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Additional Keyphrases: immunoradiometric assay • radioimmu-
nosay • monoclonal antibodies

Two-Site Assays of Bone Gia Protein (Osteocalcin) Demonstrate Immunochemical Heterogeneity of the Intact Molecule

Leonard J. Deftos, Robert L. Wolfert, Craig S. Hill, and Douglas W. Burton

We developed a panel of monoclonal antibodies to human bone gia protein (BGP; osteocalcin) peptides that span
the linear sequence of the molecule, specifically BGP 1–12 (N-terminal), BGP 15–30 (midregion), and BGP
38–49 (C-terminal). These antibodies were evaluated in various combinations of two-site formats in studies of
serum BGP concentrations. For clinical studies, we selected from a panel of antibodies the two most sensitive
antibody pairs for the intact molecule (N-C); we also used a polyclonal RIA based on BGP-C. For the two-site
format, we used two N-terminal antibodies, 029 and 052, adsorbed to polystyrene beads, and radiiodinated a
C-terminal antibody, 663. The standard for each of the

1 Department of Medicine, San Diego VA Medical Center, the
University of California, San Diego, CA. Address for correspond-
dence: 3350 La Jolla Village Drive, Mail Code V-111C, San Diego,
CA 92161.
2 Hybrittech, Inc., San Diego, CA.
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The measurement of circulating concentrations of bone gla protein (BGP; osteocalcin) has become a clinically useful procedure in a wide variety of skeletal disorders (1–3). Results from the several laboratories that have developed and applied BGP assays are in general agreement that the protein is increased in many skeletal diseases (4–13). However, differences have also appeared in the BGP measurements performed by different laboratories (12–14). Some of these differences have been attributed to technical aspects of different assay procedures, whereas others have been attributed to immunochemical heterogeneity of circulating BGP (6, 7, 12–14). To evaluate these differences, especially those attributed to BGP heterogeneity, we developed two-site immunoradiometric assays of BGP and applied them to clinical studies.

Materials and Methods

Monoclonal antibodies were produced against synthetic peptides of human BGP that span the linear sequence of the 49-residue protein, BGP 1–12 (N-terminal), BGP 15–30 (midregion), and BGP 38–49 (C-terminal) by using previously described protocols (15, 18). The peptides (Bachem, Torrance, CA) were coupled to keyhole limpet hemocyanin and injected into BALB/C and Swiss mice (15). The mice were immunized with Freund’s adjuvant for the initial injection, followed by incomplete Freund’s adjuvant at day 14. Booster injections with peptide in phosphate-buffered saline (50 mmol/L phosphate, 150 mmol/L NaCl; PBS) were repeated at 14-day intervals. Fusion of P3 myeloma and spleen cells from immunized animals was accomplished by previously described techniques (15). Serum titers and initial screening of clones were performed by immunoassay procedures as previously described (15). Hybridomas that were identified by binding studies with 125I-labeled BGP to be secreting BGP-reactive antibodies were grown in mouse ascites and evaluated in two-site formats (15).

The details of the assay procedures have been previously detailed and are only summarized here (15–18). Each of the antibodies was purified from its ascites by chromatography with Staphylococcus Protein A (Calbiochem, San Diego, CA). Purified antibody was radioiodinated and adsorbed to 8-mm polystyrene beads (Hoover, Cumming, GA), as previously described (15). In brief, the antibodies were adsorbed to the beads at a concentration of 1 mg/L in sodium bicarbonate buffer, pH 9.6, for 24 h at 4 °C. The beads were washed three times with PBS, pH 7.2, and stored in this at 4 °C in the presence of sodium azide (0.1 g/L) for as long as 4 weeks until use. Assay incubations were conducted at 4 °C as previously described (18). Serum samples and standard were added to the assay incubation in different amounts for quantification. All incubations were performed at 4 °C for 36 h in PBS, pH 7.4, containing bovine serum albumin (Pentex, Kankakee, IL), 5 g/L. The BGP standard was purified from human bone and its purity was confirmed by HPLC and automated Edman analysis (1, 2, 17). At the end of the incubation, the beads were washed and the bound radioactivity was counted (16).

Results

The antibody evaluation procedures identified monoclonal antibodies to each of the BGP peptides that span the linear sequence of the molecule, specifically BGP 1–12 (N), BGP 15–30 (M), and BGP 38–49 (C). There was no cross-reactivity among the antibodies to the different peptides, and each recognized the purified human BGP standard (2). Various antibody pairs evaluated in two-site formats identified the human BGP (Figure 1). These assay formats gave the results for a screening panel of human samples summarized in Table 1. Considering the different performances of the assays, we then selected the two best-performing antibody pairs (Figure 1) for the intact molecule, 029–663 and 052–663, to assay the serum of normal subjects, patients with renal failure, and patients with Paget disease; we also assayed these samples with a polyclonal RIA based on BGP-C (1, 5) (Table 2). The two intact assays were significantly (P < 0.01) correlated (r = 0.94), but there was greater than a twofold difference in their absolute values (Figure 2). The polyclonal RIA significantly correlated with each (r = 0.83, 0.77) of the intact assays, but it, too, gave serum values for BGP that differed from the results of the other two assays (Figure 3). The inter- and intra-assay CVs were <15% and 10%, respectively, for all three assays.

Discussion

Radioimmunoassays of BGP are clinically useful procedures in assessing and managing patients with various metabolic bone diseases (1–3). Serum BGP concen-
Table 1. Screening Evaluation of Two-Site Immunoradiometric Assay Formats for Measuring BGP in Pilot Serum Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>N1-C1*</th>
<th>N2-C1</th>
<th>C2-M</th>
<th>C3-H1</th>
<th>C4-H1</th>
<th>C1-H1</th>
<th>C5-H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.6±</td>
<td>4.2</td>
<td>5.0</td>
<td>5.0</td>
<td>3.1</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>4.6</td>
<td>5.2</td>
<td>—</td>
<td>—</td>
<td>7.2</td>
<td>1.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Normal</td>
<td>3.0</td>
<td>2.9</td>
<td>—</td>
<td>—</td>
<td>2.8</td>
<td>3.7</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Renal failure</td>
<td>10.3</td>
<td>9.9</td>
<td>6.2</td>
<td>4.6</td>
<td>12.0</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Renal failure</td>
<td>9.6</td>
<td>9.4</td>
<td>—</td>
<td>—</td>
<td>12.3</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Paget disease</td>
<td>105</td>
<td>94</td>
<td>&gt;70</td>
<td>&gt;80</td>
<td>86</td>
<td>66</td>
<td></td>
</tr>
</tbody>
</table>

*Antibody designations according to Fig. 1: N1 = 029, N2 = 052, N3 = 045, C1 = 663, C2 = 009, C3 = 009, C4 = 660, C5 = 022, M = 060. For each of the indicated antibody pairs, the first antibody was adsorbed to polystyrene and the second was radiolabeled (15, 16); e.g., in the first column, N1 (BPM029) was adsorbed to polystyrene and C1 (BPB663) was radiolabeled.

Table 2. BGP Concentrations Measured by Two Two-Site Assays for the Intact Molecule and by a Polyclonal Antibody Assay (1)

<table>
<thead>
<tr>
<th>BGP, μg/L, mean (SE)</th>
<th>n</th>
<th>N1-C1</th>
<th>N2-C1</th>
<th>Polyclonal (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>10</td>
<td>7.04</td>
<td>21.45</td>
<td>2.6 (0.17)</td>
</tr>
<tr>
<td>Renal failure</td>
<td>15</td>
<td>24.5</td>
<td>43.75</td>
<td>12.63 (1.5)</td>
</tr>
<tr>
<td>Paget disease</td>
<td>5</td>
<td>12.3</td>
<td>30.46</td>
<td>5.16 (0.77)</td>
</tr>
</tbody>
</table>

Antibody designations: N1 = 029, N2 = 052, C1 = 663 (see Fig. 1 and Table 1).

Fig. 2. Comparison of the measurement of BGP in serum samples from human subjects by the two immunoradiometric assays described in Table 1
Slope is 1.40, y intercept 10.22. Note the difference in scale for the two assays

Fig. 3. Comparison of the measurements of BGP by the two assays described in Table 1 and the polyclonal assay (1)
Top: slope is 3.11, y intercept is 2.62; bottom: 4.33 and 0.23. Antibody BPB663 was used as tracer for both assays

Concentrations are increased in patients with bone diseases characterized by increased osteoblastic activity, such as Paget disease, osteomalacia, osteitis fibrosa, and renal osteodystrophy, and in some patients with skeletal metastases (4–8). In many, but not all, of these disorders, serum BGP measurements correlate with other indices of bone formation, e.g., serum alkaline phosphatase, bone histomorphometry, and skeletal imaging procedures (4, 5). Thus, the early clinical studies of BGP as measured by RIA (1, 3, 5) established the utility of this protein in skeletal assessment: it is increased in diseases characterized by increased bone formation; unlike serum alkaline phosphatase activity, it is specific for bone and it responds rapidly (within hours) to perturbations in skeletal homeostasis. There are, however, exceptions to these generalizations. In patients with skeletal metastases, the concentrations of serum BGP are not consistently increased (19). Patients with Paget disease often show no correlation between serum BGP and serum alkaline phosphatase (5, 18, 20). Inconsistent results have been reported about the effects of alcohol and other drugs on BGP (21, 22). Variable BGP results have been reported in patients with osteoporosis (both high and low turnover), and contradictory reports con-
continue to appear about the effects of age on serum BGP measurements, with normal, increased, and decreased results being reported in response to these two variables (2, 3, 12–14). Some laboratories have observed a circadian pattern for serum BGP, whereas others have not (23–25). Similarly inconsistent measurements have been reported for BGP across the menopause, across the menstrual cycle, and during pregnancy (21, 26). Although some of the published discrepancies involving BGP may represent clinical differences, others apparently represent technical differences among the various assays (12, 13). Newer assay formats may help to resolve some of these problems (14, 27).

Some of the differences in BGP measurements from different laboratories can be attributed to the immunochemical heterogeneity of the circulating form of the protein (6, 7, 12–14). Our results offer further evidence for such heterogeneity, even when the intact molecule is being measured (Figure 1). In a screening assay with monoclonal antibodies, different assay formats gave different results for the same panel of serum samples (Table 1). Results of a polyclonal assay also differed from the results of monoclonal-based assays (Table 2). Furthermore, two-site assays based on monoclonal antibodies to the same N- and C-terminal BGP peptides also gave different assay results when compared with each other (Figure 2) and with a polyclonal antibody assay (Figure 3). Thus, quantitative differences for serum BGP are obtained with antibodies that are directed against the same peptide fragment of BGP. Presumably, the different antibodies recognize different epitopes within the peptide antigen used for their generation. However, despite the differences in absolute values of serum BGP that these assays demonstrate, there is good correlation among them (Figures 2 and 3). Of course, some of the differences observed are possibly attributable to assay matrix components and to differential recognition by the antibodies between the synthetic peptide and the corresponding peptide in the intact molecule. Nevertheless, with appropriate interpretation, the measurement of serum BGP remains a useful clinical tool in skeletal assessment despite the heterogeneity of the molecule in serum (12, 13). Furthermore, the availability of region-specific antibodies for BGP should help elucidate the structural basis for the immunochemical heterogeneity of this protein in serum (28).

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