markers IL-6 and hsCRP. The recently identified Osteoprotegerin(OPG)/RANKL/TRAIL pathway has been implicated in vascular calcification, but data on levels in PAD and effect of co-existent DM are lacking.
Materials and Methods: 4 groups of patients were recruited - 26 with PAD and DM, 35 with DM alone, 22 with PAD alone, and 21 healthy individuals. Serum OPG, RANKL, TRAIL, hsCRP and IL-6 were measured using commercial ELISA assays. Presence and severity of PAD was defined using ankle brachial index (ABI).
Results: Serum OPG (7.4 ± 0.3 vs.5.8 ± 0.2 pmol/l, $p<0.0001$), TRAIL (95.5 ± 5.2 ng/ml vs. 76.2 ± 4.4 ng/ml, $p=0.006$), hsCRP (2.6 ± 0.3 vs. 1.8 ± 0.3 mg/l, $p=0.048$), and IL-6 (4.1 ± 0.4 vs. 2.9 ± 0.4 pg/ml, $p=0.06$) were higher in patients with PAD. There was no difference in RANKL. Only OPG was significantly higher in PAD and DM (7.2 ± 0.3 pmol/l) and PAD alone (7.7 ± 0.4 pmol/l) compared to DM only (5.8 ± 0.3 pmol/l) and healthy controls (5.6 ± 0.4 pmol/l), $p<0.01$, but OPG was no higher in those with DM plus PAD versus those with PAD alone ($p<0.3$). Only OPG was associated with PAD severity, correlating negatively with ABI ($r=-0.26$, $p=0.03$), independent of age, gender, glycaemic status, hsCRP and IL-6.
Conclusions: PAD is associated with higher serum OPG, regardless of the co-existence of DM. This finding, in addition to its correlation with severity of PAD, suggests that OPG may be a novel marker for the presence and severity of PAD, possibly by reflecting the degree of underlying vascular calcification.

Peripheral arterial disease (PAD) is prevalent in diabetes, reported in 30% of patients with diabetes over the age of 50 years [1]. The pathogenesis of PAD is complex but a significant factor in the development of PAD both in patients with and without diabetes is vascular calcification. The presence of vascular calcification in PAD is associated with arterial stiffness, plaque rupture, non-compressible blood vessels and a 3 to 4 fold higher risk of cardiovascular morbidity and mortality [2].

Arterial wall calcification was first recognised nearly 100 years ago, but our understanding that this may be an active, rather than a passive, carefully regulated process has only developed in recent times with the identification of pathways such as OPG/RANKL/TRAIL. Osteoprotegerin (OPG), a protein of the tumour necrosis factor (TNF) receptor family is produced by a variety of tissues including bone marrow stromal cells, immune system cells, vascular smooth muscle and endothelial cells. OPG acts as a decoy receptor for Receptor Activator for Nuclear factor Kappa beta Ligand (RANKL), preventing the stimulation and maturation of osteoclast precursors instigated by normal binding of RANKL to its constitutive receptor and as a soluble receptor for tumour necrosis factor related apoptosis-inducing ligand (TRAIL) [3]. The role of OPG/RANKL/TRAIL within the vasculature and in PAD is not clear. OPG knockout mice present with osteoporosis and marked aortic and renal arterial calcification [4], which is reversed after the administration of recombinant OPG [5,6]. In humans, serum OPG correlates with the degree of underlying coronary artery calcification in patients with DM [7], severity of coronary artery disease [8–10], and is an independent predictor of cardiovascular mortality in patients with and without diabetes [11–14].

Studies measuring serum OPG in PAD patients have shown conflicting results, some papers have shown no difference in serum

Abbreviations: PAD, Peripheral Arterial Disease; DM, Type 2 Diabetes Mellitus; IL-6, Interleukin-6; hsCRP, high sensitivity C-Reactive Protein; RANKL, Receptor Activator for Nuclear Factor Kappa-Beta Ligand; TRAIL, Tumour necrosis factor Related Apoptosis-Inducing Ligand; TNF, Tumour Necrosis Factor; ABI, Ankle Brachial Index; OGTT, Oral Glucose Tolerance Test; BMI, Body Mass Index; BMD, Bone Mineral Density; VSMC, Vascular Smooth Muscle Cell.

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OPG levels in patients with and without PAD [15–18], but all of these studies included variable numbers of patients with diabetes, which in itself is associated with elevated levels of OPG [19–24], while others have shown a positive correlation between OPG and severity of PAD [16,25]. The heterogenous nature of these studies is evidenced by the fact that diabetes patients were included within both the PAD [15–17] and non-PAD "control" groups [18,25]. Only one study has measured RANKL in patients with peripheral arterial atherosclerosis, of whom 50% had diabetes, and found it to be no different to controls [15]. TRAIL which appears to play a role in plaque stability and regression has not to our knowledge ever been measured in patients with PAD.

The aim of our study therefore was to determine whether serum levels of OPG, RANKL and TRAIL are higher in patients with PAD compared to matched healthy controls, whether the diabetic state in PAD, a recognised risk factor for vascular calcification, further increases serum levels of OPG and whether OPG correlates with severity of PAD or with well established serum biomarkers of vascular inflammation such as interleukin 6 (IL-6) and high sensitivity c-reactive protein (hsCRP) in PAD patients.

Materials and Methods

Patients with documented type 2 diabetes and peripheral arterial disease were recruited from the diabetes and vascular surgery clinics in Beaumont Hospital, Dublin, Ireland. The healthy control subjects were recruited from Dublin City University, Ireland. The control group had no symptoms of intermittent claudication, normal peripheral foot pulses, no evidence of PAD on ankle brachial index (ABI) measurement, had normal glucose tolerance, as measured by a 75 gm oral glucose tolerance test (OGTT), and all completed an ECG-exercise stress test satisfactorily. Exclusion criteria consisted of malignancy, renal impairment (serum creatinine >120 μmol/l), type 1 diabetes, pregnancy, any disorder of calcium metabolism (ie hyper- or hypocalcaemia), previous diagnosis of osteoporosis or use of medications affecting bone metabolism (ie calcium, vitamin D, bisphosphonates, oestrogen preparations, strontium, parathyroid hormone), a recent (within previous 6 months) macrovascular event (defined as an acute coronary syndrome, transient ischaemic attack, stroke, lower limb ischaemic event or any vascular interventional procedure), a recent or current fracture or foot ulcer/osteomyelitis. Approval was obtained from the Research Ethics Committees at Beaumont Hospital and Dublin City University and all participants provided informed written consent.

Experimental Procedures

Patients attended between 8am and 9am, having fasted since midnight. A full clinical history and physical examination was performed in all patients. Blood pressure was taken as the average of two sedentary readings taken 15 minutes apart measured on a digital sphygmomanometer (A&D Medical). BMI was calculated as the weight (kg) divided by the height (m^2). Waist circumference was defined as the circumference around the mid-point between the lowest rib and anterior superior iliac spine.

Blood was drawn, centrifuged and serum stored at -80 °C for subsequent analysis. OPG, RANKL, TRAIL and IL-6 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits. OPG (Biomedica, Vienna) had an intra- and inter- assay variations of <6%, with a minimal detection limit of 0.014 pmol/l. The RANKL ELISA (Biomedica) measured total serum RANKL – ie that bound to OPG in addition to the free component, and the intra- and inter-assay variations were <6%, with a minimal detection limit of 0.02 pmol/l. TRAIL (R&D Systems, Oxford, UK) had an intra- and inter-assay variations of <5%, with a minimal detection limit of 2.86 ng/ml. IL-6 (Quantine HS R&D Systems) had an intra- and inter-assay variation of <5%, and minimal detection limit of 0.039 pg/ml. HsCRP was measured on the Randox Daytona analyser (Randox, Antrim, Northern Ireland). Plasma glucose, glycated haemoglobin (HbA1c), urea and electrolytes, calcium, albumin, triglycerides, total, LDL, and HDL cholesterol were measured in Beaumont Hospital using standard laboratory techniques.

On a different day to blood sampling, ABI measurements were performed in the non-invasive vascular unit on all patients with the SciMed Qvl system. Systolic blood pressures of both brachial, both dorsalis pedis and both posterior tibial arteries were measured. The ABI was calculated for each limb as the ratio of the higher ankle (dorsalis pedis or posterior tibial) pressure divided by the higher arm (brachial) pressure. The lowest index was used to define PAD. In those in whom ABIs were normal, toe pressures were measured according to the TASC II guidelines [26]. A diagnosis of PAD was made in a patient with a previous lower limb amputation (n = 3), lower limb angioplasty or bypass (n = 12), a falsely elevated ABI due to vascular calcification (ABI >1.3 *and* toe pressures <0.6) (n = 8), or an ABI <0.9 (n = 48). Severity of PAD was based on ABI value. Depending on the PAD and DM status of individual patients, four groups were created: those with PAD and DM (Group 1), those with DM alone (Group 2), those with PAD alone (Group 3), and those with neither DM nor PAD (Group 4).

Bone mineral density (BMD) was measured using the GE Lunar Prodigy 2 DXA scanner (GE Medical Systems, UK). The mean of the lumber spine (L1 – L4) and the total BMD at the femur were used to classify patients according to WHO criteria [27]. A total of 56.7% of participants underwent DXA scanning (42.3%, 45.7%, 50%, and 100% in groups 1, 2, 3, and 4 respectively).

Statistical analysis

Normally-distributed data are expressed as means ± standard error of the mean (SEM), and non-parametric data are expressed as median (range). Non-parametrically distributed data (fasting plasma glucose, IL-6, and hsCRP) were log-transformed for the purpose of analysis. Differences between groups were assessed using the unpaired Student *t*-test. Correlation coefficients were calculated using Spearman's method. ANCOVA was used to adjust for differences in age, gender and glycaemic status between groups. For correlation of OPG and ABI value, those patients with artificially elevated ABI due to non-compressible vessels were excluded from analysis. Statistical analysis was carried out using SPSS statistical package (version 15.0; SPSS Inc., Chicago IL, USA) and statistical significance was set at $p<0.05$.

Results

There were 26 patients with PAD and DM (Group 1), 35 with DM and no PAD (Group 2), 22 with PAD and no DM (Group 3), and 21 healthy controls with neither DM nor PAD (Group 4). Demographic characteristics of the 4 groups are displayed in Table 1. All groups were matched for BMI and BMD. Healthy controls (Group 4) were younger, had lower blood pressure, lower use of statins, aspirin and medications affecting the renin-angiotensin system, lower fasting plasma glucose, higher fasting cholesterol and a greater proportion of females than the other groups.

In the diabetes patients (n = 61), the median duration of diabetes was 7(range 1-35) years with a HbA1c (mean ± SEM) of 7.0 ± 0.9%. 9 (14.8%) were diet-controlled, 36 (59%) were on oral hypoglycaemics and 16 (26.2%) were prescribed insulin (either alone or in conjunction with oral meds). Only 2 patients were on a thiazolidinedione and no patients were on drugs affecting the incretin system. Of those with PAD (n = 48), mean ABI was 0.7 ± 0.2. There were 8 patients (17%) with elevated ABIs due to vascular calcification, of whom 7 had diabetes. There was a previous history of peripheral lower limb