HIGH–MOLECULAR-WEIGHT AGGREGATES IN REPACKAGED BEVACIZUMAB

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Purpose: The antivascular endothelial growth factor agents ranibizumab and bevacizumab are used to treat ocular neovascular diseases. There have been recent reports of sustained elevation of intraocular pressure after use of either agent, which we hypothesize could be because of high–molecular-weight aggregates.

Methods: Enzyme-linked immunosorbent assay, size exclusion chromatography, and polyacrylamide gel electrophoresis were used to analyze repackaged bevacizumab syringes obtained from three outside compounding pharmacies and samples obtained directly from the original vial. Microflow imaging was used to examine particulate material within samples.

Results: All syringes contained statistically similar amounts of protein, consisting of immunoglobulin (IgG) heavy and light chains (polyacrylamide gel electrophoresis). However, two of the three compounding pharmacies' batches had significantly less functional IgG in the solution (enzyme-linked immunosorbent assay). Additionally, the compounding pharmacies with the lowest IgG (~50%) also contained 10-fold the number of micron-sized particulate matter as measured by microflow imaging.

Conclusion: There are significant differences in IgG concentration measured from repackaged bevacizumab syringes. A trend exists for an increase in micron-sized protein aggregates with the decrease in IgG concentration. Large particulate matter within some samples may lead to obstruction of aqueous outflow and subsequent elevation in intraocular pressure. Additional studies are warranted to explore these findings.

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The most commonly used antivascular endothelial growth factor agents for ophthalmic neovascular diseases are bevacizumab and ranibizumab. Ranibizumab (Lucentis, Genentech, Inc., South San Francisco, CA), a Fab fragment of a recombinant humanized immunoglobulin-1 (IgG1) kappa isotype murine monoclonal antibody, is Food and Drug Administration-approved specifically for treating wet age-related macular degeneration.[1] Bevacizumab (Avastin, Genentech, Inc.), a humanized monoclonal antibody, is Food and Drug Administration-approved for treating colorectal cancer and is commonly used as an off-label treatment for ocular neovascular disease.[2] Although the use of intravitreal bevacizumab (IVB) for ocular disease did not go through the Food and Drug Administration approval process, published reports suggest that it is safe and apparently effective at treating wet age-related macular degeneration.[3,4]

Despite the apparent safety profile of both IVB and ranibizumab, there have been recent reports of elevated intraocular pressure (IOP) after single or multiple injections of either agent.[5–8] The number of reported cases of IOP spikes has, overwhelmingly, involved IVB prepared in plastic syringes by one or more repackaging formularies with fewer cases involving ranibizumab obtained directly from the manufacturer.[6–7] In most cases, the affected patients had no

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history of ocular hypertension or glaucoma and were noted to have stable IOP before intravitreal injections. Often medical therapy, laser trabeculoplasty, and/or filtration surgery was required to decrease IOP to safe levels. In this report, we seek to investigate the hypothesis that repackaged bevacizumab may contain aggregated proteins or other particulate contaminants that could potentially lead to alterations in aqueous humor outflow facility with subsequent spikes in IOP.

Materials and Methods

Bevacizumab

Repackaged bevacizumab (either 1.25 mg/0.05 mL or 2.5 mg/0.10 mL) was ordered from 3 external compounding pharmacies (CPs) and arrived in plastic syringes. Bevacizumab in its original glass vial was ordered through the University of Colorado Hospital (UCH) outpatient pharmacy and was drawn into plastic syringes immediately before assays were performed in our laboratory. Comparisons were made between the groups (n = 5 each) of syringes.

Protein Content

Total protein content was determined by the bicinchoninic acid method (Micro BCA Protein Assay Kit, Pierce/Thermo Scientific, Rockford, IL). Bevacizumab preparations (25 mg/mL) were diluted 1:100 (0.25 mg/mL) in phosphate-buffered saline (8 g/L sodium chloride, 2.16 g/L sodium phosphate dibasic heptahydrate, 0.2 g/L potassium phosphate monobasic, and pH 7.4). A standard curve (0.025–1.5 mg/ mL) was prepared from a serial dilution of purified human IgG (Pierce/Thermo Scientific). The final 562 nm absorbance was determined using a Synergy 4 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Protein concentrations were determined by fitting absorbance data to a linear regression of the standard curve absorbances. In each syringe, bevacizumab protein content was determined from the average of triplicate assays.

Immunoglobulin Content

Total IgG concentration was determined using the Easy-Titer Human IgG (H+L) Assay Kit (Pierce/ Thermo Scientific). Bevacizumab formulations (25 mg/mL) were diluted $1{:}10^5$ (250 ng/mL) in the supplied dilution buffer. A standard curve from 15.6 ng/mL to 500 ng/mL was prepared from a serial dilution of purified human IgG in dilution buffer. The final 410 absorbance was determined using a Synergy 4 Multi-Mode Microplate Reader. IgG concentrations were determined by fitting absorbance data to a semi-log regression of the standard curve absorbances. In each syringe, bevacizumab IgG content was determined from the average of duplicate assays.

Protein Molecular Weight Determination by Polyacrylamide Gel Electrophoresis

Bevacizumab samples were separated on NuPAGE Novex 10% Bis-Tris Gels (Invitrogen) using MOPS running buffer (Invitrogen). Three micrograms of each sample were prepared in NuPAGE LDS sample buffer (Invitrogen) with or without dithiothreitol as a reducing agent and heated to 70°C for 10 minutes before loading. Gels were run for ~1.5 hours at 150 W and stained with Coomassie Blue R-250 to visualize protein bands.

Size Exclusion Chromatography

Chromatography of bevacizumab samples was performed by fast protein liquid chromatography using a Superose 6 10/300 GL column (GE Healthcare Bio-Sciences, Piscataway, NJ) operated at a flow rate of 0.5 mL/minutes of phosphate-buffered saline. Protein elution was monitored with an inline ultraviolet absorbance detector, and fractions were collected throughout. Protein standards included blue dextran (2,000 kD), thyroglobulin (669 kD), ferritin (450 kD), aldolase (158 kD), and ovalbumin (45 kD).

Microflow Imaging

Microflow imaging (MFI) (DPA 4100, Brightwell Technologies Inc., Ontario, Canada) determines particle size and number by imaging a fluid as it passes through flow cells using a digital camera and software filters. Bevacizumab samples were diluted 10-fold with bevacizumab placebo (50 mmol/L sodium phosphate, pH 6.25, 60 mg/mL α,α-trehalose dihydrate, and 0.4 mg/mL polysorbate 20) before injection into the MFI flow cell. For control/baseline particle measurements, 50 μL of bevacizumab placebo formulation was drawn into a 1-mL tuberculin syringe, expelled into an Eppendorf tube, and diluted 10-fold with more bevacizumab placebo formulation before injection.

Results

The protein content in the 5 syringes obtained from the university pharmacy (UCH) and the 3 CPs (CP1, CP2, and CP3) had an average protein content of 29.5, 29.2, 30.6, and 30.6 mg/mL, respectively. The data are shown in Table 1. By using analysis of variance for comparison, there was no statistically significant

Table 1. Assay of Bevacizumab Content

Source	Protein (mg/mL)	IgG (mg/mL)
UCH	29.5 ± 0.8	23.8 ± 1.4
CP1	29.2 ± 1.2	21.4 ± 3.1
CP2	30.6 ± 0.2	18.5 ± 3.1*
CP3	30.6 ± 1.7	12.1 ± 1.2†

*$P < 0.01$.
†$P < 0.000001$.

difference in total protein content among any of the groups of syringes ($P = 0.14$). In contrast, statistically significant differences were noted in the concentration of IgG between the UCH-obtained syringes and both CP2 ($P < 0.01$) and CP3 ($P < 0.000001$) but not between UCH and CP1 ($P = 0.15$). The average amount of IgG contained in the 5 syringes from the various pharmacies was determined to be: 23.8 mg/mL (UCH), 21.4 mg/mL (CP1), 18.5 mg/mL (CP2), and 12.1 mg/mL (CP3) (Table 1). Compared with the labeled concentration of 25 mg/mL, the concentration of bevacizumab varied from −13% to 1% in the syringes from the UCH pharmacy, from −23% to 7% in the syringes from CP1, from −47% to −14% in the syringes from CP2, and from −59% to −48% in the syringes from CP3 (n = 5 for each).

We first sought to resolve differences in protein content and IgG content by determining whether protein degradation had occurred in these samples. As shown in Figure 1, all samples of bevacizumab from the 4 pharmacies resolved into the heavy and light IgG chains (50 and 25 kD) as expected for IgG samples treated with a reducing agent such as dithiothreitol. No other bands were detected, and the intensity of the bands was approximately equal from lane to lane. These results suggest that lower IgG levels measured in some samples did not arise from protein fragmentation during repackaging and storage.

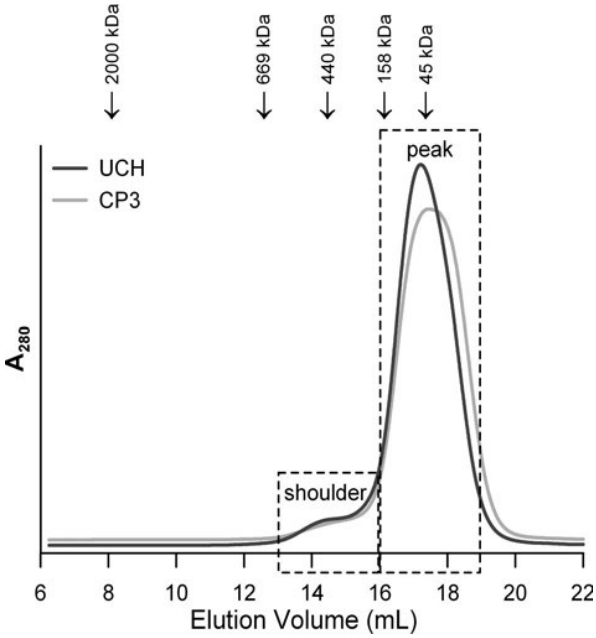

Fig. 2. Size exclusion chromatography of bevacizumab samples. Fifty micrograms of bevacizumab from UCH and CP3 were loaded onto a Superose 6 chromatography column. Elution volumes for protein standards are indicated by arrows above the graph. Fractions corresponding to the major peak and leading shoulder are indicated in boxes. Because of interaction with the Superose chromatography matrix, IgG molecules such as bevacizumab elute at a position corresponding to ~50 kD.

Second, we used size exclusion chromatography to determine if large protein aggregates may have formed in the samples from the different CPs. Figure 2 shows size exclusion chromatography elution profiles of bevacizumab obtained from UCH (which shows normal IgG content) compared with a sample obtained from CP3 (which showed only ~50% of the normal IgG content). All bevacizumab samples demonstrated a major peak eluting at a volume expected for IgG

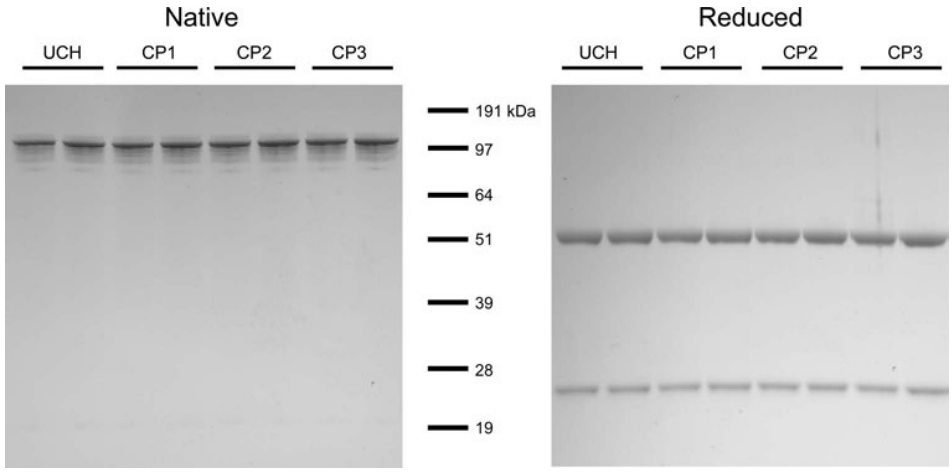

Fig. 1. Polyacrylamide gel electrophoresis of bevacizumab samples. Three micrograms from two syringes from each source of bevacizumab was run under native and reducing conditions. A single band was seen in the native gel, representing the full sized IgG (~150 kD). On denaturing, only the heavy (~50 kD) and light (~25 kD) IgG chains could be detected.

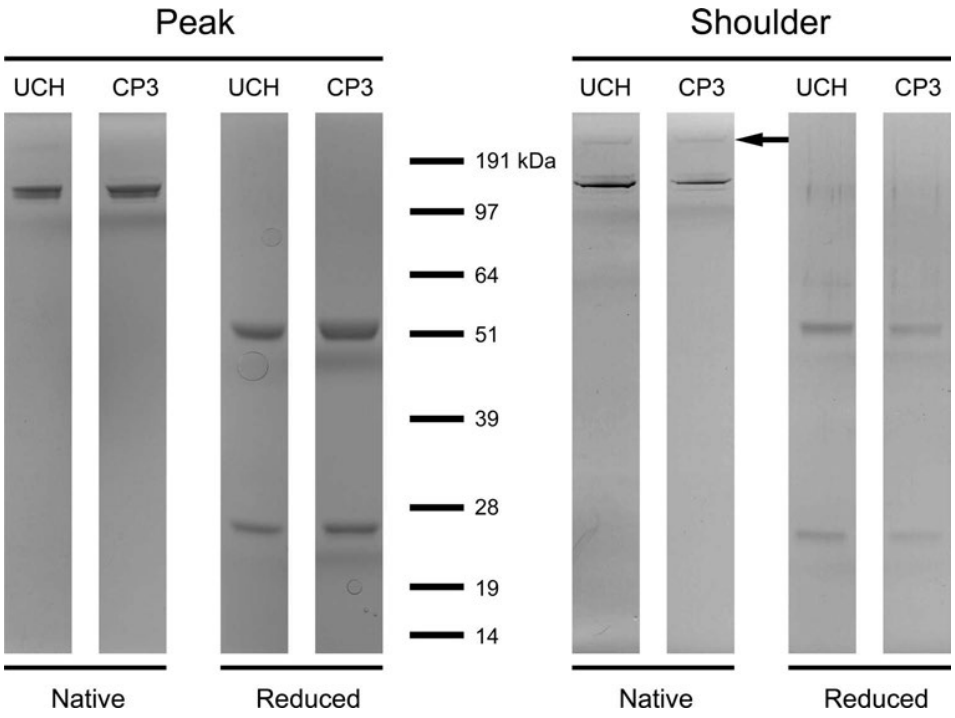

Fig. 3. Polyacrylamide gel electrophoresis of size exclusion chromatography fractions. Denaturing and nondenaturing polyacrylamide gel electrophoreses were performed on peak (left) and shoulder (right) fractions from the Superose separated UCH and CP3 bevacizumab samples. Peak fractions appeared identical to the gels of starting material. Shoulder fractions showed a high molecular weight aggregate (arrow) that resolves into heavy (~50 kD) and light (~25 kD) IgG chains on addition of a reducing agent.

when analyzed under these conditions. In addition, a relatively minor shoulder at the leading edge of the peak was observed with both samples (Figure 2).

Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions confirmed that the peak and shoulder fractions contained IgG as evidenced by the characteristic pattern of heavy and light chains (Figure 3). When samples were analyzed without pretreatment with a reducing agent, aggregates of apparently higher molecular weight were noted (arrow, Figure 3) in fractions from the ''shoulder'' samples but not the major peak. Because these higher molecular weight forms do not exist in the presence of a reducing agent but are stable by electrophoresis, they most likely arise from intermolecular disulfide crosslinks between two or more IgG molecules.

In an effort to determine whether differences exist in the number and size of particles in different syringe groups, MFI was performed on four samples—one directly from the vial obtained from the UCH pharmacy and one each from all three CPs. The number of 0.7 μm to 400 μm-sized particles in the single UCH sample tested was determined to be 61,000/mL, whereas the number of particles in the bevacizumab placebo alone (no protein) was 37,000/mL. The total number of particles from CP1 and CP2 was 57,000 and 73,000, respectively. The total number of particles in the CP3 sample was 510,000/mL. These findings show that CP3 had almost 10 times the number of large (>1 μm) particulate matter when compared with the bevacizumab sample taken directly from the vial. Some of the particulate matter in CP3 reached a diameter of ~19 μm.

Discussion

Reports of persistent IOP spikes after single or multiple injections of bevacizumab have increased since the introduction of antivascular endothelial growth factor agents for treating wet age-related macular degeneration.[5–8] We focused attention on bevacizumab because this agent is involved in most reported cases of IOP complications. Our investigation into potential causes of these IOP spikes showed significant variability in the concentration of bevacizumab IgG in some samples, although the total protein concentration was at expected levels in all samples examined. This led us to investigate the possibility that differences in levels of protein aggregation exist between the various sources of bevacizumab. As shown by size exclusion chromatography and polyacrylamide gel electrophoresis analysis, all samples examined contained a population of high–molecular-weight aggregates that likely represented dimers or trimers of bevacizumab IgG monomers. To quantify the number of particles between different syringes, the

more sensitive MFI method was performed. This approach showed a substantially higher number of >1-μm particles in the material sourced from a CP when compared with material not prepared by a CP. The CP3 sample, which contained the highest number of >1-μm particles, was the same material that had the lowest concentration of the bevacizumab IgG. Of all the analytical studies shown here, MFI is the only 1 sensitive enough to detect these larger particles because: 1) although micron-sized particles contain millions of proteins, they represent a trace amount of the total protein and would be nearly invisible by polyacrylamide gel electrophoresis; 2) the size of these particles would preclude them from entering the size exclusion chromatography column; and 3) in addition to protein, some of the particles could be resulting from nonprotein materials such as silicone oil from the syringes. These findings require further investigation.

The introduction of ranibizumab-antibody-based therapeutics for ophthalmic disease is a relatively new phenomenon.[10] It spawned the off-label use of bevacizumab and created for the first time a need for CP-based repackaging of a recombinant protein therapy. With this new therapeutic group comes the need for a better understanding of how these agents should be handled by the CP and physicians alike. The importance of precise and reproducible handling of repackaged bevacizumab was introduced in the original Bascom Palmer protocol for using bevacizumab for intraocular injections, which covered storage conditions and expiration date based on work published by Bakri et al.[11] Bakri et al found that the stability of bevacizumab repackaged into syringes documented a substantial loss of activity after <3 months (~8.8% loss) of refrigerated storage. Often in storage studies of therapeutic proteins, aggregate formation accounts for such loss of activity.[12,13] Conversion of ~10% of the monomeric protein into aggregates and particles could result in injection of a relatively high level of nonfunctional protein into the eye. Therefore, storage stability assessment for repackaged bevacizumab should be based on the degradation profile as well as retained biologic activity.

Aggregation of therapeutic proteins is ubiquitous during all stages of production from initial fermentation to purification and to filling of syringes and vials.[12,13] Furthermore, aggregation occurs readily during shipping, storage, and even delivery of the drug product.[12,13] Aggregation can be stimulated by conditions such as warming above refrigerator temperatures, freeze–thawing, and excursions in pH.[12,13] An extremely potent cause of aggregation is exposure to interfaces such as the air–water, liquid–solid, and liquid–silicone oil interfaces.[14–16]

Furthermore, protein molecules readily adhere to microparticles of foreign materials (such as glass from vials or syringes, rubber from stoppers, stainless steel from filling pumps, and silicone oil used to lubricate syringes) creating protein particles and potentially seeding further aggregates.[16,17]

Of particular importance for the current example of bevacizumab repackaging, a formulation that was originally developed to maintain protein stability in its original glass vial, is whether a plastic syringe is an appropriate container for preventing protein aggregation and particle formation. In fact, there are examples in which a formulation of a protein therapeutic, Food and Drug Administration-approved for storage in a glass vial, failed to prevent protein aggregation when the same formulation was used in a prefilled syringe.[16] One of the main reasons for formulation failure in these conditions is that, in a syringe, the protein solution is exposed to materials and surfaces such as silicone oil that it may not encounter in a vial.[16,17] Another important issue is that storage of a protein solution in a plastic syringe can extract leachable material from the syringe barrel and the rubber plunger tip. A range of materials can enter the protein solution, including ions such as zinc, plasticizers, and other organic molecules.[18,19] These compounds can induce protein aggregation as well as potentiate adverse effects in patients such as immunogenicity.[19] Therefore, properly developed therapeutic protein products are not stored in plastic containers.[18]

In conclusion, our findings indicate that there are significant differences in IgG concentration between groups of IVB syringes. The reasons for these differences require further studies, but a trend exists for an increase in micron-sized protein aggregates with the decrease in IgG concentration. Although the clinical use of IVB has demonstrated a broad therapeutic index of safety, it is unclear how the variability in expected concentration/dose that we identified may affect clinical outcomes. Lower than expected concentrations may diminish the robustness of efficacy or, alternatively, higher concentrations may lead to toxic effects or higher rates of uveitis.[20] Obstruction of the outflow pathway by particulate matter with subsequent elevation in IOP is a well-known phenomenon both in disease states and in experimental glaucoma models.[21,22] The presence of protein aggregates and a high number of large molecules in some syringes of bevacizumab obtained from CPs, not seen in samples taken directly from original vials, could potentially lead to obstruction of the outflow pathways. We cannot at this time directly correlate protein aggregates with subsequent elevation in IOP that is resistant to medical therapy, and we cannot hypothesize whether single or

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