ELISA Methodology for Detection of Modified Osteoprotegerin in Clinical Studies, De Chen,* Nihal A. Sarikaya, Han Gunn, Steven W. Martin, and John D. Young (Department of Pharmacokinetics and Drug Metabolism, Amgen, Inc., One Amgen Center Dr., Thousand Oaks, CA 91320; * author for correspondence: fax 805-499-4953, e-mail dchen@amgen.com)

Osteoprotegerin (OPG), also known as osteoclast inhibitory factor, is a soluble receptor of the tumor necrosis factor receptor superfamily. The protein is secreted as a covalent, disulfide-linked homodimer, which is the predominant extracellular form (1), and is expressed in multiple tissues (1–3). OPG-mediated pathways might have a role in osteoporosis (3–6) because estrogen increases OPG gene expression (4, 5). OPG maintains the structure of healthy bone and inhibits osteoclast activation and differentiation (3, 7). In the vascular system, OPG inhibits pathological calcification in the media intima (3). OPG has been proposed for therapy of osteopenic disorders, such as postmenopausal osteoporosis, Paget disease, rheumatoid arthritis, hypercalcemia, and lytic bone metastases (8).

Initially, we developed an antibody-based ELISA method with an anti-human OPG monoclonal antibody for capture and an anti-human OPG polyclonal antibody for detection. Yano et al. (9) raised the concern for us that we may not detect the active dimeric OPG with antibody capture because they reported that serum OPG increased with age and that the monomer was the predominant form of OPG in human serum. Although they used a different antibody-dependent ELISA method (monoclonal capture and detection), the results reported by Yano et al. (9) do not correspond with the work performed at Amgen (1, 3–5, 7, 8, 10–12). We reasoned that OPG ligand (OPGL) (2, 7, 8, 10–16), also known as osteoclast differentiation factor, is a potential alternative capture protein for an OPG assay. In an attempt to develop an assay that would measure all bioactive form(s) of OPG, we developed an ELISA assay that uses OPGL as the capture protein. To avoid problems posed by batch-to-batch variability of human serum pools for use as assay diluent, assay development was used to define a serum substitute.

Assay development was performed with AMGN-0007, a modified OPG. Calibrators and quality-control (QC) materials were prepared in human serum or serum substitute. Whereas calibrators were serially diluted, QC materials were prepared individually. Calibration curves were prepared using calibrators containing 0.020–500 μg/L AMGN-0007. Each calibration curve contained at least nine points, including the zero calibrator.

OPGL, AMGN-0007, and murine monoclonal antibody were purified essentially as described previously (1, 7). OPGL was coated onto 96-well microtiter plates (Costar). Plates were blocked with 2 mL/L I-Block (Tropix) and 5 mL/L Tween 20 (Pierce) in phosphate-buffered saline (PBS). Assay buffer, calibrators, and QC materials were added to the wells. After all unbound substances were removed by washing, murine anti-human OPG monoclonal antibody was added to the wells. After another wash, goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP; Zymed) was added to the wells. After the final wash, KPL TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories) was added to the wells. The colorimetric reaction was stopped with 0.812 mol/L phosphoric acid. The color intensity was measured at 450–650 nm with a ThermoMax Microtiter Plate Reader (Molecular Devices).

The full-length OPG homodimer (OPG-FLD) and the full-length OPG monomer (OPG-FLM) were purified from conditioned medium with Sepharose columns and concentrated into PBS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to confirm size and purity (95%) of the monomeric and dimeric OPG. Calibrators and QC materials were prepared for each OPG analog: AMGN-0007, OPG-FLD, and OPG-FLM. The assay was performed as described above, except that we used HRP-conjugated murine anti-human OPG monoclonal antibody.

Prepared in PBS, human serum substitute buffers contained 30 mL/L human serum albumin (HSA; Bioreclamation, Inc.) and various concentrations (0–500 mL/L) of fetal bovine serum (FBS; Sigma Chemical Co.). Calibrators and QC materials were prepared and assayed as described above.

OPGL was immobilized on the surface of the microtiter plate. AMGN-0007 was then added to the plate at concentrations of 0.244–31.25 μg/L, the calibration curve range. The analyte was detected with murine anti-human OPG monoclonal antibody plus goat anti-mouse IgG-HRP. Defined as two times the zero calibrator signal, the detection limit was 0.244 μg/L. Other assay configurations, such as monoclonal capture with polyclonal detection and ligand capture with polyclonal detection, were studied and demonstrated low signal-to-noise ratios throughout the calibration curve. For the dynamic range of interest, OPGL capture followed by monoclonal detection produced the best signal-to-noise ratio throughout the calibration curve; most likely, the result was attributable to the specificity of both the ligand and the monoclonal antibody for AMGN-0007.

The abilities of OPG analogs to bind to solid-phase-bound OPG were compared (Fig. 1). The signal at 50 μg/L OPG analog for OPG-FLD (2.352 absorbance units) was very similar to that of AMGN-0007 (2.411 absorbance units), whereas OPG-FLM had signal strength of 1.693 absorbance units. Compared with AMGN-0007 and OPG-FLD, OPG-FLM demonstrated a curve shift to the right.
and a lower signal strength throughout the calibration curve. Although monomeric OPG did bind to OPGL, the ligand appeared to have greater affinity for dimeric OPG. Results from a previous study using pulse-chase labeling and immunoprecipitation of extracts from Chinese hamster ovary cells transfected with OPG suggested that the primary extracellular form of OPG is the homodimer (17). Tomoyasu et al. (17) also determined that dimeric OPG was secreted in greater amounts than monomeric OPG in mammalian cell systems and was biologically more active than monomeric OPG. In addition, they suggested that dimeric OPG might bind to heparin and be transported to the target sites more rapid than monomeric OPG (17); this may explain the observations of Yano et al. (9).

Plapp et al. (18) suggested that QC materials be composed of the same matrix as the specimen. With human serum as diluent, problems may arise for the following reasons: (a) the presence of infectious agents that cannot be screened; (b) batch-to-batch variability attributable to endogenous factors (19); and (c) a finite shelf life that may fall short of the time spans of clinical studies. To minimize the variability for pharmacokinetic profile studies, a buffer that could be substituted for human serum as an assay diluent was sought. HSA makes up 30–55 g/L of serum (20, 21). Therefore, development was focused on the normal concentration of albumin to maintain a total protein content similar to the low end of the HSA reference interval. Weber et al. (19) suggested that the addition of bovine serum might eliminate interference of heterophilic antibodies. After comparing the absorbance and slope obtained for bovine serum with those obtained for human serum, we chose 30 mL/L HSA with 100 mL/L FBS as the serum substitute and diluent for AMGN-0007 assays. Differences in the assay signals obtained for the serum substitute and human serum were tested for statistical significance using one-way ANOVA (Table 1). No significant differences (P >0.05) were observed among the signals obtained at different concentrations of human serum in substitute diluent, suggesting that the human serum substitute was an adequate substitute for human serum as assay diluent.

In conclusion, we developed an ELISA AMGN-0007, with OPGL for analyte capture and a monoclonal antibody specific for detection, and found that an albumin-based diluent can be used. The ELISA will be useful for analyzing samples from clinical trials as well as for monitoring therapeutic efforts for osteopenic diseases. Finally, this tool may enable the development of an assay to measure endogenous OPG concentrations.

Table 1. ANOVA results for QC materials prepared in different diluents.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>QC 1 (20 μg/L)</th>
<th>QC 2 (5 μg/L)</th>
<th>QC 3 (1 μg/L)</th>
<th>QC 4 (0.7 μg/L)</th>
<th>QC 5 (0.5 μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mL/L HSA + 100 mL/L FBS in PBS</td>
<td>21.930</td>
<td>5.035</td>
<td>0.891</td>
<td>0.600</td>
<td>0.426</td>
</tr>
<tr>
<td>1 mL/L human serum in substitute diluent</td>
<td>22.850</td>
<td>5.186</td>
<td>0.928</td>
<td>0.600</td>
<td>0.426</td>
</tr>
<tr>
<td>10 mL/L human serum in substitute diluent</td>
<td>21.660</td>
<td>4.862</td>
<td>0.880</td>
<td>0.649</td>
<td>0.401</td>
</tr>
<tr>
<td>100 mL/L human serum in substitute diluent</td>
<td>21.250</td>
<td>4.775</td>
<td>0.880</td>
<td>0.612</td>
<td>0.401</td>
</tr>
<tr>
<td>Human serum only</td>
<td>21.740</td>
<td>4.580</td>
<td>0.880</td>
<td>0.637</td>
<td>0.426</td>
</tr>
<tr>
<td>F</td>
<td>3.741</td>
<td>4.902</td>
<td>0.424</td>
<td>0.534</td>
<td>0.870</td>
</tr>
<tr>
<td>P</td>
<td>0.090</td>
<td>0.056</td>
<td>0.787</td>
<td>0.718</td>
<td>0.541</td>
</tr>
<tr>
<td>F_{c,s} (critical)</td>
<td>5.192</td>
<td>5.192</td>
<td>5.192</td>
<td>5.192</td>
<td>5.192</td>
</tr>
</tbody>
</table>

*QC materials and calibrators were prepared with AMGN-0007 in substitute diluent, human serum, or human serum combined with substitute diluent. Assay range was 0.484–62 μg/L.*

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References

Cysteine is the only sulfhydryl-containing amino acid in proteins and is the thiol residue in glutathione (1). In addition to its importance in the storage and transport of cysteine, glutathione plays a pivotal role in detoxification by the action of glutathione S-transferases and in scavenging free (oxygen) radicals by the action of glutathione peroxidases (1).

Thiol metabolism may be altered during pregnancy. In healthy pregnant women, plasma concentrations of cysteine, glutathione, and homocysteine are decreased, whereas increased homocysteine and cysteine concentrations are seen in pathologic conditions, such as pre-eclampsia, in which oxidative stress may play an important role (2–4).

Thiols may have important physiological functions in fetal metabolism. Although protein and amino acid turnover in the human placenta has been studied extensively (5–7), few data concerning fetal concentrations of thiols and placental maternal-fetal thiol interactions are currently available (8). During normal pregnancy, fetal growth depends on the supply of nutrients from the mother, and a clear correlation between maternal and fetal amino acid and homocysteine concentrations has been shown (6–8). Decreased concentrations of amino acids in the umbilical artery, as compared with the umbilical vein, have been interpreted as an uptake of amino acids into fetal tissues where they may be used in protein biosynthesis or as sources of energy (5). We studied fetal and maternal thiol plasma concentrations in normal pregnancies to determine reference concentrations for cysteine, homocysteine, and cysteinylglycine in arterial and venous umbilical cord plasma to gain insight into maternal-fetal thiol interactions.

Arterial and venous umbilical cord blood samples from 320 consecutive neonates were drawn in preheparinized tubes immediately after birth (cat. no. 260545; Kemper Medical) from March 1997 to January 1998 at the Department of Obstetrics/Gynecology of the ‘Nij Smellinghe’ Hospital. Approval for this study was granted by the Institutional Review Board of the hospital. Blood gas values were assessed on an ABL-330 analyzer (Radiometer Nederland). Samples with a pH difference between arterial and venous umbilical cord blood <0.02 pH units and samples from neonates with a gestational age <37 weeks, an umbilical artery pH <7.20, a birth weight below the 10th percentile according to Kloosterman (9), or who were the offspring of women with a diastolic blood pressure >90 mmHg during gestation were excluded. Antecubital maternal venous blood of 35 women was collected in parallel with the umbilical cord samples after elective caesarian delivery or vaginal birth. Samples were taken in heparinized tubes (cat. no. 367684; Becton and Dickinson) <4 h before elective caesarian delivery or <15 min after vaginal birth. Blood was centrifuged within 10 min at 1200g for 10 min at room temperature. Plasma samples were stored at −30 °C for ~2 years until analysis. Plasma concentrations of total cysteine (tCys), total homocysteine (tHcy), and total cysteinylglycine (tCysGly) in 195 umbilical cord (102 males and 92 females; no gender was recorded for 1 neonate) and 35 maternal samples were determined by HPLC as described previously (2).

After log transformation to approach normalization, we analyzed the data by the paired t-test to assess statistical differences between maternal venous blood and arterial