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Research paper

UV photodegradation of murine growth hormone: Chemical analysis and immunogenicity consequences

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ABSTRACT

During manufacturing, therapeutic proteins may be exposed to ultraviolet (UV) radiation. Such exposure is of concern because UV radiation may cause photooxidative damage to proteins, which in turn could lead to physical changes such as aggregation and enhanced immunogenicity. We exposed murine growth hormone (mGH) to controlled doses of UV radiation, and examined the resulting chemical, physical and immunogenic changes in the protein. mGH chemical structure was analyzed by mass spectrometry after UV irradiation. Photooxidation products detected by mass spectrometry included methionine sulfoxide formed at Met[127] and Met[149] residues, and, tentatively assigned by MS/MS analysis, ether cross-links between original Ser[78] and Cys[188], and Cys[206] and Ser[213], and a thioether cross-link between Cys[17] and Cys[78] residues, transformation of Cys[189] into Ala, and various hydrolytic fragments. Physical damage to UV-irradiated mGH was monitored by infrared spectrometry, chromatographic analyses, and particle counting by micro-flow imaging. UV radiation caused mGH to aggregate, forming insoluble microparticles containing mGH with non-native secondary structure. When administered subcutaneously to Balb/c or Nude Balb/c mice, UV-irradiated mGH provoked antibodies that cross-reacted with unmodified mGH in a fashion consistent with a T-cell dependent immune response. In wild-type Balb/c mice, titers for anti-mGH IgG_1 antibodies increased with increasing UV radiation doses.

1. Introduction

Therapeutic proteins are known to aggregate in response to common stresses that may be encountered throughout their product life cycle. Ultraviolet (UV) light is one such stress to which therapeutic proteins may be exposed during processing, storage and consumer use. In addition, it has been proposed that biotechnology products may be exposed to UV irradiation for viral decontamination [1]. UV exposure can also occur during storage and consumer use if products are not completely isolated from light.

Proteins may be chemically damaged when exposed to light (for a review of light-induced chemical damage pathways, see Kerwin et al., 2007) [2]. In turn, chemically damaged protein molecules may have a greater propensity to aggregate [3,4]. Chemically degraded proteins may be immunogenic [5,6] and protein impurities at levels <1% may induce immune responses [7]. Furthermore, aggregated proteins have long been known to be immunogenic in humans [8–11], and more recently aggregation of therapeutic proteins and immunogenicity has been associated in a number of studies conducted in mice [7,12,13]. Another recent study [14] showed that exposure of naive human peripheral blood mononuclear cells to aggregated therapeutic antibodies stimulated their innate immune response.

We hypothesize that exposure of therapeutic proteins to UV light could lead not only to their photodegradation, but also to aggregation and concomitant enhancements in their immunogenic potential.

In this study, we exposed murine growth hormone (mGH) to UV light, characterized the products of photodegradation by mass spectroscopy and measured the resulting levels of protein

Abbreviations: mGH, murine growth hormone; UV, ultraviolet; MS, mass Spectrometry; Met, methionine; Cys, cysteine; Ser, serinine; Ala, alanine; Asn, asparagine; Gln, glutamine.

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aggregates. In addition, we performed *in vivo* testing of the immunogenicity of samples of mGH exposed to UV at $\lambda = 254$ nm for various intervals of time by measuring levels of antibodies produced that were cross-reactive against unmodified mGH. To gain insight into the role of T-cells in the immune responses to photodegraded mGH, we tested the immunogenicity in both Balb/c and T-cell deficient Nude Balb/c mice.

2. Materials and methods

2.1. Materials

mGH was produced recombinantly in *Escherichia coli* and purified as described previously [15]. Limulus Amebocyte Lysate (LAL) tests were performed to ensure that the purified mGH had endotoxin levels below the preclinical USP endotoxin limit of 5 EU/kg [16]. VETone™ saline for injection, lot 7J004 (MWI Veterinary Supply, Meridian, ID) was purchased from the University of Colorado apothecary. 4-hydroxy-3-nitrophenylacetic hapten conjugated to aminoethylcarboxymethyl-Ficoll (NP28-Ficoll) (F1420-10 lot 021292-03), 4-hydroxy-3-nitrophenylacetic hapten conjugated to chicken gamma globulin (NP39-CGG) (N5055-5, lot 028021-06) and 4-hydroxy-3-nitrophenylacetic hapten conjugated to bovine serum albumin (NP_{14}-BSA) (N5050-10, lot 025060-04) were purchased from Biosearch Technologies (Novato, CA). Goat anti-mouse IgG1 (ab9165, lot 862097), goat anti-mouse IgG3 (ab9166, lot 892368), goat anti-mouse IgM (ab9167, lot 815738) and HRP conjugated rabbit anti-goat IgG (ab6741, lot 871673) were purchased from Abcam (Cambridge, MA). 3,3′,5,5′ tetramethylbenzidine was purchased from KPL (Gaithersburg, MD). All other reagents were from Fisher Scientific (Pittsburgh, PA).

2.2. Sample preparation

A stock preparation of mGH (0.26 mg/ml) in acetate buffer pH 4.75 was stored at 4 °C and was used to prepare all samples of mGH. For photo-irradiation, the samples were placed in quartz tubes. The headspaces above the samples were gently flushed with Ar prior to photoirradiation. mGH was exposed to ultraviolet light at $\lambda = 254$ nm with a dose rate of 4.93×10^{-8} einstein s^{-1} (UVLMS-38 EL Series 3-UV lamps, 8-W, UVP Upland, CA) for 10, 30 and 60 min intervals. All samples were kept at 4 °C or aliquoted and stored at −20 °C. The geometry of a representative sample was a cylinder with a diameter of 1 cm and a height of 1 cm. The irradiated surface area (half of the sides + top of the cylinder) was 2.35 cm^2. An 8 W lamp emits 2.22×10^{19} photons s^{-1}. Our actinometry measurements show that only 4.93×10^{-8} einstein s^{-1} (or 2.97×10^{16} photons s^{-1}) are going through the solution. Therefore the solution is exposed to 0.012 W per 2.35 cm^2, or 51 W m^{-2}. Thus, 10, 30, and 60 min of exposure correspond to 8.5 W h m^{-2}, 25.5 W h m^{-2}, 51 W h m^{-2}, respectively. The limit of irradiance in the ICH Q1B is 200 W h m^{-2}.

2.3. Size exclusion high performance liquid chromatography

Size-exclusion high performance liquid chromatography (SE-HPLC) analysis was conducted using a Superdex™ 75 10/300 GL column on an Agilent 1100 series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were centrifuged at 1700*g* for 5 min prior to injection. Triplicate 100 µl injections of each sample were analyzed. Isocratic chromatography was performed at room temperature with a flow rate of 0.8 ml/min using phosphate buffered saline pH 7.4 as the mobile phase. UV absorbance at 280 nm was monitored using the Agilent UV diode array detector for 50 min. The chromatograms were analyzed in Chemstation software (Agilent Technologies, Inc., Santa Clara, CA, USA) by integration to determine areas for respective peaks. Peak area percentages are reported relative to stock sample peak areas as calculated by:

$$\frac{\text{Area}_{\text{peak}}}{\text{Area}_{\text{stock mGH,total}}} \times 100 \quad (1)$$

Peak area percentages of insoluble aggregates determined by mass balance:

$$\frac{\text{Area}_{\text{stock mGH,total}} - \text{Area}_{\text{preparation,total}}}{\text{Area}_{\text{stock mGH,total}}} \times 100 \quad (2)$$

95% confidence intervals were calculated from the triplicate injections of each sample on the SE-HPLC. SE-HPLC analysis was performed throughout the course of the study to ensure that aggregate content did not change during intermediate storage before animal studies or further characterization experiments were conducted.

2.4. Particle analysis

Particle analysis was performed using Micro-Flow Imaging™ on a DPA 4100 (Brightwell Technologies, Inc., Ottawa, Ontario, Canada) as described earlier [7]. Briefly, in order to obtain a clean baseline and optimize illumination, water was filtered through a 0.2 µm filter and used to flush the system prior to sample analysis. Three aliquots (0.5 ml) of each preparation were analyzed at a flow rate of 0.1 ml/min through a high magnification flow cell using the "set-point 3" configuration, which allows detection of particles 1–50 µm. Prior to analysis, samples were slowly inverted 10 times to ensure suspension of particles. Negative controls using protein-free buffer were also analyzed to eliminate any buffer influence on particle detection. The data obtained were number counts per volume per 0.25 µm diameter size bins.

2.5. Infrared spectroscopy

Infrared spectra for insoluble aggregates of mGH formed by ultraviolet exposure were acquired using a Bomem™ IR spectrometer (Quebec, Canada) and a deuterated triglycine sulfate KBr detector. A variable path length CaF_2 cell was used for all the measurements. Suspensions were centrifuged at 1700*g* for 5 min and the supernatant removed. Aggregates were resuspended in buffer to ensure analysis of aggregates only. Buffer corrections were made by subtracting spectra measured on buffer samples. The methods used to obtain and analyze spectra have been described previously [17]. All mathematical manipulations of spectra were performed in GRAMS software (Thermo Electron Corp., Waltham, MA).

2.6. Reverse-phase high performance liquid chromatography

Reverse-phase high performance liquid chromatography (RP-HPLC) experiments were conducted using a Jupiter 300 Å 5 µm C4 column (Phenomenex, Torrance, CA) on an Agilent 1100 series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were centrifuged at 1700*g* for 5 min prior to injection. 100 µl injections of each sample were analyzed in triplicate. Mobile phase A was water containing 0.1% trifluoroacetic acid, and mobile phase B was acetonitrile containing 0.1% trifluoroacetic acid. The column was equilibrated for 10 min at 100% mobile phase A followed by a linear gradient from 0% to 100% mobile phase B over 45 min; followed by 10 min of 100% B to wash any remaining protein from the column. UV absorbance at 214 nm was monitored using the Agilent UV diode array detector for 65 min. The chromatograms were analyzed in Chemstation software