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Effects of Growth Hormone on the Differentiation of Mouse B-Lymphoid Precursors

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Abstract. Growth hormone (GH) has been known to enhance immune responses directly or through insulin-like growth factor-I (IGF-I). The present study aimed to clarify the roles of GH in the differentiation of B-lineage precursors. In short-term bone marrow cultures, which contained stem cells and early B-lineage cells, GH (10 μg/L) treatment for one day decreased the percentages of stem cells (0.5-fold) and increased those of B-lineage cells (1.4-fold). Furthermore, GH changed the expressions of transcription factors for B cell progenitors differentiation such as paired box gene-5 (Pax-5), immunoglobulin-associated-α (Ig-α)/CD79a, Ig-β/CD79b, and IGF-I. Thus, a physiological concentration of GH stimulated the differentiation of B-lymphoid precursors from bone marrow stem cells. Since mRNAs of both GH and GH receptor were present in stem cells and B-cell precursors in bone marrow, GH may modulate B-lymphoid precursors development in an autocrine or paracrine manner in bone marrows.

Keywords: growth hormone, bone marrow, stem cell, B cell, insulin-like growth factor-I

Introduction

There is increasing evidence to suggest that growth hormone (GH) is involved in immunoregulation (for reviews, see Refs. 1 and 2). In several animal species, GH deficiency resulted in small hypo-cellular primary and secondary lymphoid organs with abnormal morphology (3 – 6). Reconstitution with GH, or a combination of GH and thyroxine, increased the cell numbers and restored normal morphology in lymphoid organs in GH-deficient animals (5, 7), suggesting that GH is important for normal development of the immune system. Treatment with GH enhances interleukin (IL)-6, IL-1, and granulocyte-macrophage-colony stimulating factor (GM-CSF) production by mouse thymocytes and exerts a modulatory role in thymic hormone production, particularly in the secreation of thymulin (8 – 11). Besides these effects on T cell functions, GH appears to effect B-cell differentiation (12, 13). For example, snell bagg dwarf mice lacking GH and other neuroendcrine mediators were found to be deficient in pre-B cells in their bone marrow, suggesting that GH or other neuroendocrine mediators play a role in early B-cell development. Since little is known about the roles of GH in bone marrow, we examined the effects of GH on early B-cell development using short-term bone marrow cultures, in which hematopoietic stem cells differentiate to pre-B cells (14, 15).

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Materials and Methods

Animals

Male C57BL/6 mice of 8 weeks of age were purchased from Japan SLC (Shizuoka). Mice were handled in accordance with the "Rules of Animal Experimentation Committee, Kansai Medical University".

Bone marrow cultures

Bone marrow cultures were established and maintained as described by Whitlock and Witte (14) and Whitlock et al. (15). Briefly, bone marrow cells from the four major leg bones were placed in culture at a density of 5×10^9 cells/L in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 5% heat-inactivated CELLect GOLD fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 2×10^{-3} M L-glutamine, 1×10^5 U/L penicillin, 100 mg/L streptomycin, and 5×10^{-2} M of 2-mercaptoethanol. Bone marrow cells were cultured

for 4 days and then stimulated with rat GH (NIDDK-rGH-I-6, 10 μg/L; NJDDL-NIH, Bethesda, MD, USA) for 1, 3, and 7 days. Rat GH recognizes the murine GH recepror (GHR) (16). After stimulation for definite periods, cells were harvested, gently washed, and subjected to the following experiments. As controls, unstimulated cells were used.

Cell surface staining, flow cytometric analysis and cell sorting

Cells were harvested from cultures and washed three times with cold washing buffer containing phosphate-buffered saline (PBS) with 2% heat-inactivated FBS and 0.05% sodium azide. Cells were preincubated with appropriate concentrations of anti-mouse CD16/CD32 (Fcγ III/II receptor) monoclonal antibody (mAb) for 5 min on ice to block nonspecific binding of antibodies and were then incubated with detection-mAbs for 30 min on ice. Stem cells were detected with phycoerythrin (PE)-conjugated mAb against mouse CD34, an important marker of the stem cells, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse stem cell antigen-1 (Sca-1) mAb, which reacts with stem cell antigens Ly-6A/E. B cells were detected with FITC-conjugated anti-mouse B220 mAb, which identifies all cells of B lymphocyte lineage, regardless of maturity. All antibodies were obtained from BD Biosciences Pharmingen (San Jose, CA, USA). The cells were then washed three times with the washing buffer, resuspended in PBS, and analyzed on a FACScan flow cytometer with the CellQuest program (BD Biosciences). $CD34^+$, $Sca\text{-}1^+$, or $B220^+$ cells were sorted on the EPICS ALTRA cell sorter (Beckman Coulter, Miami, FL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene expression

Total RNA was isolated from cells using TRIZOL Reagent (Invitrogen). The amounts of RNA were quantitated using UV spectroscopy (absorption at 260 nm). RT-PCR was performed with the SuperScript One-Step RT-PCR with Platinum Taq System (Invitrogen). The program for RT-PCR using in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA) was 48°C RT-reaction for 30 min, 94°C RT-inactivation reaction for 2 min, and then 30 to 40 cycles with 95°C denaturation for 30 s, 55°C annealing for 1 min, and 70°C extension for 1 min. Primer sequenses are listed in Table 1. All primers were synthesized by Invitrogen Custom Primers. The linear range of the PCR amplifi-

Table 1. Primers used for RT-PCR

Gene	Primer	Sequence	Product Size (bp)	Reference
GH	forward	5'-CAGCCTGATGTTTGGTACCTCGGA-3'	253	17
	reverse	5'-GCGGCGACACTTCATGACCCGCA-3'		
GHR	forward	5'-AGTTGGAGGAGGTGAACACCAT-3'	330	17
	reverse	5'-GGCACAAGAGATCAGCTTCCAT-3'		
IGF-I	forward	5'-GGACCAGAGACCCTTTGCGGGG-3'	210	16
	reverse	5'-GGCTGCTTTTGTAGGCTTCAGTGG-3'		
Ig-α/CD79a	forward	5'-GCCAGGGGGTCTAGAAGC-3'	310	18
	reverse	5'-TCACTTGGCACCCAGTACAA-3'		
Ig-β/CD79b	forward	5'-GGTGAGCCGGTACCAGCAATG-3'	350	19
	reverse	5'-AGTTCCGTGCCACAGCTGTCG-3'		
PU.1	forward	5'-CGGATGACTTGGTTACTTACG-3'	293	19
	reverse	5'-GTAGGAAACCTGGTGACTGAG-3'		
Pax-5/BSAP	forward	5'-CTACAGGCTCCGTGACGCAG-3'	439	20
	reverse	5'-TCTCGGCCTGTGACAATAGG-3'		
GATA-1	forward	5'-GGAATTCGGGCCCCTTGTGAGGCCAGAGAG-3'	375	20
	reverse	5'-CGGGGTACCTCACGCTCCAGCCAGATTCGACCC-3'		
GATA-2	forward	5'-CGGAATTCGACACACCACCCGATACCCACCTAT-3'	720	20
	reverse	5'-CGGAATTCGCCTACGCCATGGCAGTCACCATGCT-3'		
GATA-3	forward	5'-TCGGCCATTCGTACATGGAA-3'	262	20
	reverse	5'-GAGAGCCGTGGTGGATGGAC-3'		
β-actin	forward	5'-GTCACCCACACTGTGCCCATCT-3'	542	17
	reverse	5'-ACAGAGTACTTGCGCTCAGGAG-3'		

cation was determined by carrying out the PCR for varying numbers of cycles on a fixed quantity of RNA (0.1 μg), so that the expression levels could be evaluated semi-quantitatively. The optimal numbers of cycles were 30 cycles for immunoglobulin-associated-α (Ig-α)/CD79a and Ig-β/CD79b; 35 cycles for GH, GHR, insulin-like growth factor-I (IGF-I), GATA-3, PU.1 and paired box gene (Pax)-5; and 40 cycles for GATA-1 and GATA-2. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. PCR product bands of the expected sizes were then analyzed using a model FluorChem IS-8000 imaging densitometer (Alpha Innotech, San Leandro, CA, USA). For quantiative comparisons, absorbency ratio of the target band over the β-actin band was normalized by the ratio in the untreated samples.

Cloning and sequencing

The RT-PCR products of GH and GHR were electrophoresed in 2% agarose gels and ethidium bromide stained bands of the PCR fragments were excised from the gel, followed by purification using GENECLEAN II Kit (BIO 101, Vista, CA, USA). Then the PCR fragments were ligated to a plasmid pCR2.1 T-vector by T4 DNA ligase and transformed into competent cells using the TA Cloning Kit (Invitrogen). The plasmid DNA was purified by an alkaline lysis procedure using the Mag-Extractor nucleic acid purification kit (Toyobo, Osaka) and sequencing of the cDNA fragments were performed using the ABI PRISM Big dye Terminatior v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a GeneAmp PCR System 9600.

Statistical analyses

The paired Student's *t*-test, Wilcoxon signed rank test, and analysis of variance (ANOVA) were used to compare differences between groups. The differences with $P<0.05$ were considered to be significant.

Results

GH and GHR mRNA expression in bone marrow cells

As shown in Fig. 1A, GH and GHR mRNAs were expressed in mouse bone marrow cells as examined by RT-PCR. The amount of GH mRNA in bone marrow was approximately one fourth of that in spleen, and GHR mRNA level in bone marrow was approximately 0.9-fold of that in spleen (Fig. 1B). Thus, both GH and GHR seem to be expressed in mouse bone marrow cells.

Effects of GH on bone marrow cultures

To test the effects of GH on B cell generation from bone marrow stem cells, we examined GH-induced changes in the populations of stem cells ($CD34^+$ or $Sca\text{-}1^+$) and B cells ($B220^+$) in bone marrow cultures

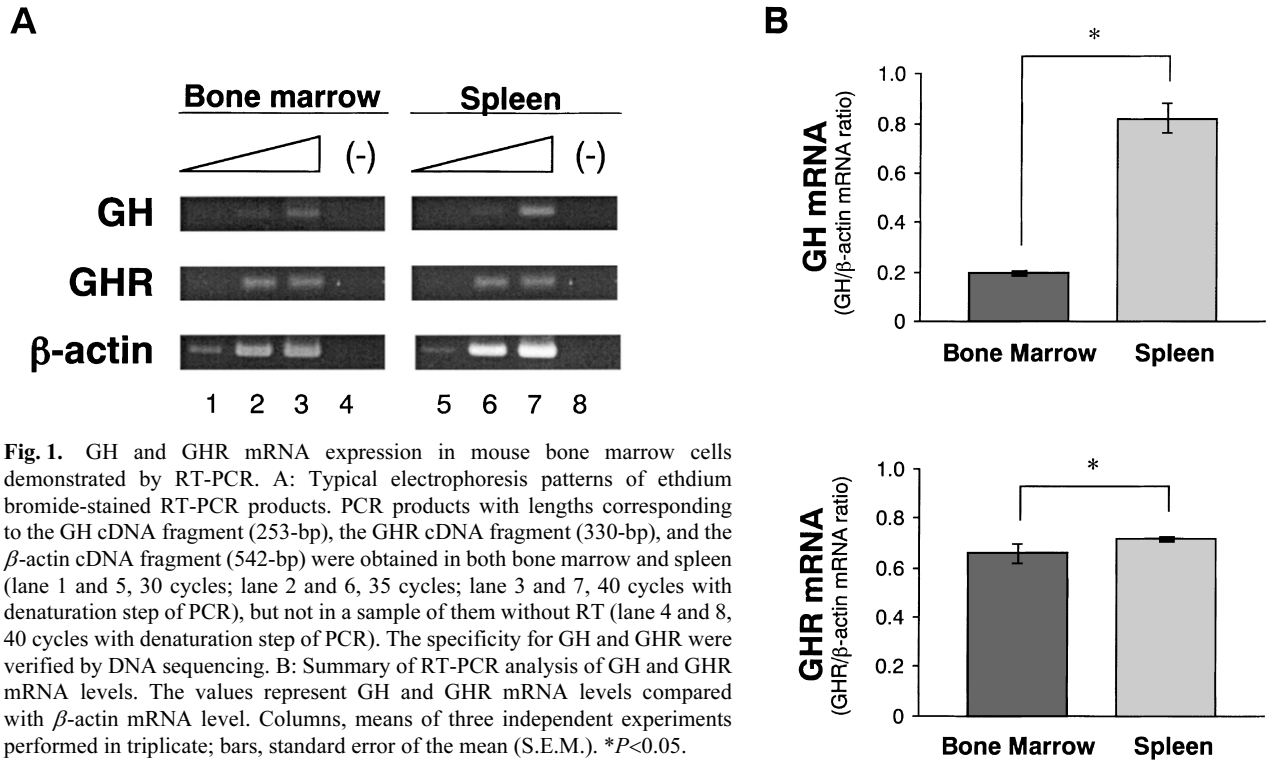

Fig. 1. GH and GHR mRNA expression in mouse bone marrow cells demonstrated by RT-PCR. A: Typical electrophoresis patterns of ethdium bromide-stained RT-PCR products. PCR products with lengths corresponding to the GH cDNA fragment (253-bp), the GHR cDNA fragment (330-bp), and the β-actin cDNA fragment (542-bp) were obtained in both bone marrow and spleen (lane 1 and 5, 30 cycles; lane 2 and 6, 35 cycles; lane 3 and 7, 40 cycles with denaturation step of PCR), but not in a sample of them without RT (lane 4 and 8, 40 cycles with denaturation step of PCR). The specificity for GH and GHR were verified by DNA sequencing. B: Summary of RT-PCR analysis of GH and GHR mRNA levels. The values represent GH and GHR mRNA levels compared with β-actin mRNA level. Columns, means of three independent experiments performed in triplicate; bars, standard error of the mean (S.E.M.). *$P<0.05$.

using flow cytometric analysis. In control culture (without GH-treatment), the population of $CD34^+$ cells decreased with time, the population of $Sca\text{-}1^+$ cells transiently increased and then decreased, and the population of $B220^+$ cells increased with time. Although GH treatment (10 μg/L, for 3 days) did not affect cell

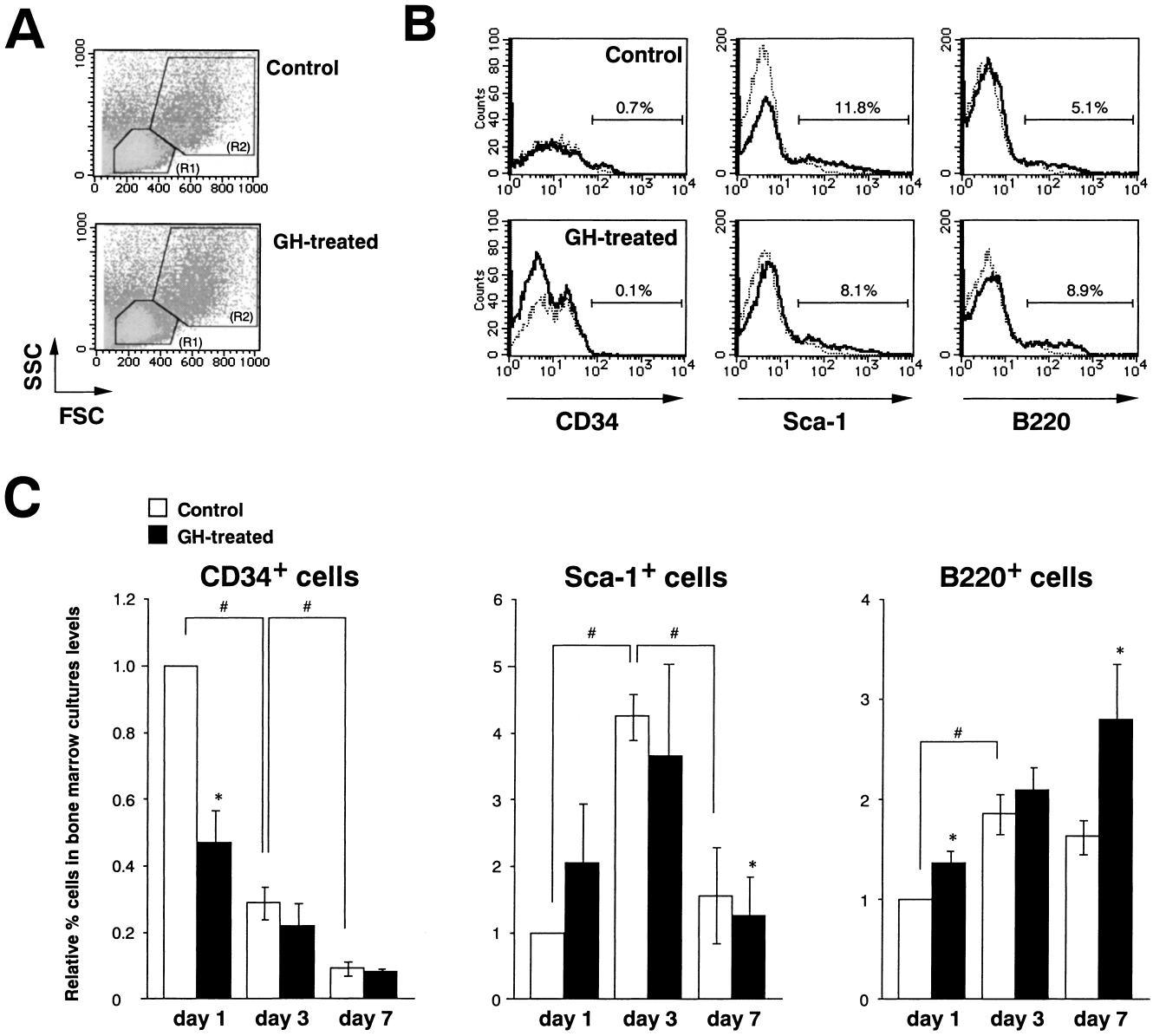

Fig. 2. Flow cytometric analyses of bone marrow culture cells. A: Typical example of forward scatter (FSC) and side scatter (SSC) analysis. The majority of cells gated on R1 are stem cells and lymphocytes, and those gated on R2 are stromal cells. No significant differences were observed in FSC and SSC between bone marrow cultures with GH treatment for one day and controls. B: Flow cytometric analyses for surface CD34, Sca-1, and B220 molecules on the bone marrow culture cells. The samples stained with monoclonal antibodies (solid lines) are shown in comparison with negative controls without monoclonal antibodies (dotted lines). After GH treatment (10 μg/L), bone marrow cultures showed decreased expression of CD34 (stem cell marker protein) and increased expression of B220 (B cell marker protein). C: Summary for the results of analyzing the percentages of $CD34^+$, $Sca\text{-}1^+$, or $B220^+$ cell populations in bone marrow cultures obtained by flow cytometry. The values represent the relative level of the percentages of $CD34^+$, $Sca\text{-}1^+$, or $B220^+$ cell populations in GH-treated cultures and $Sca\text{-}1^+$ and $B220^+$ cell populations in without GH (control) cultures compared with the $CD34^+$ cell populations in control cultures, which was defined as 1.0. The decreased percentages of $CD34^+$ cells were only a tendency in GH treatment-cultures compared with controls at day 1, 3, and 7. The percentages of $Sca\text{-}1^+$ cells were increased in GH treatment for 1 day and were decreased in GH treatment for 3 and 7 days compared with controls. In contrast, the percentages of $B220^+$ cells increased in GH treatment-cultures compared with controls at day 1, 3, and 7. Columns, means of five independent experiments performed in triplicate; bars, S.E.M. $^*P<0.05$, compared with the control (without GH); $^{\#}P<0.05$.

granularity and sizes as assessed by forward scatter (FSC) and side scatter (SSC) (Fig. 2A), expression of CD34 and Sca-1 proteins decreased and expression of B220 proteins increased after GH treatment (Fig. 2B). As summarized in Fig. 2C, the population of $CD34^+$ cells significantly decreased on day 1 of GH-treatment, suggesting that GH was inefficient in maintaining the survival/proliferation machinery of $CD34^+$ cells in bone marrow cultures. The population of $Sca\text{-}1^+$ cells increased on day 1 of GH-treatment and decreased on day 7, suggesting that GH was efficient in differentiation from $CD34^+$ cells to $CD34^-Sca\text{-}1^+$ cells and inefficient in maintaining the survival/proliferation machinery of $Sca\text{-}1^+$ cells in bone marrow cultures. The population of $B220^+$ cells significantly increased on day 1 of GH-treatment and tended to increase over the following period, suggesting that GH promoted bone marrow-drived B lineage cell differentiation.

Effects of exogenous GH on GH, GHR, and IGF-I mRNA expressions

GH reportedly regulates the expressions of GH and GHR mRNAs/proteins in several tissues including liver and neurons (21 – 24). To examine the effects of GH on the expression of GH and GHR mRNA themselves in bone marrow cultures, GH-induced changes in GH and GHR mRNAs were examined. As shown in Fig. 3, GH mRNA levels in $CD34^+$ cells slightly but significantly decreased, while those in $Sca\text{-}1^+$ and $B220^+$ cells slightly increased by GH treatment. GH stimulation increased GHR mRNA expression in $CD34^+$, $Sca\text{-}1^+$, and $B220^+$ cells, suggesting that the regulation of GHR mRNA expression is one of the downstream events of the GH signal transduction cascade in bone marrow cells.

Since many GH effects are reportedly mediated through IGF-I (25), IGF-I mRNA levels in the bone marrow cultures were also examined. GH treatment

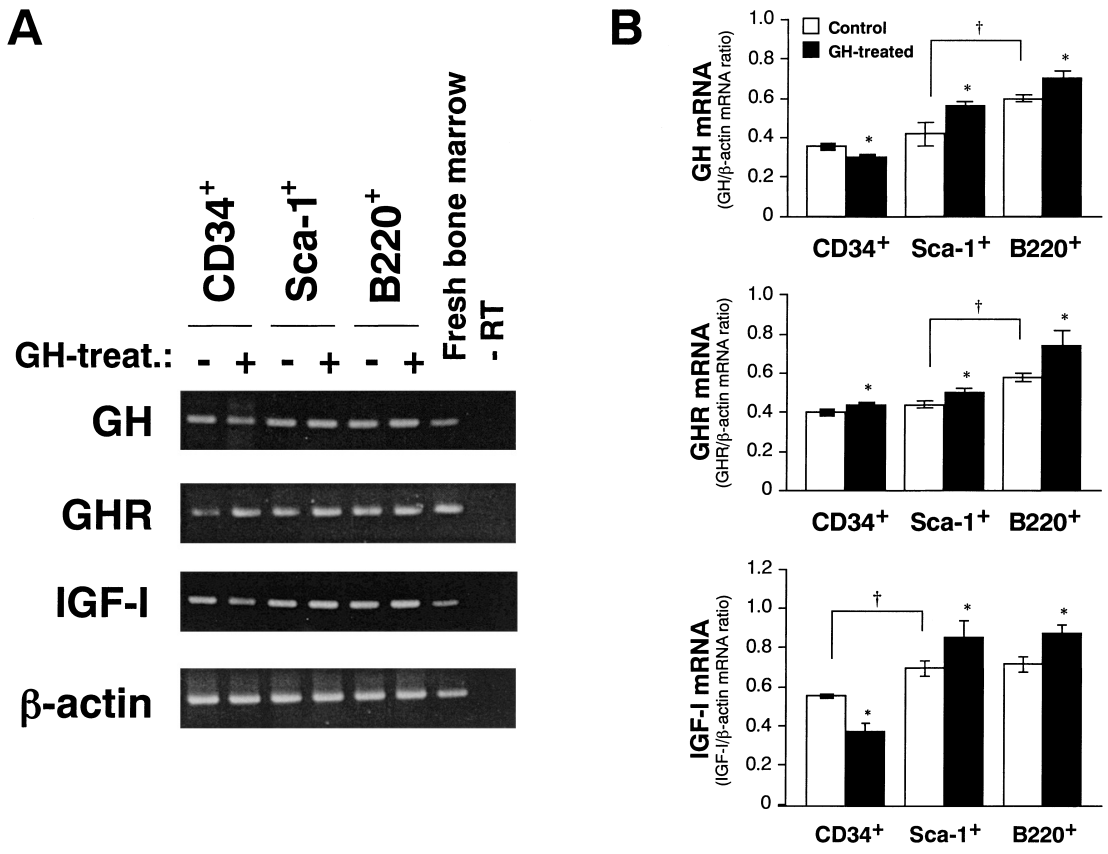

Fig. 3. GH, GHR, and IGF-I mRNA expression in mouse bone marrow cultured cells demonstrated by RT-PCR. A: Typical example of ethdium bromide-stained RT-PCR products. PCR products with lengths corresponding to the GH cDNA fragment (253-bp), GHR cDNA fragment (330-bp), IGF-I cDNA fragment (210-bp), and β-actin cDNA fragment for the control (542-bp) were obtained in samples from bone marrow cultured cells ($CD34^+$, $Sca\text{-}1^+$, and $B220^+$ cell) with or without GH treatment, and freshly prepared bone marrow (positive control), but not in the sense sample without RT (negative control). B: Summary of RT-PCR analyses of GH, GHR, and IGF-I mRNA levels in $CD34^+$, $Sca\text{-}1^+$, and $B220^+$ cells. The values represent GH, GHR, and IGF-I mRNA levels compared with β-actin mRNA level. Columns, means of three independent experiments performed in triplicate; bars, S.E.M. *P<0.05, compared with each control culture; $^\dagger P$<0.05.

increased IGF-I mRNA levels in Sca-1$^+$ cells or B220$^+$ cells, while it decreased IGF-I mRNA levels in CD34$^+$ cells compared with control cells (Fig. 3). Thus, exogenous GH stimulates GH, GHR, and IGF-I mRNA expressions in differentiated B lineage progenitors in bone marrow cells.

Effects of GH on lineage-associated gene expression in bone marrow culture cells

Execution of the early B-lineage developmental program requires the coordinate expression of a series of transcription factors that regulate sets of genes critical for progression through each differentiation stage. We evaluated the expression of lineage-associated transcription factors in bone marrow cultures (Fig. 4). Ig-α /CD79a and Ig-β/CD79b are associated with the B-cell antigen receptor and participate in B-cell antigen receptor-mediated signal transduction. Ig-α mRNA expression increased in Sca-1$^+$ or B220$^+$ cells by GH treatment. Ig-β mRNA expression decreased in CD34$^+$ or Sca-1$^+$ cells, while it increased in B220$^+$ cells by GH treatment. Transcripts for PU.1, which is known to be required for B lymphocyte lineage development, were detectable in CD34$^+$, Sca-1$^+$, and B220$^+$ cells. PU.1 mRNA level was decreased in stem cells (CD34$^+$ or Sca-1$^+$ cells) and increased in B cells (B220$^+$ cells) by GH-stimulation, suggesting that GH may accelerate B-lymphocyte development via regulation of PU.1. Pax-5 genes, also called B-cell specific activator protein (BSAP), were highly upregulated in more differentiated B-lineage progenitors, and also GH treatment significantly increased Pax-5 mRNA levels in both Sca-1$^+$ and B220$^+$ cells. GATA-3 transcription activity was constitutively higher in stem cells (CD34$^+$ or Sca-1$^+$ cells)

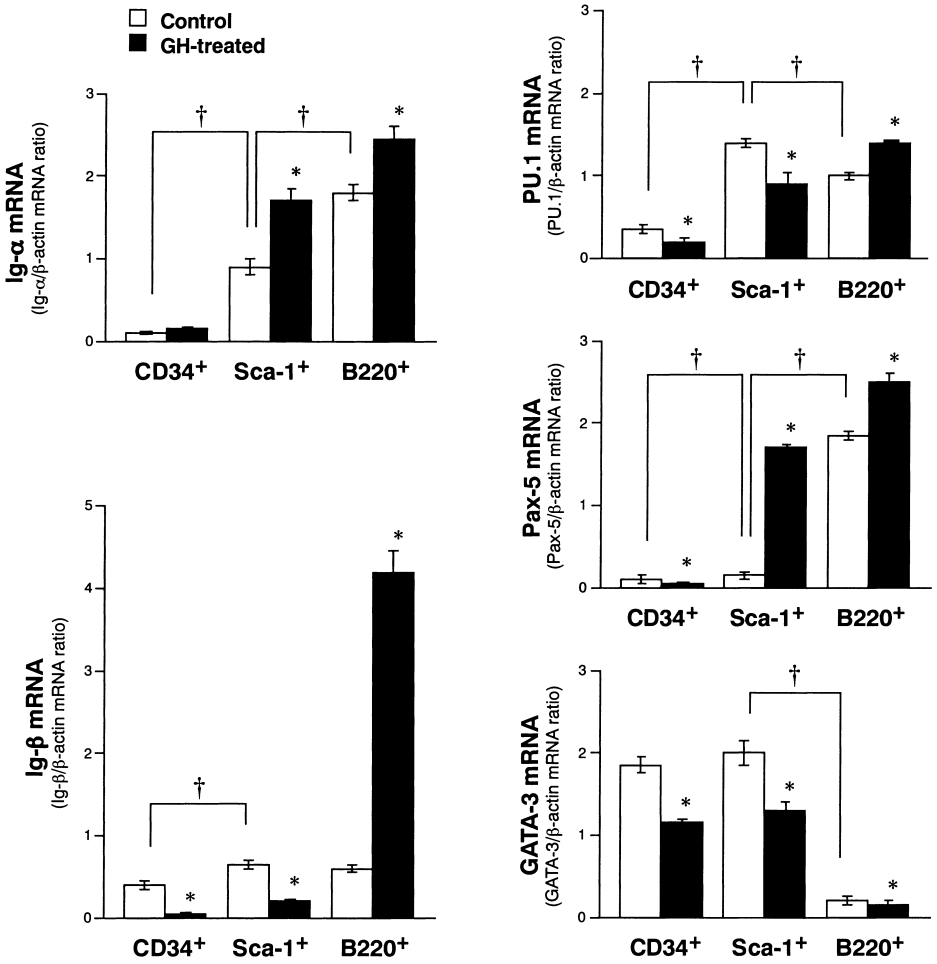

Fig. 4. Effect of GH treatment on the expression of the lymphohematopoietic genes in mouse bone marrow cultured cells. RT-PCR was used to amplify transcripts for the indicated genes, and the results were normalized according to β-actin expression. Summary of RT-PCR analysis of Ig-α, Ig-β, PU.1, Pax-5, and GATA-3 mRNA levels in CD34$^+$, Sca-1$^+$, and B220$^+$ cells. Columns, means of three independent experiments performed in triplicate; bars, S.D. *P<0.05 compared with each control culture; $^\dagger P$<0.05.

than in B cells ($B220^+$ cells) and was downregulated with GH treatment. GATA-1 and GATA-2 transcripts were undetectable in the cultures, whereas they were detected in freshly prepared bone marrow cells (data not shown). Taken together, the above observations strongly suggest that GH is one of the essential factors for B cell maturation.

Discussion

The generation and cell turnover of lymphohemopoietic lineages are very active throughout the lifespan of the individual. These processes are under the regulatory control of intercellular interactions and soluble factors acting in autocrine, paracrine, and endocrine manners. Previous reports have shown that GH is produced by lymphoid cells (26 – 30) and that GHR is expressed in several lymphoid cells including bone marrow cells (31, 32), but there are little data about the roles of GH in bone marrow. In this work, we examined the putative involvement of GH in mouse B-lineage precursor development by the short-term bone marrow cultures. These cultures, frequently referred to as Whitlock-Witte cultures, are based on an adherent monolayer derived from bone marrow stromal cells, which can support the in vitro growth and differentiation of lymphoid cells (14, 15). The present culture system is thus suitable for analysis of the phases of growth through bone marrow stem cells.

In order to elucidate the roles of GH in bone marrow cultures, we first confirmed that the GH and GHR are expressed in murine bone marrow cells (Fig. 1). Then we examined the populations of stem cells ($CD34^+$ or $Sca\text{-}1^+$) and B cells ($B220^+$) in bone marrow cultures after GH-treatment. GH induced a decline in the population of stem cells and an augmentation in the population of B cells, suggesting that GH is efficient in bone marrow-drived B lineage cell growth or differentiation.

We showed that exogenous GH induced the cell stage-dependent regulation of GH and IGF-I expression, while exogenous GH induced upregulation of the expression of GHR mRNA levels in both stem cells and B cells (Fig. 3). Although there are many studies on GH or IGF-I function, it has remained to be examined in detail how the expression of GH or IGF-I is regulated by external GH stimuli. This observation might be characteristic of a transition from the $CD34^+$ cell to the $CD34^-Sca\text{-}1^+$ cell stage. The present results supported the idea that a GH-mediated signal is involved in the regulation of GH, GHR, and IGF-I. It has been reported that IGF-I transcripts are expressed in a developmentally dependent manner, being expressed during differentiation of hemopoietic cells into multiple myeloid lineages (33). Administration of IGF-I has been shown to promote B-cell reconstitution after syngeneic bone marrow transplantation in mice (34). IGF-I also promoted the proliferation of various B-cell lymphomas in vitro (35). These results suggest a role of GH (a primary inducer of IGF-I production) in B-cell development and function in bone marrows.

Analysis for some transcription factors and B-cell specific molecules (Fig. 4) support the idea that GH promotes the early B-lymphoid progenitors differentiation in bone marrow cultures. PU.1, a member of the ETS family of transcription factors, is known to be required for B lymphocyte lineage development. Pax-5, also a B-cell specific activator protein (BSAP), is a key transcription factor in B-cell commitment, as it is critical in suppressing alternative lineage cell fates early in development (36). GATA-1 and GATA-2 transcripts were undetectable in the present bone marrow cultures, and GATA-3 transcripts were consistent with T lineage potential being particularly high in stem cells ($CD34^+$ and $Sca\text{-}1^+$ cells). The $CD34^+$ and $Sca\text{-}1^+$ cells in short-term bone marrow cultures have the typical GATA expression patterns of the "common lymphoid progenitors (CLPs)", which are clonogenic precursors of T-lymphocytes, B-lymphocytes, and natural killer cells in mouse bone marrow and fetal liver (20, 37). Collectively, our observations seem to be reasonable in accordance with the function of each transcription factor in the maturation events of early B-lymphoid cells, suggesting that GH modulates the differentiation program of mouse B-cell progenitors.

This is the first report showing direct evidence of GH-induced promoting effects of the early B-lymphoid progenitors differentiation as far as we know. B-cell lymphopoiesis is a complex process evolving through ordered stages of differentiation that are controlled by transcription factors, lineage-specific gene products (e.g., pre-B cell receptor encoding genes and Ig loci-derived clonotypes), stromal cell-progenitor interactions, and paracrine/endocrine factors (38). In this context, the study of pregnant mice has revealed a negative regulatory role of estrogens in bone marrow-derived B cell generation (39, 40). Androgen receptors are also expressed in immature B cells as well as in bone marrow stromal cells, and androgens may also modulate B cell development in male mice (41, 42). Also, IGF-I secreted by stromal cells can stimulate the differentiation to cytoplasmic μ-positive pro-B cells in short-term bone marrow cell cultures (43). Like several hormones, GH may contribute in a major way to the lymphopoietic microenvironment.

Acknowledgments

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