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Biochemical and Biophysical Research Communications 293 (2002) 13–17

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A single nucleotide polymorphism in the promoter region of the human gene for osteoprotegerin is related to vascular morphology and function

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Received 22 February 2002

Abstract

Osteoprotegerin (OPG) is a secreted member of the tumor necrosis factor receptor family, and has previously been shown to regulate bone mass by inhibiting osteoclast differentiation and activation. Recent evidence indicates that OPG also plays a role in the vascular system, since ablation of the OPG gene in mice results in calcification of the aorta and renal arteries, and association has been found between serum levels of OPG and cardiovascular mortality. This study presents a novel single nucleotide polymorphism, a T/C transition located 129 bp upstream the TATA-box of the human OPG gene, detected by sequence analysis. The OPG genotype was determined by restriction fragment length polymorphism in a cohort consisting of 59 healthy subjects. The intima-media thickness (IMT) in the common carotid artery and maximal post-ischemic forearm blood flow (FBF) were investigated. Subjects with the CC genotype showed a significantly increased IMT ($p < 0.05$) and a concommitantly reduced maximal FBF ($p < 0.01$) as compared to those with the T allele. Thus, our results show that the polymorphism in the promoter region of OPG is associated with both vascular morphology and function in apparently healthy subjects.

Keywords: Osteoprotegerin; Polymorphism; Carotid arteries/physiology; Forearm/blood supply

Osteoprotegerin (OPG) is a recently cloned member of the tumor necrosis factor receptor family and was first discovered as a bone regulating protein with the capacity to decrease bone resorption [1–3]. Expression of OPG has been found in various cell types. By in situ hybridisation, high OPG expression was found in the smooth muscle wall of the incipient part of the aorta, and in the smooth muscle wall of the renal artery of mice [4], and OPG is produced by endothelial cells [5]. By binding of $\alpha v\beta 3$ integrin to osteopontin, OPG is upregulated and seems to represent an anti-apoptotic signal for endothelial cells [5]. The two major phenotypic features of mice lacking the OPG gene are found in bone and arterial walls. The OPG $-/-$ mice exhibited a decrease in total bone density, and sustained spontaneous fractures. Also, the OPG $-/-$ mice showed extensive calcification in the medial and subintimal regions of the ascending aorta and medial and subintimal calcifications in the renal artery [4]. The exact role of OPG in the vascular system is unknown. However, it has recently been shown that elevated OPG serum levels in women are associated to an up to fourfold increase in cardiovascular mortality [6].

In a sequencing effort for single nucleotide polymorphisms (SNPs) in the promoter region of OPG, we discovered a T–C transition 129 bp upstream of the TATA-box. We decided to investigate possible associations between this SNP in the promoter region and alterations in vascular morphology and function in a population based sample of apparently healthy subjects. We assessed intima-media thickness (IMT) of the common carotid artery as a measurement of structural vascular changes, and indeed IMT is regarded as a measure of early atherosclerosis [7]. Vascular function

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00137-7

was evaluated by postischemic forearm blood flow (FBF), as an index of vasodilatory capacity [8].

Materials and methods

Subjects. The cohort, recruited from a population based study in Uppsala county, Sweden, consisted of 59 apparently healthy subjects (27 females and 32 males). The original aim of this study was to investigate the effect of gender and age on cardiovascular morphology and function [8]. All subjects gave their informed consent for participation and the local ethics committee approved the study protocol. The cohort had a mean age of 50 (range: 24–76) years, mean blood pressure 121/78 (range: 95–155/65–95) mm Hg, mean body mass index (BMI) 24.7 (range = 17.9–32.5) kg/m^2, mean serum-triglycerides 0.84 (range: 0.27–2.53) mmol/l, and mean serum cholesterol 5.4 (range: 3.43–8.51) mmol/l. Subjects with a history of any cardiovascular, metabolic, or other serious diseases were neither included in the study, nor were any users of hormones or other medications.

DNA analysis. Leukocyte DNA from each individual was extracted from 3 ml whole blood using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). A 330 bp fragment corresponding to the OPG promoter region (GenBank accession number AB008821) was amplified by polymerase chain reaction (PCR) using the following primer pair: 5′-CCCAGGGGACAGACACCAC-3′ (forward), and 5′-GCGCGCAGCACAGCAACTT-3′ (reverse). PCRs were run on a Gene Amp PCR system-9700 using Ampli-Taq Gold kits and standard reagents (Perkin–Elmer, Norwalk CT, USA.). The amplification profile for the 330 bp segment of the OPG promoter consisted of denaturation at 96 °C for 10 min, followed by 36 cycles with denaturation at 96 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 1 min, and final extension at 72 °C for 7 min.

Sequence analysis. PCRs were run with the same amplification profile and the same primer sequence as above, but primers were tagged with M13-tails. Sequence reactions for both DNA strands were performed on a ABI-877 Integrated Thermal Cycler using ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Norwalk, CT, USA) followed by sequencing on a DNA automated fluorescence sequencer, ABI Prism 310 Genetic Analyser (Perkin–Elmer, Norwalk, CT, USA).

PCR-restriction fragment length polymorphism analysis. The PCR fragments were digested with 10 U of *Hin*c-II (Life Technologies, Stockholm, Sweden) at 37 °C for 2 h followed by staining with ethidium bromide and separation on a 1.5% agarose gel.

Intima-media thickness measurements of the common carotid artery. The IMT was assessed by a high-resolution B-mode ultrasonography (Acuson XP/128 with a 7.5 MHz transducer; Mountain View, CA, USA) of the right and the left common carotid artery 1–2 cm proximal to the carotid bulb. The measurements used in the present study were the mean of the IMT of the far wall of the right and left common carotid artery. Six individuals had technically inadequate IMT measurements and were therefore excluded.

Forearm blood flow measurements. Forearm blood flow (FBF) was measured as follows: a mercury insilastic gauge was placed at the upper third of the forearm and the strain gauge was coupled to a calibrated pletysmograph. Venous occlusion was achieved by a blood pressure cuff applied proximal to the elbow, and occluded forearm circulation for 5 min. Determinations of forearm blood flow were made 5 s after deflation of the cuff, by the mean of five consecutive recordings. Three individuals had technically inadequate FBF measurements and were therefore excluded.

Statistical analysis. All statistical calculations were performed using Stat View 4.5 software (SAS Institute, Cary, NC, USA). Data were evaluated by ANOVA and p-values <0.05 was accepted as the level of significance.

Results

Sequence analysis revealed a novel base pair substitution from a thymidine (T) to a cytosine (C) located at position 950 in the promoter region of OPG according to the published sequence by Morinaga et al. [9] (Fig. 1). This polymorphism is situated 129 bp upstream the TATA-box. The transition introduced a restriction recognition site for *Hin*c-II. Thus, cleavage of the 330 bp PCR product of the OPG promoter with the restriction enzyme *Hin*c-II enabled us to determine the genotype of each individual in the cohort. The distribution of the OPG alleles in the study cohort of vascular morphology and function was as follows: 17/59 (29%) were homozygous for the TT genotype, 28/59 (47%) were heterozygous, and 14/59 (24%) were homozygous for the CC genotype. The OPG allele frequencies are in agreement with Hardy–Weinberg ratios. We found no significant differences between the genotype groups with respect to age, gender, BMI, and smoking, or age at menopause in the cohort.

Subjects being homozygotic for the C allele exhibited a significantly lower maximal FBF during hyperemia, as compared to the heterozygotic (TC) and individuals homozygotic for T (Fig. 2A). Furthermore, when comparing the three groups for common carotid artery IMT, the CC genotypes had an increased IMT, as compared to the other genotypes (Fig. 2B). IMT and maximal FBF during hyperemia were inversely correlated ($r = -0.46$, $p < 0.001$). These results show a statistically significant association between our recently discovered C–T SNP in the promoter region of OPG gene and measurements of early atherosclerosis (IMT), as well as vasodilatory function in a Swedish population-based cohort of healthy individuals.

Discussion

This study shows a significant association between an SNP in the promoter region of the OPG gene and structural vascular changes indicative of early atherosclerosis (IMT) and to maximal post-ischemic vasodilatation in apparently healthy subjects.

The vascular endothelium is a major modulator of vascular smooth muscle tone and proliferation [10–12] by the production of various vasoactive substances [13–15]. Thus, the vascular endothelium may play a key role in the development of atherosclerosis, and subsequent cardiovascular disease. It has been shown that OPG is expressed in vascular endothelial cells and may be involved in inflammatory functions of these cells since nuclear factor κ-B (NFκB) activation, the target nuclear transcription factor of OPG, leads to upregulation of inflammatory mediators and leukocyte adhesion molecules [5]. Also, transgenic OPG delivered from mid-gestation through adulthood prevents arterial

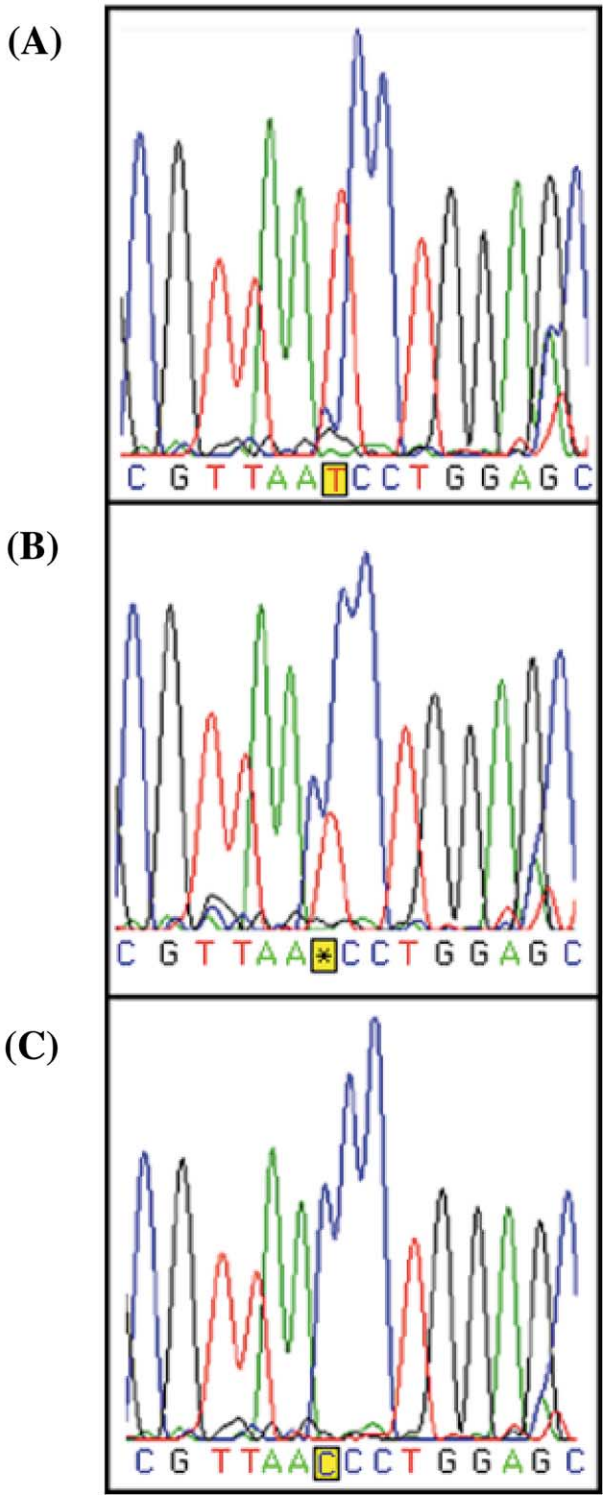

Fig. 1. DNA sequences of the polymorphic region in the OPG promoter. A 330 bp fragment of the OPG promoter was amplified by PCR. Sequence analysis revealed a single nucleotide polymorphism at position 950 of the OPG gene published by Morinaga et al. [9]. The underlined base corresponds to the nucleotide of interest. (A) DNA sequence from an individual homozygous for the TT genotype. (B) DNA sequence from a heterozygous, TC individual. (C) DNA sequence from an individual homozygous for the CC genotype.

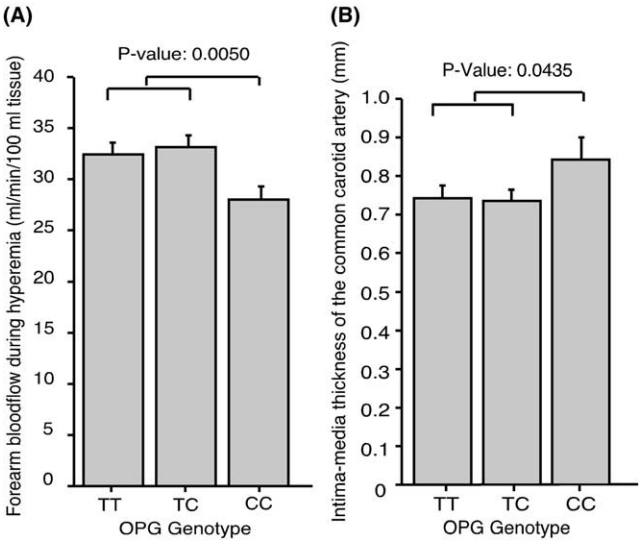

Fig. 2. Association of vascular morphology and function and OPG genotypes. Data are presented as means ± SEM for the three genotypic subgroups. Statistical analysis was performed using ANOVA. p-Values <0.05 were considered as significant. (A) Distribution of FBF during hyperemia and OPG genotypes. Group TT consisting of 14 (26%) of the individuals, TC 25 (47%) of the individuals and CC 14 (26%). Forearm blood flow (FBF) was measured with venous occlusion plethysmography as an index of vasodilatation. Individuals homozygous for C (absence of T) had a significantly lower FBF compared to individuals with a T present, $p = 0.0050$. (B) Distribution of IMT and OPG genotypes. Group TT consisting of 16 (29%) of the individuals, TC 28 (50%) of the individuals and CC 12 (21%).The intima-media thickness (IMT) was assessed by a high-resolution B-mode ultrasonography of the right and left common carotid artery. Individuals homozygous for C (absence of T) had a significantly higher IMT compared to individuals with a T present, $p = 0.0435$.

calcification in OPG −/− mice [16] and in rats, the calcification of arteries induced by warfarin or high doses of vitamin D could be prevented by OPG injections [17]. OPG serum levels also seem to be associated with cardiovascular disease in humans. In a recent study, serum OPG levels in 490 postmenopausal women were not associated with baseline bone mineral density or fractures, but to cardiovascular mortality. This finding indicates that the OPG may be involved in, or is a marker for atherosclerosis and/or vascular calcification [6]. Thus, serum OPG levels appear to be associated with cardiovascular morbidity and mortality in men. However, there may be differences between species. Although the aorta and renal arteries are sites frequently affected by atherosclerosis in humans, the calcification in the OPG −/− mouse arteries have features differing from these seen in humans. The lesions seen in the knockout mice exhibit no fat deposition, and the onset of arterial calcification occurs early in the mice, as opposed to late in the atherosclerotic process of humans [16]. These differences may be due to species specific effects, or putative compensatory mechanisms in the genetically altered OPG −/− mice.

In the present study, IMT of the common carotid artery was used as an index of early atherosclerosis. It has been shown that IMT is related to the prevalence of atherosclerotic plaques in the carotid artery, as well as in the coronary circulation [18]. Furthermore, IMT has been shown to be a powerful predictor of cardiovascular events, such as stroke and myocardial infarction [19]. Maximal FBF during hyperemia is a measurement of the vasodilatory capacity of the arterioles in the forearm. This measurement was previously thought to reflect structural changes in the small arteries [20], but it has recently been shown that this measure also is governed by the production of nitric oxide (NO) [21]. NO is known as the major vasodilatatory substance secreted by the endothelium. NO also opposes vascular inflammation, thrombosis, formation, and vascular hypertrophy, favouring vessel patency [22]. Our findings that this promoter SNP in the OPG gene relates to both IMT and maximal FBF during hyperemia, suggests associations with both structural changes visible at ultrasound and functional changes assessed by vasodilatation.

An association between cardiovascular disorders and bone metabolism disturbances is common, especially in the elderly [23,24]. Presence of atherosclerosis and arterial calcifications in osteoporotic patients is the commonest of these associations, and there is a close relation between the clinical course of arterial calcification with that of osteoporosis [25]. Since previous studies have concentrated on OPG involvement in the regulation of bone resorption, the results of our study are interesting in that we find an association to the vascular structure and function. This warrants more studies on the role of OPG in cardiovascular disease, in cohorts of different age and studying different outcomes such as hypertension, myocardial infarction, and vascular pathology, as well as BMD and fractures.

Acknowledgments

The work was supported by grants from the Torsten and Ragnar Söderberg Foundation, Stiftelsen för geriatrisk forskning (Foundation for Geriatric Research), the Swedish Rheumatism Association, the Swedish Cancer Society, Ingabritt and Arne Lundbergs Research Foundation, The Swedish Society of Medicine and Karolinska Institute.

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