Proteomic Profiling of Growth Hormone-Responsive Proteins in Human Peripheral Blood Leukocytes

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Context: GH is a known modulator of the immune system, but the effect of exogenous GH administration on white blood cell proteins has not been investigated. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a powerful platform for the study of GH effects on immune system proteins.

Objective: Our objective was to explore a novel approach for the detection of GH-responsive proteins in human leukocytes by proteomic analysis using SELDI-TOF MS.

Design: We conducted a randomized double-blind, placebo-controlled GH administration study of 8 wk treatment followed by 6 wk washout. Pre- and posttreatment samples from 30 subjects were used for biomarker discovery.

Setting: The study was performed at a clinical research facility.

Participants: We studied 30 recreationally trained healthy athletes.

Intervention: Subjects received either recombinant human GH (2 mg/d sc; n = 22) or placebo (n = 8) for 8 wk.

Main Outcome Measures: Proteomic profiles were determined using CM10 weak cation-exchange protein chips, and some GH-regulated proteins were purified and identified by mass spectrometry and/or immunoblotting.

Results: SELDI-TOF analysis revealed a number of GH-regulated peptides/proteins in the 3- to 22-kDa range that are either up- or down-regulated by GH. Several of these may be useful as biomarkers of GH action. The calcium-binding, proinflammatory calgranulins S100A8, S100A9, and S100A12 were all significantly down-regulated in response to GH treatment.

Conclusion: This study illustrates the novel use of human leukocyte proteomic profiling by SELDI-TOF MS and reveals the negative regulation of proinflammatory S100 proteins by GH in human white blood cells. (***J Clin Endocrinol Metab* 94: 3038–3043, 2009**)

GH is expressed primarily in the pituitary gland, but both the hormone and its receptors are also found in cells of the immune system (1–4). GH acts on the liver and other tissues to increase circulating levels of IGF-I, IGF-binding protein-3, and the acid labile subunit, which together form a ternary complex responsible for much of the circulating IGF transport (5–7). Substantial evidence is emerging that GH is also an important modulator of the immune system (8, 9). Phagocytic functions of neutrophils and monocytes are impaired in GH-deficient subjects and improve after GH replacement therapy (10). The natural killer activity of lymphocytes is reduced in the GH-deficient state and rectified by GH treatment, which also protects lymphocytes against apoptosis induced by glucocorticoids (8). GH receptors have been identified in lymphocytes and macrophages, and GH

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Abbreviations: AUC, Area under the curve; m/z, mass/charge; PKC, protein kinase C; ROC, receiver operating characteristic; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry.

stimulates the proliferation of these cells *in vitro* (8, 11). Many effects of GH are also mediated by IGF-I, and some leukocytes also express IGF-I (12, 13). Thus the constituents of leukocytes are targets of GH action, both directly and via immunoregulatory effects. However, to date, there has been no proteomic analysis of GH-regulated proteins in human white blood cells.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a proteomic technique in which proteins are bound to proprietary protein chips with different types of adsorptive surfaces, *e.g.* hydrophobic, cation-exchange, or anion-exchange. SELDI-TOF MS can be used to analyze peptide and protein expression patterns in a variety of clinical and biological samples, and biomarker discovery can be achieved by comparing the protein profiles obtained from control and patient groups to elucidate differences in protein expression (14–17). Because SELDI analysis provides biochemical information about detected proteins (*i.e.* mass and adsorption conditions), more specialized protein biochemistry and mass spectrometry can then be used to identify unknown marker proteins for a particular condition. To define new circulating markers of GH administration, we and others have previously investigated serum proteomic profiling using SELDI-TOF MS and have found novel serum biomarkers of human GH action (18, 19).

In this study, we used SELDI-TOF MS to develop a novel method for the detection of GH-responsive proteins using proteomic profiling of human peripheral blood leukocytes. We discovered a number of GH-dependent peaks including those representing proteins of the calcium-binding S100 family. Our results have the potential to shed new light on the relationship between GH and the immune system and may be of benefit in the diagnosis of growth disorders.

Subjects and Methods

Study subjects

Healthy subjects (12 males, 18 females), recruited as part of a collaborative World Anti-Doping Agency-funded intervention study (20) aimed at developing a GH doping test, were administered 2 mg/d GH (eight males, 14 females) or placebo (four males, four females) for 8 wk followed by a 6-wk washout period. GH (Somatropin, 1 mg/ml) and matched GH placebo were provided by Novo Nordisk (Bagsvaerd, Denmark). GH or placebo was self-administered sc, with subjects instructed to administer the injection at night. To minimize side effects, the GH dose was increased from 1 mg/d (first week) to 1.5 mg/d (second week) and then to the final dose of 2 mg/d for 6 wk, as previously reported in detail (20). All subjects provided written informed consent, and the study was approved by the St. Vincent's Hospital Human Research Ethics Committee.

Protein chip surface capture

Leukocytes were prepared from fresh whole blood samples, anticoagulated with EDTA, by isolating the buffy coat layer after centrifugation at 3500 rpm for 10 min at 4 C. White blood cells were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), protein extracts being obtained from the interphase and lower phenol phase after first removing the upper aqueous phase according to the manufacturer's instructions. DNA was precipitated using ethanol and the remaining ethanol-phenol supernatants were stored at −80 C before protein analysis. In preliminary studies, protein extracts were analyzed on several different protein chip surfaces (strong anion-exchange, weak cation-exchange, hydrophobic, and immobilized metal affinity loaded with Cu^{2+}) and under various adsorption and washing conditions. The CM10 weak cation-exchange chip was chosen for further study. The array spots were preequilibrated with the binding buffer (100 mM NH_4 acetate, pH 6) for 10 min. Leukocyte extracts were diluted with the binding buffer (1:5), and the diluted samples (5 μl per spot) were pipetted onto the chips. All samples were run in duplicate. After incubation for 60 min, unbound proteins were removed by washing with binding buffer (three times, 5 μl each). After two rinses with deionized H_2O, 1 μl of matrix solution (20 mg/ml sinapinic acid, 50% acetonitrile, 0.5% trifluoroacetic acid) was applied twice and air dried for 10 min. The protein chip arrays were then read in a ProteinChip reader (PBSIIc; Bio-Rad, Hercules, CA).

Data analysis of SELDI-TOF MS

The initial study involved 88 mass spectra from 22 subjects, each sampled before GH (wk 0) and after wk 4 of GH treatment, analyzed in duplicate. Protein peak intensities in all spectra were normalized to the total ion current over mass/charge (m/z) values from 3,000–70,000 Da using Bio-Rad software (version 3.2.2). The SELDI-TOF MS was externally calibrated using the $[M+H]^+$ ion peaks of bovine insulin (5,734.51m/z), equine cytochrome c (12,361.96 m/z), equine apomyoglobin (16,952.27 m/z), bovine carbonic anhydrase (29,023.70 m/z), rabbit muscle aldolase (39,212.28 m/z), glucose-6-phosphate dehydrogenase (57,432.72 m/z), and bovine albumin (66,430.09 m/z; all standards from Sigma-Aldrich Corp., St. Louis, MO). In the 88 spectra, 77 peaks common to all spectra were identified, and for each peak in each sample, intensity values for duplicate analyses were averaged. Additional samples from eight placebo-treated subjects, sampled at wk 0 and 4, were also examined in duplicate, yielding 32 additional spectra, to achieve a total of 120 mass spectra for the 30 subjects. The coefficient of variation for the average of duplicate peak intensity values, calculated by ANOVA, was 19.5%.

To discover GH-regulated proteins, univariate analysis of samples from GH-treated subjects (Mann-Whitney *U* test, SPSS version 15.0.1; SPSS Inc., Chicago. IL) was used to identify all protein peaks that significantly distinguished between the two conditions (before and after GH treatment). A receiver operating characteristic (ROC) curve was constructed to evaluate the predictive power of each significant peak. Peaks selected for further study were those that 1) showed a highly significantly difference ($P < 0.001$) between pre- and post-GH treatment samples and 2) could individually discriminate between the two groups with a ROC area-under-the-curve (AUC) value of at least 0.900.

Time course of GH action

Analysis of leukocyte samples at various sampling times was performed for the 22 GH-treated subjects. Samples taken at wk 0, 4, and 8 of the GH administration period and wk 6 of the washout period (*i.e.* 14 wk after commencement of the study) were subjected to SELDI-TOF analysis using the CM10 chip method as described above.

Protein purification and identification of GH-responsive proteins in human white blood cells

Human leukocyte extracts were fractionated with an anion-exchange resin (Q ceramic HyperD F; Pall Corp., Ann Arbor, MI) using stepwise pH elution between pH 9 and pH 4 in a 96-well filter plate format (AcroPrep; Pall). The filter plates were loaded with 100 μl pretreated resin (washed with five bed volumes of 50 mM Tris-HCl, pH 9). The buffer was removed using a multiwell plate vacuum manifold (Pall). Protein extracts were diluted 1:5 in binding buffer containing 50 mM Tris-HCl (pH 9) and were loaded onto a 96-well plate at 4 C for 20 min with shaking. This was followed by stepwise elution. Proteins of interest in eluted samples were monitored by SELDI-TOF MS on NP20 (normal-phase) chips. The semipurified proteins were further purified by reverse-phase liquid chromatography on a C_{18} column (250 × 4.6 mm; 300-Å

pore size; Phenomenex, Torrance, CA) eluted with a 30-min linear gradient from 15–60% acetonitrile in 0.1% trifluoroacetic acid at 1.5 ml/min. Fractions were examined on 12% SDS-PAGE detected with SYPRO ruby protein stain. Purified bands of interest were excised from the gel and digested with trypsin for further protein identification by peptide mass fingerprinting and MS/MS at the Bio-analytical Mass Spectrometry Facility, University of New South Wales, Sydney, Australia.

Western blot analysis

Leukocyte extracts (2 μl) were diluted 1:4 in PBS and run on 12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen), transferred to Invitrolon polyvinylidene difluoride membranes (Invitrogen), and incubated with anti-S100A8 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 15 μl diluted in 3 ml), anti-S100A9 polyclonal antibody (Santa Cruz; 15 μl diluted in 3 ml), or anti-S100A12 polyclonal antibody (Abcam, Cambridge, MA; 3 μl diluted in 3 ml) and then incubated with goat antirabbit IgG horseradish peroxidase as secondary antibody (1.5 μl diluted in 3 ml; Santa Cruz) to specifically detect S100A8, S100A9, or S100A12. Proteins were revealed with an enhanced chemiluminescence detection method according to the manufacturer's instructions (GE Healthcare Biosciences, Rydalmere, New South Wales, Australia). Paired *t* tests were used for between-group analyses (SPSS version 16.0).

Results

SELDI-TOF MS data analysis

Using CM10 chips, we initially identified 77 peaks common to 88 profiles (22 subjects treated with GH, before and after GH, in duplicate) derived from leukocyte extracts. Figure 1 shows two regions of representative SELDI protein spectra, in the m/z ranges 2500–4500 and 10,000–15,000. Examples of differential protein expression between GH-treated (wk 4) and untreated (wk 0) subjects are indicated. Figure 1A shows a peak up-regulated after 4 wk GH treatment; two peaks down-regulated by GH are indicated in Fig. 1B. Duplicate peak intensities were averaged for all 77 common peaks in all samples. Univariate analysis and ROC analysis identified nine of these 77 peaks that 1) showed a highly significantly difference ($P < 0.001$) between pre- and post-GH treatment samples and 2) could individually discriminate between the two groups with a ROC AUC value of at least 0.900 (Table 1). Values from samples taken at wk 0 and 4 of GH treatment for a representative five of these nine peaks for both GH-treatment and the placebo groups are shown in Fig. 2. Figure 2A indicates a peak at m/z 3480, which was up-regulated over 3-fold after 4 wk GH treatment. Figure 2, B–E, illustrates four peaks, all of which were significantly suppressed by GH treatment. Also shown in Fig. 2 are relative intensities of the same five peaks in samples from subjects treated for 4 wk with placebo. The peak intensities of samples from wk 4 are expressed relative to values for corresponding samples taken at wk 0. For all five peaks significantly affected by GH, treatment with placebo had no effect.

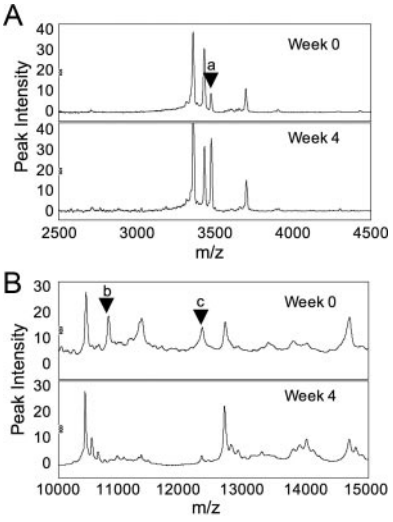

FIG. 1. SELDI-TOF MS protein expression profiling of human peripheral blood leukocyte extracts. A, A segment of the protein mass profile obtained on CM10 chips between m/z 2500 and 4500 indicating a peak at m/z 3480 (a) up-regulated by GH treatment (wk 4 *vs.* wk 0). B, A segment of the protein profile between m/z 10,000 and 15,000; *arrows* represent peaks suppressed by GH treatment (wk 4 *vs.* wk 0) at m/z values of 10,829 (b) and 12,310 (c).

TABLE 1. Protein peaks differentially expressed between GH-treated and untreated subjects

m/z	Effect of GH	ROC-AUC
3480	↑	0.901
6888	↓	0.950
7763	↓	0.981
9053	↓	0.969
10438	↓	0.998
10829	↓	0.949
11346	↓	0.963
12715	↓	1.000
21277	↓	0.930

↑, Up-regulated by GH; ↓, down-regulated by GH.

Time course of GH effect

For the nine peaks indicated in Table 1, the time course of change in protein peak intensity over 8 wk GH treatment and the following 6-wk washout period was examined on CM10 chips. The mean profiles shown in Fig. 3 represent all 22 subjects (eight males, 14 females) before commencement of GH treatment at wk 0, GH-treated at wk 4 and 8, and after washout at wk 14. Surprisingly, several of the peaks that showed highly significant changes at 4 wk treatment had reverted to near-baseline values by wk 8 treatment, suggesting that the changes did not directly indicate GH dependence. In contrast, a few of the peaks were still markedly different from baseline at 8 wk treatment (*e.g.* m/z 10,438 and 10,829), and remarkably, the peak at m/z 10,438 remained highly suppressed even after the 6-wk washout period.

Identification of GH-responsive proteins

To identify the GH-down-regulated peaks at m/z 10,438 and 10,829, human leukocyte extracts were initially purified by anion exchange. Protein extracts were dissolved and loaded in binding buffer at pH 9 and eluted by sequentially decreasing the pH (Fig. 4). Fractions were monitored on an NP20 (hydrophilic) chip to detect peaks of the expected mass in the protein mass profile of each chromatography fraction. Figure 4A demonstrates SELDI protein profiles representing proteins eluted at pH 8, pH 7, pH 6, pH 5, pH 4, and 30% acetonitrile. Peaks of interest were eluted at pH 5 and subsequently purified by C_{18} reverse-phase liquid chromatography. Fractions from the C_{18}

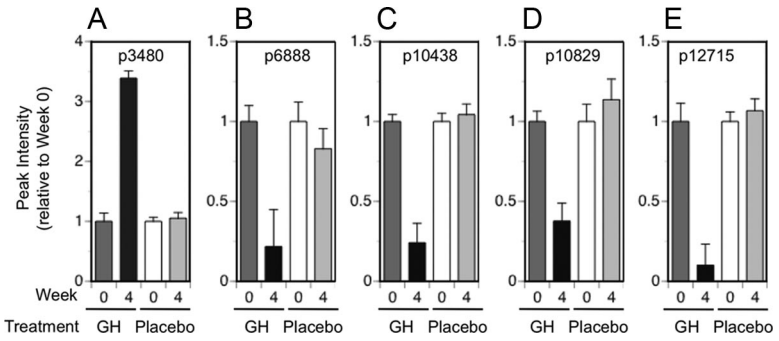

FIG. 2. Effect of GH treatment on intensities of five protein peaks. Mean peak intensities from SELDI-TOF MS analysis of five leukocyte protein peaks at m/z 3480 (A), 6888 (B), 10,438 (C), 10,829 (D), and 12,715 (E) were determined before treatment (wk 0) or after 4 wk treatment with GH (22 subjects) or placebo (eight subjects). For each peak, the mean intensity (± SE) at 4 wk is expressed relative to the corresponding mean intensity at wk 0.

column were then examined on 12% SDS-PAGE detected with SYPRO ruby protein stain; three fractions are shown in Fig. 4B. The band in lane 2 at approximately 11 kDa indicated by an *arrow* was excised from the gel and digested with trypsin. Two proteins were identified in this band by peptide mass fingerprinting and tandem MS: S100A8 (calculated monoisotopic mass 10,828) and S100A12 (calculated monoisotopic mass 10,437) (21).

Validation by identified protein markers

The inverse GH regulation of S100A8 was confirmed using Western blotting. Figure 5, A and B, demonstrates in extracts from four subjects that monomeric S100A8 was significantly down-regulated after 4 wk GH treatment ($P < 0.036$), consistent with the results found by SELDI-TOF MS. The related protein S100A9 was also examined in these samples because it is known to heterodimerize with S100A8 (22). S100A9 similarly showed significant down-regulation by GH in the same four subjects (Fig. 5, A and C; $P < 0.035$). Finally, S100A12 was also analyzed by immunoblotting, confirming significant down-regulation by GH (Fig. 5, A and D; $P < 0.045$).

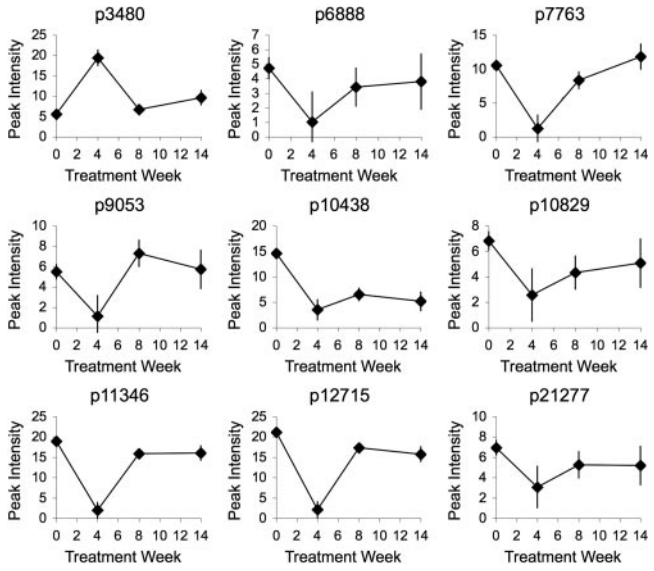

FIG. 3. Time course of GH treatment on GH-responsive peaks. For each of the nine GH-responsive peaks described in Table 1, the time course of change in peak intensity was determined over 8 wk GH treatment and the following 6-wk washout period. Mean data (± SE) are from 22 subjects sampled at wk 0, 4, and 8 of GH treatment and after 6 wk washout at wk 14.

Discussion

In this study, we used SELDI-TOF MS to detect GH-responsive proteins in human peripheral blood leukocytes. Comparison of human leukocyte protein profiles from subjects before or after 4 wk GH treatment revealed nine GH-responsive protein peaks, each of which could distinguish the pre- and post-GH samples with high statistical significance and which could potentially have utility as single biomarkers of GH action. However, time-course analyses over 8 wk GH treatment and 6 wk washout showed that for the majority of GH-responsive proteins, the GH-dependent change was short-lived and peak intensities had reverted to pretreatment levels by 8 wk treatment, possibly indicating an adaptive response to continuing high GH levels. Although it is unclear why the response of these proteins to GH treatment was transient, they may, nevertheless, be involved in mediating some effects of GH. Two notable exceptions were peaks at m/z values 10,438 and 10,829, both down-regulated by GH, which remained suppressed after 8 wk treatment. Indeed, the 10.4-kDa protein was still about 70% down-regulated even after a 6-wk washout period, suggesting that it may warrant further investigation as a circulating biomarker of GH action.

The proteins forming ions with m/z values 10,438 and 10,829 were identified by tandem MS after off-line purification as S100A12 (calgranulin C, 10.4 kDa) and S100A8 (calgranulin A, 10.8 kDa), respectively. Immunoblot analysis of both proteins and of the S100A8 dimerization partner S100A9 (calgranulin B) revealed that they were all negatively regulated by GH in white blood cells. Although intact S100A9 has a predicted mass of approximately 13.2 kDa, a truncated form with a mass of 12.7 kDa, determined by matrix-assisted laser desorption/ionization

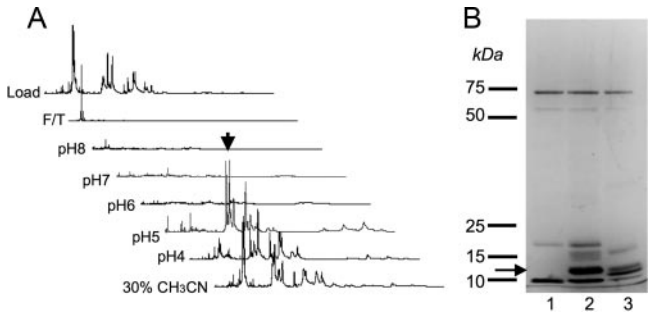

FIG. 4. Purification and identification of a putative GH-responsive protein. A, Human leukocyte extracts were purified using Q HyperD F resin-based chromatography (Pall). Protein profiles are shown in the approximate m/z range 3,500–30,000 for the initial extract (Load), unbound flow-through fraction (F/T), and fractions eluting at pH 8, 7, 6, 5, and 4 as indicated. The filter plate was finally washed with 30% acetonitrile (CH_3CN). The *arrow* indicates the 10.8-kDa protein eluted from this fractionation procedure at pH 5. B, After the semipurified proteins were further purified by C_{18} reverse-phase liquid chromatography, selected fractions were subjected to SDS-PAGE. Lanes 1–3, fractions 27–29 eluted from C_{18} reverse-phase column. The *arrow* indicates the purified protein band at 10.8 kDa.

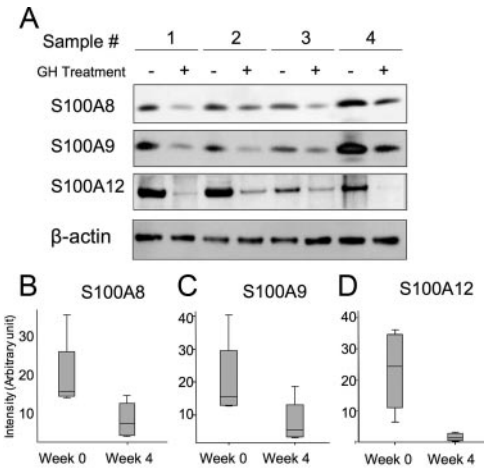

FIG. 5. Validation by Western blotting analysis. A, The identified GH-responsive protein S100A8 was verified by immunoblot probed with anti-S100A8 polyclonal antibody (*top*). Leukocyte extracts from four subjects were examined before (−) and after 4 wk (+) GH treatment. The related proteins S100A9 and S100A12 were also examined in these samples (*middle*). The sample loading control is β-actin (*bottom*). B–D, Densitometric analysis of the data in A for S100A8, S100A9, and S100A12, respectively, showing significant down-regulation of all three proteins by GH ($P < 0.05$). *Boxes* indicate the median and 25th and 75th percentiles; *vertical lines* indicate the range of data values.

mass spectrometry, has been described in human granulocytes (23). This form is believed to lack amino acid residues 1–4 (24) and is presumed to account for the negatively GH-regulated peak at m/z 12,715 seen in our study. Serum levels of all three calgranulins have previously been identified by SELDI-TOF MS as potential biomarkers in arthritis (25), but our identification of their potential utility as white blood cell biomarkers of GH action is novel.

The calgranulins belong to the EF-hand S100 superfamily of calcium-binding proteins. Members of this family usually form homo- or heterodimers, which may associate with higher-order oligomers in a calcium-dependent manner (26, 27). The heterodimer of S100A8 and S100A9, also known as calprotectin (22), represents the major calcium-binding proteins in neutrophils and monocytes (28). Both proteins play a role in calcium-induced signaling where they are involved in the organization of cytoskeletal structures during transendothelial migration of activated phagocytes. Whereas S100A8 is assumed to be the active component of the S100A8/S100A9 complex, S100A9 predominantly seems to function as regulatory subunit.

S100A8/S100A9 and S100A12 are classified as DAMP (damage-associated molecular pattern) molecules, recognized as important proinflammatory factors (29, 30), which act at least in part through interaction with Toll-like receptor 4 (31) and the receptor for advanced glycation endproducts (RAGE) (32). They are specifically expressed in circulating neutrophils and early differentiation stages of monocytes, as well as in keratinocytes and epithelial cells under inflammatory conditions (33). Among the members of the S100 family, S100A12 is most closely homologous to S100A9, but it is believed not to form part of the S100A8/S100A9 complex (34). Inflammatory disorders such as cystic fibrosis, inflammatory bowel disease, and rheumatoid arthritis have been shown to be associated with elevated serum levels of calgranulins (35, 36).

Exogenous GH is reported to have an antiinflammatory effect in subjects with Crohn's disease (37) or abdominal obesity (38) and to reduce inflammatory cardiovascular risk markers in GH-deficient subjects (39), although it did not alter inflammatory cytokine release in response to major surgery (40). Our novel observation, discovered using protein chip proteomics, that GH administration suppresses the proinflammatory calgranulins S100A8/S100A9 and S100A12 in human peripheral blood leukocytes, may provide an explanation for the antiinflammatory effect of GH. Similarly, the suppression of S100A8 and S100A9 by glucocorticoids in a skin inflammation model (41) may be linked to the antiinflammatory effects of these agents, although this depends on the cell type or other inflammatory mediators because dexamethasone enhanced S100A8/A9 mRNA levels in human monocytes and macrophages (42). Interestingly, protein kinase C (PKC) inhibition was shown to increase S100A8 gene expression, implying negative regulation by PKC (42). Because protein kinase C can mediate some GH effects in monocytes (43), it is conceivable that PKC activation is also involved in the suppression of calgranulins by GH.

This study demonstrated the novel use of SELDI-TOF MS to discover GH-dependent molecules in human peripheral blood leukocytes. We identified a group of inflammation-related proteins (calgranulins) as GH responsive in white blood cells. This observation contributes to our understanding of the antiinflammatory effect of GH, although our study did not measure other inflammatory markers or explicitly determine whether the inflammatory response in these subjects was suppressed. Our findings could also potentially have important diagnostic implications for growth disorders such as GH deficiency and acromegaly. However, the study was performed on a small cohort and more GH-treated and control subjects would need to be tested before the calgranulins could be considered as clinically useful biomarkers of GH action. Furthermore, the mechanism of functional interaction between GH administration and the expression of these inflammation-related proteins in leukocytes remains to be elucidated.

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