Immunogenicity of Aggregates of Recombinant Human Growth Hormone in Mouse Models

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ABSTRACT: Aggregation of recombinant therapeutic protein products is a concern due to their potential to induce immune responses. We examined the immunogenicity of protein aggregates in commercial formulations of recombinant human growth hormone produced by freeze-thawing or agitation, two stresses commonly encountered during manufacturing, shipping and handling of therapeutic protein products. In addition, we subjected each preparation to high-pressure treatment to reduce the size and concentration of aggregates present in the samples. Aggregates existing in a commercial formulation, as well as aggregates induced by freeze-thawing and agitation stresses enhanced immunogenicity in one or more mouse models. The use of high-pressure treatment to reduce size and concentrations of aggregates within recombinant human growth hormone formulations reduced their overall immunogenicity in agreement with the "immunon" hypothesis. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:3247–3264, 2009

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INTRODUCTION

Therapeutic proteins are susceptible to aggregation in response to a wide variety of stresses encountered during their manufacture, storage and delivery to patients.[1] In turn, aggregates of therapeutic proteins may compromise their safety and efficacy.[2–5] The primary safety concern is that aggregates in therapeutic protein products may induce immune responses,[6,7] which can have consequences ranging from reduction of product efficacy to patient fatality.[8] In extreme cases, parenterally administered aggregates can induce a severe allergic reaction resulting in anaphylactic shock.[9,10] Also, antibodies formed against aggregated protein molecules have the potential to cross-react with the native protein as well.[5] This cross-reaction with the native protein may reduce the efficacy of the therapeutic due to a faster clearance of the protein or neutralization of the protein. In addition to the neutralization of the exogenous native therapeutic protein, cases have shown that antibodies raised against recombinant therapeutic human proteins can potentially recognize endogenous human proteins.[11–13]

Stresses that frequently provoke protein aggregation such as agitation[14] or freezing[15] are common in the manufacturing and shipping of therapeutic proteins. Agitation (and the resulting exposure of proteins to interfaces such as the air-liquid interface) can result in aggregation during

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manufacturing, shipping and handling of the product.[16] Likewise, protein bulk drug substance is commonly frozen as a storage step in manufacturing process. Additionally, accidental freezing is a risk, particularly during refrigerated storage of therapeutic formulations intended for home use.[17] Aggregates produced as a result of different stresses may exhibit different size distributions and their component proteins may contain different secondary and tertiary structures,[18] which presumably expose different epitopes and thus potentially provoke different immune responses.[19] Previous studies reported the immunogenicity of aggregates formed in interferon α2 formulations.[20,21] In the previous study, aggregates were generated by oxidation with hydrogen peroxide, metal-catalyzed oxidization, cross-linking with glutaraldehyde, or exposure to extreme pH. Conditions that result in aggregation via oxidation or exposure to extreme pH may be encountered in industrial processes, but aggregation of therapeutic proteins is more frequently the result of stresses incurred during freeze-thawing and agitation. Thus, the current study focuses on aggregates formed during agitation and freeze-thawing of recombinant human growth hormone (rhGH) and their potential impacts on the immunogenicity of the protein. We also demonstrate the use of high (~2 kbar) hydrostatic pressures as a method to disaggregate the protein[22,23] with a resultant decrease in immune response.

Due to a lack of sophisticated models and a need for greater understanding of human immune function, pre-clinical predictability of immunogenicity to recombinant human therapeutic proteins is problematic.[24] Preclinical immunogenicity studies frequently rely on murine models, in part because mice are relatively inexpensive and low maintenance and are readily available. Naïve mice inherently develop immune responses to foreign proteins (such as therapeutic human proteins). However, murine models may demonstrate enhanced immune responses to more immunogenic samples, and provide a means by which to assess relative immunogenicity of various types of aggregates of a given protein.[25] Alternatively, Hermeling et al.[26] recently developed a transgenic mouse model in which the mice were genetically altered to produce a human protein in order to eliminate the innate immune response to that protein, but the relevance of these models to prediction of responses in humans is also still uncertain.

In this study we used three murine models to measure the immunogenic response to protein aggregates produced by agitation or freeze-thawing stresses in two commercial formulations of rhGH. Aggregates were characterized for size and conformation of the component protein molecules. Two murine models used, naïve adult and transgenic, are similar to models used in previous work.[25,26] The third murine model is a neonatally primed model in which mice are sensitized to the rhGH in the neonatal stage.[27–29] The neonatally primed model was chosen to mimic the effect of low concentration pre-existing antibodies to a therapeutic protein. It has been reported that antibodies formed during treatment with a protein therapeutic can be found in the patient in some cases as long as 59 months after discontinuing treatment with that therapeutic.[30–32] The presence of antibodies to a therapeutic in a patient after cessation of therapy could pose unknown risks if the patient were to relapse and need additional treatment with that therapeutic.

MATERIALS AND METHODS

Materials

The two commercial formulations of rhGH Nordiflex® (Novo Nordisk®, Bagsvaerd, Denmark) and Saizen® (Serono, Rockland, MA), were purchased from the University of Colorado apothecary, and are hereafter referred to as Product A and Product B, respectively. Sterile water for injection (SWFI) (Hospira, Inc., Lake Forest, IL) and 0.9% sodium chloride for injection (Hospira, Inc.) were also purchased form the University of Colorado apothecary. Histidine and mannitol were purchased from JT Baker (Phillipsburg, NJ). Pluronic F-68 was purchased from Spectrum Chemicals (New Brunswick, NJ). Phenol was obtained from Sigma Chemicals (St. Louis, MO).

Sample Preparation

For samples produced from the liquid rhGH formulation Product A, 15 mg/1.5 mL vials were used for sample preparation. The rhGH was diluted to a concentration of 1 mg/mL. One of two diluents was used: (1) a solution of identical composition to the product A formulation buffer: 1.13 mg/mL histidine, 3 mg/mL pluronic F-68,