EXPERIMENTAL STUDY

Human lymphocytes express *hGH-N* gene transcripts of 22 kDa, 20 kDa and minor forms of GH, but not *hGH-V* gene

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Abstract

Expression of human growth hormone (hGH) in lymphocytes was examined by reverse transcription polymerase chain reaction (RT-PCR) in five normal subjects. Transcripts of *hGH-N* gene, but not *hGH-V* gene, were detected. Sequence analysis revealed four kinds of transcripts: 22 kDa GH, 20 kDa GH and two other forms of variant GH. The 20 kDa GH transcript was generated by alternative splicing within exon 3, resulting in a 45 bp deletion. One of the variant GH transcripts was also generated by alternative splicing within exon 3, but at a different site, resulting in a 73 bp deletion. Because of a frameshift, this variant GH transcript may encode a 6.6 kDa protein (truncated GH) that structurally differs from that of 22 kDa GH after residue 31. In the other variant GH mRNA, exons 3 and 4 were completely skipped. The proportions of expression of 22 kDa GH, 20 kDa GH and the truncated GH were 60.9 ± 13.6 ($\pm$ S.D.)%, 32.7 ± 14.1 % and 6.4 ± 1.1 % ($n=5$), respectively, by comparative RT-PCR.

We conclude that human lymphocytes, like the pituitary gland, express *hGH-N* gene transcripts of mainly 22 kDa GH, but also 20 kDa GH and minor variant forms of GH.

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Introduction

Increasing evidence suggests that the immune and neuroendocrine systems interact with each other: cells of the immune system produce neuroendocrine hormones, those of the neuroendocrine system produce cytokines, and the immune and neuroendocrine systems share the same receptors (1). We and other investigators have reported that lymphocytes synthesize and secrete growth hormone (GH) the antigenicity, molecular weight and bioactivity of which are similar to those of pituitary GH (2–9). Because lymphocytes possess GH receptors (10), GH produced by lymphocytes may act in an autocrine or paracrine fashion. Hypophysectomized rats show an increased susceptibility to salmonella infection-induced lethality, and the survival rate is increased by GH treatment (11), suggesting that GH has an important role in immune function. GH also enhances natural killer cell activity (12), stimulates lymphoproliferation (13) and increases superoxide anion generation (14).

There are two human GH genes, termed the *hGH-N* gene and *hGH-V* gene, which are mainly expressed in the pituitary gland and the placenta respectively (15). The corresponding proteins are highly homologous and differ only by 13 out of 191 amino acids. The *hGH-N* gene has five exons and four introns, and the primary

transcript is spliced into two different mature mRNAs, most of the mRNA encoding 22 kDa GH and 5–10% of mRNA encoding 20 kDa GH. Although there are several reports describing the expression of GH mRNA in the immune system (7–9), little is known about the heterogeneity of the immune cell-derived GH transcript(s). In the present study, we examined *hGH-N* and *hGH-V* gene expression in human lymphocytes and the ratio of various GH transcripts, using quantitative RT-PCR techniques in samples from normal volunteers.

Materials and methods

Cell preparation and RNA isolation

Venous blood (20 ml) was taken from five normal adult volunteers (four men and a woman, ages 31–47 years) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) as described previously (2). Placenta samples were obtained from women who had normal deliveries. Total RNA was isolated using a monophasic solution of phenol and guanidine isothiocyanate, followed by extraction and precipitation with isopropyl alcohol.

Table 1 Sequences of primers for *hGH-N* and *hGH-V* transcripts. Nucleotides underlined in primers for *hGH-V* transcripts indicate differences from the sequence of *hGH-N* cDNA.

Sense primer	Antisense primer
<i>hGH-N</i> -S1 5'-CAGGCTTTTGTACAACGCTATG-3'	<i>hGH-N</i> -AS1 5'-GTTCTTGAGTAGTGCGTCATCGTT-3'
	<i>hGH-N</i> -AS2 5'-TTGGAGGGTGTGCGGAATAGACT-3'
<i>hGH-V</i> -S1 5'-GTTTGAAGAAGCCTATATCCIG-3'	<i>hGH-V</i> -AS1 5'-CTCTAGGTCCTTCAGGIGGCG-3'
	<i>hGH-V</i> -AS2 5'-GTTAGATTCTGCTGCGTTTICA-3'

Reverse transcription-polymerase chain reaction (RT-PCR)

GH primers (GH-N-S1 and GH-N-AS1) flanking the last 73 nucleotides of exon 2, full exons 3 and 4 and the first 99 nucleotides of exon 5 were designed on the basis of the sequence of the *hGH-N* gene (15) (Table 1). The amplified fragment of the 22 kDa GH cDNA was 457 bp in length. Because nucleotide sequences of *hGH-N* and *hGH-V* cDNAs are very similar (16), *hGH-V*-specific primers were carefully selected so that as many different nucleotides, especially at the 3' end of the primers, were included (Table 1). The length of amplified *hGH-V* cDNA was 265 bp for primer pairs of *hGH-V*-S1 and *hGH-V*-AS1, and 127 bp for those of *hGH-V*-S1 and *hGH-V*-AS2. RNA samples were treated with DNase I (Gibco BRL, Rockville, MD, USA) to remove trace amounts of genomic DNA. RT-PCR was performed with a commercially available RT-PCR system (Promega, Madison, WI, USA) in 50 µl 10 mmol/l Tris-HCl buffer containing 1 µmol/l each sense and antisense primer, 0.1 U/µl avian myeloblastosis virus reverse transcriptase, 0.1 U/µl *Thermus flavus* DNA polymerase, 0.2 mmol/l dNTP, 1.2 mmol/l MgSO₄ and 0.1–1 µg RNA. Thirty-eight cycles of PCR were carried out in a Gene Amp PCR System 9600 (Perkin-Elmer Co., Foster City, CA, USA) under the following conditions: denaturing at 94 °C for 40 s, annealing at 64 °C for 40 s and extension at 72 °C for 40 s.

Cloning and sequencing

The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide, the bands excised from the gel, and the cDNA fragments cloned using the TA cloning kit (Invitrogen Co., Carlsbad, CA, USA). Sequencing of the cDNA was performed with a Thermo Sequence dye terminator cycle sequencing pre-mix kit (Amersham Life Sci. Inc., Cleveland, OH, USA) in a Gene Amp PCR System 9600.

Quantitative RT-PCR

To separate the bands of PCR products more accurately, we designed another set of primers for *hGH-N* gene transcripts (*hGH-N*-S1 and *hGH-N*-AS2) to produce shorter PCR products, and analyzed them by polyacrylamide gel electrophoresis and fluorescence detection

(Table 1). The predicted length of the 22 kDa GH cDNA fragment was 168 bp. RT was performed as described above and 27–38 cycles of PCR were carried out at intervals of 1–2 cycles. The PCR products were electrophoresed on 10% polyacrylamide gel and the bands of PCR fragments were stained with Vistra green (Amersham, Buckinghamshire, UK) for 20 min. The fluorescence intensity of each band was measured by a Fluor Imager 595 (Molecular Dynamics, Sunnyvale, CA, USA), which is an optical scanner that detects light emitted from fluorescent samples and produces a digital image. The wavelengths of excitation and emission were 488 and 530 nm respectively. The data were then analyzed by Image Quant, a software program from Molecular Dynamics. To estimate the initial amount of the template, regression equations of the form: $y = a \times b^n$, where y is the fluorescence intensity and n is the number of cycles, were fitted to the data in the linear portion of the semilogarithmic graphs. Constants a and b of the equation reflected the amount of the original template and the efficiency of amplification every cycle respectively (17). Because the fluorescence intensity is proportional to the size of cDNA, the ratio of fluorescence intensity of each band was corrected by the molecular weight to yield the molar ratio. The data are presented as the mean $\pm$ s.d.

Results

Figure 1 shows agarose gel electrophoresis of PCR products using *hGH-N*-S1 and *hGH-N*-AS1 primers. Although the bands were broad, PCR products with the predicted size (457 bp) were detected in lymphocytes from all five normal volunteers and unexpected shorter bands (172 bp) were observed in two of them. To confirm that the PCR products originated from *hGH-N* transcripts, we performed sequence analysis. Sequencing of 20 clones obtained from cDNA bands of around 457 bp revealed three kinds of transcripts: 14 clones of 22 kDa GH cDNA, three clones of 20 kDa GH cDNA and three clones of variant GH cDNA. Transcript of the 20 kDa GH was generated by alternative splicing within exon 3, resulting in a 45 bp deletion, while the variant GH transcript was also produced by alternative splicing within exon 3, but at a different site (Fig. 2). Seventy-three base pairs from the start of exon 3 were deleted in

Figure 1 Agarose gel electrophoresis of PCR products followed by ethidium bromide staining. RNA samples (0.1–1 μ g) from five normal volunteers were reverse transcribed and 38 cycles of PCR reactions were performed using *hGH-N-S1* in exon 2 and *hGH-N-AS1* in exon 5. Lanes 1 and 7: 100-bp DNA markers; lanes 2–6: volunteers 1–5, respectively. Although the bands were broad, PCR products of the predicted size (457 bp) were detected in all five normal volunteers and shorter bands (172 bp) were observed in lane 4 (volunteer 3) and lane 5 (volunteer 4).

this cDNA. Because the splicing was between positions 1 and 2 of the original glutamic acid codon (GAG), a frameshift occurred and a stop codon (UAG) appeared within exon 4 in this mRNA. This GH transcript was speculated to encode a 6.6 kDa protein with 48 amino acids, the structure of which differs from 22 kDa and 20 kDa GH after residue 31 (truncated GH). Sequencing

of shorter PCR products (172 bp) disclosed another variant GH transcript that completely skipped exons 3 and 4 (Fig. 2).

To determine the ratio of expression of 22 kDa, 20 kDa and truncated GH in human lymphocytes, we performed comparative RT-PCR. Because separation of larger-size PCR products on agarose gel electrophoresis was not adequate, we carried out RT-PCR using another set of primers (*hGH-N-S1* and *hGH-N-AS2*), generating a 168 bp cDNA fragment for 22 kDa GH, 123 bp for the 20 kDa GH and 95 bp for the truncated GH, and separated them by polyacrylamide gel electrophoresis. As shown in Fig. 3a, three bands with the predicted sizes were detected and the fluorescence intensity of each band increased with the increase in cycles. Figure 3b shows the reaction cycle–fluorescence intensity curves of each band. The slopes of the linear portion of the curves (log *b*) were similar, indicating similar efficiencies of PCR amplification for the three transcripts. The *y* axis intersection (log *a*) represents the initial level of transcript. The ratios of expression of the 22 kDa, 20 kDa and truncated GH were determined in five normal subjects. They varied among individuals; the ratio is shown in Table 2.

Figure 4 shows agarose gel electrophoresis of PCR products using two different primer pairs for *hGH-V* gene transcripts. Although *hGH-V* gene expression was clearly detected in the placenta with both primer

GH DNA

Figure 2 Structure of the *hGH-N* gene and four forms of its transcripts. Human lymphocytes expressed transcripts of 22 kDa GH, 20 kDa GH and two forms of variant GH.

Figure 3 (a) Representative pattern of polyacrylamide gel electrophoresis of PCR products followed by Vistra green staining. Twenty-seven to 38 cycles of PCR were carried out at intervals of 1–2 cycles using *hGH-N-S1* in exon 2 and *hGH-N-AS2* in exon 3. Three bands with predicted sizes matching 22 kDa GH, 20 kDa GH and the truncated GH transcripts were detected. The fluorescence intensity of each band was measured by FluorImager. (b) Reaction cycle–fluorescence intensity curves of each band (●, 22 kDa GH; ■, 20 kDa GH; ▲, truncated GH) shown in Fig. 3a. The fluorescence intensity reached a plateau in all three transcripts. Regression equations of the form: $y = a \times b^n$, where y is the fluorescence intensity and n is the number of cycles, were fitted to the data in the linear portion of the semilogarithmic graphs. Each constant a and b represents the amount of the original template and the efficiency of amplification of PCR respectively. Note that the slopes ($\log b$) of each band (efficiency of PCR) were almost the same for the three transcripts.

pairs, the expression was not observed in human lymphocytes.

Discussion

In this study, we demonstrated that human lymphocytes express *hGH-N* gene, but not *hGH-V* gene. Because the nucleotide sequences of the two genes are very similar – that is, 95% of the entire open reading frames are identical – cross-reaction of *hGH-V* primers with *hGH-N* transcripts becomes a problem when examining the expression of *hGH-V* gene by RT-PCR. Therefore, we carefully chose primers that include as many mismatches as possible, especially at the 3' end, so that the cross-reactivity with *hGH-N* transcripts could be minimized. Our results are in contrast to those presented in a previous report that showed the presence of both *hGH-N* and *hGH-V* transcripts by RT-PCR in human peripheral blood mononuclear cells (18). Although the reasons for the discrepancy are unclear, there have been some reports supporting our findings. Kooijman *et al.* (19) revealed that human neutrophils express *hGH-N* gene transcript, but not *hGH-V* gene transcript, using RT-PCR followed by restriction analysis. Palmetshofer *et al.* (20) showed that lymphoid and myeloid cell lines express *hGH-N*-derived mRNAs, but not *hGH-V* mRNAs, using RT-PCR followed by sequencing.

In the pituitary gland, the main transcript of the *hGH-N* gene encodes a protein with a molecular weight of 22 kDa (22 kDa GH). An alternatively spliced mRNA that lacks 45 nucleotides of exon 3 is generated in small amounts (5–10%) (15). Four additional alternative splicing products of the *hGH-N* gene have been detected in pituitary tumor tissue (20, 21). In three of these mRNAs, exon 3, exons 3 and 4, and exons 2, 3 and 4 are completely skipped. The other mRNA arises from an alternative splicing within exon 3, but at a different site from that for 20 kDa GH. This codes for a truncated GH with 48 amino acids that structurally differs from 22 kDa GH after residue 31 because of a frameshift. In this study, we demonstrated that human lymphocytes also have alternatively spliced mRNAs. One of these mRNA encodes a 20 kDa GH, as in the pituitary gland.

Table 2 Proportions of the expression of 22 kDa GH, 20 kDa GH and a truncated GH in lymphocytes from normal subjects.

Subject	Sex	Age (yr)	Transcripts (%)		
			22 kDa GH	20 kDa GH	Truncated GH
1	M	41	76.5	16.9	6.6
2	F	31	63.0	32.1	4.9
3	M	47	37.1	57.1	5.8
4	M	31	57.3	36.3	6.4
5	M	33	70.6	21.1	8.3
Mean $\pm$ s.d.			60.9 $\pm$ 13.6	32.7 $\pm$ 14.1	6.4 $\pm$ 1.1

Figure 4 Agarose gel electrophoresis of PCR products followed by ethidium bromide staining. RNA samples (0.1–1 µg) from lymphocytes of five normal volunteers and placenta tissue from one woman were reverse transcribed and 38 cycles of PCR reactions were performed using *hGH-V-S1* and *hGH-V-AS1* (a) and *hGH-V-S1* and *hGH-V-AS2* (b). Lanes 1 and 8: 100 bp DNA markers; lane 2: placenta tissue; lanes 3–7: lymphocytes in volunteers 1–5, respectively. Although *hGH-V* gene expression was clearly detected in the placenta with both primer pairs, the expression was not observed in human lymphocytes.

This is in agreement with a previous report that described the presence of a 20 kDa GH transcript of *hGH-N* gene in lymphoid and myeloid cell lines (20). However, Wu *et al.* (22) found no *hGH-N* transcripts other than that of 22 kDa GH. This discrepancy can be explained by the positions of primers for RT-PCR: as Wu *et al.* designed a sense primer flanking exon 2 and exon 3, 20 kDa GH transcript, which lacks the first 45 nucleotides of exon 3, could not be amplified by RT-PCR using this primer. Variant GH transcript lacking 73 nucleotides of exon 3 was first demonstrated in the immune cells, and may correspond to the transcript encoding a truncated GH in pituitary tumor tissue (21). The other variant GH transcript that completely skipped exons 3 and 4 was previously reported in the pituitary gland and lymphoid and myeloid cell lines (20). We showed that human lymphocytes from some individuals express the variant GH transcript, but others do not. The reasons for the individual variations and the biological significance are unknown.

In this study, we designed primers sharing 22 kDa, 20 kDa and truncated GH transcripts, to enable a

comparative RT-PCR to be performed for the determination of their ratios. Efficiency of PCR depends on many factors, including primers, annealing temperature, Mg^{2+} concentration, DNA polymerase and RNA samples (23). Because we examined the three transcripts in an RNA sample by RT-PCR under the same conditions and using the same primers, the efficiency of PCR amplification was assumed to be equal, enabling comparison of the ratio of each transcript. Indeed, the slopes of the linear portion of reaction cycle–PCR product curves were almost equal among the three transcripts.

Although other minor *hGH-N* transcripts have also been reported using RT-PCR with different primers in lymphoid and myeloid cell lines (20), GH transcripts expressed in human lymphocytes were *hGH-N* gene-derived ones of mainly 22 kDa GH, followed by 20 kDa GH and minor variant forms of GH, as in the pituitary gland.

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