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Osteoprotegerin autoantibodies do not predict low bone mineral density in middle-aged women

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ABSTRACT

Purpose: Autoantibodies against osteoprotegerin (OPG) have been associated with osteoporosis. The aim was to develop an immunoassay for OPG autoantibodies and test their diagnostic usefulness of identifying women general population with low bone mineral density.

Methods: Included were 698 women at mean age 55.1 years (range 50.4–60.6) randomly selected from the general population. Measurement of wrist bone mineral density (g/cm^2) was performed of the non-dominant wrist by dual-energy X-ray absorptiometry (DXA). A T-score < -2.5 was defined as having a low bone mineral density. Measurements of OPG autoantibodies were carried by radiobinding assays. Cut-off levels for a positive value were determined from the deviation from normality in the distribution of 398 healthy blood donors representing the 99.7th percentile.

Results: Forty-five of the 698 (6.6%) women were IgG-OPG positive compared with 2 of 398 (0.5%) controls ($p < 0.0001$) and 35 of the 698 (5.0%) women had a T-score < -2.5. There was no difference in bone mineral density between IgG-OPG positive (median 0.439 (range 0.315–0.547) g/cm^2) women and IgG-OPG negative (median 0.435 (range 0.176–0.652) g/cm^2) women ($p = 0.3956$). Furthermore, there was neither a correlation between IgG-OPG levels and bone mineral density ($r_s = 0.1896$; $p = 0.2068$) nor T-score ($r_s = 0.1889$; $p = 0.2086$). Diagnostic sensitivity and specificity of IgG-OPG for low bone mineral density were 5.7% and 92.9%, and positive and negative predictive values were 7.4% and 90.8%, respectively.

Conclusion: Elevated OPG autoantibody levels do not predict low bone mineral density in middle-aged women selected from the general population.

1. Introduction

Osteoprotegerin (OPG) was discovered in 1997 as a novel protein involved in the regulation of bone density (Simonet et al., 1997). OPG belongs to the tumor necrosis factor receptor superfamily member 11B (TNFRSF11B), which acts as a cytokine receptor for receptor activator of nuclear factor kappa B ligand (RANKL), and is furthermore essential for the regulation of bone remodeling by maintaining the correct balance between bone resorption and bone formation (Delmas, 2008). In 2009, autoantibodies against OPG were discovered in a subset of patients with severe osteoporosis (Riches et al., 2009). Although, their role in the pathogenesis are still unclear, it has been suggested that OPG autoantibodies may neutralize OPG thereby leading to increased osteoclast activity causing bone resorption (Riches et al., 2009).

Radiobinding assays (RBA), or immunoprecipitation assays, measure antibodies bound to low amount of tracer radioactive antigen in a solution and antibodies directed against both linear and conformational epitopes are precipitated by conjugated beads (Grubin et al., 1994). By coupled in vitro transcription/translation of human plasmid cDNA, potentially any antigen in presence of radioactively labeled methionine can be used for the detection of autoantibodies and protocols can be further standardized for clinical practice. RBAs are both sensitive and specific for the detection of various autoantibodies related to human disease such as type 1 diabetes (Grubin et al., 1994) and celiac disease (Agardh et al., 2005), and moreover, proven suitable as a method of screening large populations (Bjorck et al., 2010). Still, there is paucity of RBAs for the assessment of OPG autoantibodies and no study has yet evaluated their usefulness as a method of screening the general population for osteoporosis.

The aims of this study were to develop an OPG autoantibody immunoassay and test the diagnostic usefulness of IgG-OPG to predict low bone mineral density (BMD) in middle-aged post-menopausal women

Abbreviations: BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry, DXA; OPG, osteprotegerin; RBA, radiobinding assays

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selected from the general population. We hypothesized that IgG-OPG levels correlated with BMD and thus could be utilized as a potential screening marker of osteoporosis in the general population.

2. Material and methods

2.1. Subjects

A total of 10,766 women living in a defined area of Southern Sweden were identified through a population register and were asked to participate in the Women's Health in the Lund Area (WHILA) study between December 1, 1995 and February 3, 2000, and inclusion criteria and detailed characteristics are described elsewhere (Lidfeldt et al., 2002). Screening for osteoporosis was performed in 6917 post-menopausal women at mean age 56 years (range 50–64 years) from who a subset of blood samples were available from 698 women at mean age of 55.1 years (range 50.4–60.6) randomly selected for this study. As control subjects, we analyzed serum samples from 398 healthy blood donors (260 males, 133 females, 5 with unknown sex) at mean age 44.0 years of age. The Ethics committee at Malmö/Lund University approved the study (EPN 2011/9).

2.2. Bone mineral density (BMD) measurements

Measurement of wrist BMD was performed of the non-dominant wrist (at the 8 mm distal position) using dual-energy X-ray absorptiometry (DXT 200; Osteometer MediTech, Inc., Hawthorne, CA, USA) as previously described (Lidfeldt et al., 2002). In short, a phantom for daily calibration of the instrument was used, and one technician performed all measurements. BMD (g/cm^2) was automatically compared with a "reference" population furnished by the instrument supplier, giving T-scores, defined as (BMDo - BMDm)/SD, where BMDo is the obtained BMD, BMDm is the mean value for 20-year-old Danish female controls, and SD is the standard deviation in the same reference population. For the purpose of this study, we defined low BMD as T-score < -2.5 and moderately low BMD as T-score between $-2.5 <$ and < -1.0, respectively.

2.3. Sub-cloning of OPG insert (TNFRSF11Bv2) and preparation of the pThOPG vector

The cDNA construct (TNFRSF11Bv2) prepared based on the sequence in the National Center for Biotechnology Information (NCBI) was from normal pigmented retinal epithelium human eye. The fragment was purchased in the pJ201 vector from DNA 2.0 Inc. (DNA 2.0 Inc. Menlo Park, CA) was cloned into the pTnT vector (Promega, Madison, WI). The pTnT™ vector was designed with multiple cloning sites to support highly efficient expression of cloned genes, was used for in vitro coupled transcription translation system in the presence of 35S-methionine. The cDNA construct (TNFRSF11Bv2) fragment was cut from the pJ201 vector (2 μg) (DNA2.0 Inc.) with EcoRI and NotI (FastDigestTM, Fermentas Sweden AB, Helsingborg, Sweden) using the FastDigest buffer 10 (Fermentas) using the restriction buffer, NEB2 10 × (New England Biolabs, Inc.). The linearized pTnT™vector and OPG construct were separated and analyzed by gel electrophoresis in 1% agarose and the two bands extracted using a gel purification kit (QIAquick Gel Extraction Kit, QIAGEN AB, Solna, Sweden) according to the manufacturer's instructions. Subcloning efficiency DH5α competent cells were used for transformation according to manufacturer's instructions (Invitrogen AB, Stockholm, Sweden). The pThOPG plasmid DNA was extracted using the QiaPrep Spin MiniPrep Kit (QIAGEN AB). The insert was sequenced by GATC Biotech AG (Konstanz, Germany) using the 5′-TTA CGC CAG CCCGGATCC-3′ and 5′-AAG GCT AGA GTA CTT AAT ACG A-3′ as the reverse and forward primers, respectively (DNA Technology A/S, Risskov, Denmark).

2.4. Coupled in vitro transcription–translation of the OPG plasmid (pThOPG)

The coupled in vitro transcription translation of the pThOPG vector were assembled in a single step one reaction mixture containing 2 μg of pThOPG (reaction concentration, 0,5 μg/μL), 50 μL TNT®rabbit reticulocyte lysate, 4 μL TNT®reaction buffer, 2 μL amino acid mixture without methionine, 2 μL RNasin® Ribonuclease inhibitor, 2 μL SP6 RNA Polymerase (all from Promega, Madison, WI), 4 μL [^{35}S]-methionine (EasyTagTM, 1175 Ci/mmol Perkin Elmer, Boston) and nuclease-free water to a final volume of 100 μL. The reaction mixture was incubated for 90 min at 30 °C with shaking (300 rpm) in a ThermoMixer® comfort, Eppendorf, AG Hamburg, Germany). The translation product was subjected to a filtration Illustra™ NAP™-5 Columns (GE Healthcare, Buckinghamshire, UK) for removal of unincorporated label. The incorporation of [^{35}S]-methionine radioactivity into the OPG peak fraction was counted in a β-counter (1450 MicroBeta®TriLux Microplate Scintillation and Luminescence Counter, (PerkinElmer Turku, Finland). and the concentration was determined (cpm/μL). The concentration of pThOPG was pre titrated for optimal protein expression for one reaction. The sum of the incorporated product, compared with total sum unincorporated product was calculated (cpm/μL). The incorporation yield of the [^{35}S]-methionine radioactive label into the pTnT-rht OPG was calculated to an average 20%.

2.5. IgG-OPG autoantibody radiobinding assays (RBA)

Measurements of IgG-OPG autoantibodies were carried out by RBA as previously described for tissue transglutaminase autoantibodies with slight modifications (Kjelleras et al., 2011). In short, duplicate samples of 2.5 μL undiluted serum and a standard of rabbit anti human Osteoprotegerin (catalog no ab9986, Abcam, Cambridge, UK) diluted 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, and 1:5120 in antigen buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 0.15% (v/v) Tween 20, 0.1% (w/v) BSA) and 60 μL labeled OPG-antigen at a final concentration of 425 ± 25 cpm/μL in antigen buffer were added in V-bottom 96-well plates (catalog no 442587, 96-well MicroWell™ PP plates, Nunc A/S, Roskilde, Denmark) and incubated over night at 4 °C on a plate shake (950 rpm) (Wallac Delfia® Plate Shake Perkin Elmer, Turku, Finland). A total of 50 μL of the serum and labeled OPG-antigen mixture was then transferred to a 96-well MultiScreen-DV Filter Plate (catalog nr MSDVN6B50, Merck Millipore S.A.S, Molsheim, France) (pre-coated with antigen buffer over night at 4 °C) and 50 μL 20% Protein A Sepharose 4B conjugated in antigen buffer (catalog nr 10-1090, Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA) was added and incubated on a plate shake (950 rpm) for 90 min at 4 °C. The plate was then washed 8 times in antigen buffer using a microplate washer (ELx50TM Microplate Washer, BioTek Winooski, VT). 50 μL of scintillation cocktail OptiPhase Supermix (PerkinElmer Health Inc., Waltham, MA) was added. The antibody-antigen and Protein A Sepharose bound radioactivity was counted 1 min (cpm) in a β-counter.

2.6. Statistical analysis

The SPSS 18® statistical package (SPSS Inc. Chicago, IL) was used for statistical analysis. A p value 0.05 was considered as significant. Pearson Chi square test of independence (and Yates' correction for continuity value when applied) was used to assess differences in frequencies of autoantibody positivity. Levels of IgG-OPG were expressed as U/mL and calculated from standard curves. Background signal (antigen buffer) was subtracted from samples and standard curves (Fig. 1). Cut-off levels for a positive value were determined using quantile-quantile (QQ) plots to identify deviation from normality in the distribution of the 398 controls and set at > 3.0 U/mL representing the 99.7th percentile of 398 healthy blood donors (Fig. 2).