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Pharmacology

Identification of a cDNA for a human high-molecular-weight B-cell growth factor

(cytokine/interleukin/growth factor/lymphoma/leukemia)

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ABSTRACT **Proliferation is necessary for many of the phenotypic changes that occur during B-cell maturation. Further differentiation of mature B cells into plasma cells or memory B cells requires additional rounds of proliferation. In this manuscript, we describe a cDNA for a human B-cell growth factor we call high-molecular-weight B-cell growth factor (HMW-BCGF). Purified HMW-BCGF has been shown to induce B-cell proliferation, inhibit immunoglobulin secretion, and selectively expand certain B-cell subpopulations. Studies using antibodies to HMW-BCGF and its receptor have suggested that HMW-BCGF, while produced by T cells and some malignant B cells, acts predominantly on normal and malignant B cells. The HMW-BCGF cDNA was identified by expression cloning using a monoclonal antibody and polyclonal antisera to HMW-BCGF. Protein produced from the cDNA induced B-cell proliferation, inhibited immunoglobulin secretion, and was recognized in immunoblots by anti-HMW-BCGF antibodies. The amino acid sequence of HMW-BCGF deduced from the cDNA predicts a secreted protein of 53 kDa with three potential N-linked glycosylation sites. The identification of this cDNA will allow further studies examining physiologic roles of this cytokine. We propose to call it interleukin 14.**

Various steps in B-cell maturation and differentiation depend upon proliferation, including rearrangement of heavy- and light-chain genes, switching from use of one to another class of heavy-chain gene, and terminal differentiation into plasma cells (1–7). The signals regulating B-cell proliferation are, however, poorly understood. Interleukin 7 (IL-7) appears to be important for proliferation during certain stages of B-cell maturation (8–10). Various recombinant cytokines including IL-2, IL-3, IL-4, IL-5, IL-10, tumor necrosis factor, and interferons have been described to induce B-cell proliferation of mature B lymphocytes in different human and murine systems (11–24). However, the fact that all of these cytokines have multiple activities on different cell types (25, 26) and many of these cytokines have different activities on human compared with murine B cells (19, 20, 27–29) has raised significant controversy concerning the necessity for and existence of specific human B-cell growth factors.

We have previously described a cytokine produced by malignant B cells, as well as normal and malignant T cells, which we have called high-molecular-weight B-cell growth factor (HMW-BCGF). We classified HMW-BCGF as a human B-cell growth factor based on its ability to induce proliferation of activated B cells, its inability to stimulate resting B cells, and its inability to induce antibody synthesis or secretion by B cells (30–33). HMW-BCGF also has been found to inhibit antibody secretion (34). Among blood leukocytes and tumors derived from them, only B-lineage cells have receptors for HMW-BCGF (33, 35, 36) (F. M. Uckun, R.J.F., and J.L.A., unpublished data). Preliminary data suggest that HMW-BCGF may select and/or expand a subpopulation of normal memory B cells (30, 34, 37).

To precisely characterize HMW-BCGF both structurally and functionally, we undertook identification of its cDNA. This manuscript describes a cDNA from which we have produced recombinant human HMW-BCGF.§

MATERIALS AND METHODS

Cells. Human B and T cells were purified from peripheral blood, as described (34). The Namalva cell line (31) was subcloned to identify clones producing high levels of BCGF.

cDNA Library Synthesis and Screening. mRNA was prepared from Namalva cells stimulated with phytohemagglutinin (PHA) for 8 hr, reverse-transcribed into cDNA, and ligated into the λZAP vector (Stratagene) according to standard protocols (38). The cDNA library was screened with the monoclonal antibody BCGF/1/C2 (32) and a rabbit antiserum to HMW-BCGF (39) according to established protocols (38). The cDNA described was positive with both BCGF/1/C2 and anti-HMW-BCGF antiserum. The HMW-BCGF cDNA was excised from λZAP using helper phage and recircularized to form a Bluescript plasmid, as described (40). The HMW-BCGF plasmid was then sequenced using double-stranded DNA sequencing by the method of Sanger *et al.* (41).

Purification and Sequencing of Native HMW-BCGF. Fifty-liter batches of HMW-BCGF were prepared from Namalva cells stimulated with PHA at 1 μg/ml in serum-free medium (31). Cell supernatants were concentrated by positive-pressure filtration with membranes that exclude molecules >10,000 Da, absorbed with anti-fetal bovine serum Sepharose (32), and then separated by hydroxyapatite chromatography (42). Fractions with BCGF activity were pooled and separated by fluid-phase isoelectric focusing with a Rotofor (Bio-Rad) cell. Rotofor fractions with BCGF activity were separated by two-dimensional SDS/PAGE, transblotted to poly(vinylidene difluoride) paper, and stained with Coomasie blue. Two spots of 60 kDa and isoelectric points of 6.7 and 7.8, respectively, were obtained. N-terminal amino acid sequencing was performed by Edman degradation. In addition, cyanogen bromide digestion was done directly on the

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Abbreviations: HMW-BCGF, high-molecular-weight B-cell growth factor; PHA, phytohemagglutinin; IPTG, isopropyl β-D-thiogalactoside; IL, interleukin.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L15344).

poly(vinylidene difluoride) paper, and the generated fragments were sequenced simultaneously by Edman degration, such that multiple amino acids were obtained at each cycle. The cDNA described here encodes the pI 7.8 protein.

B-Cell Functional Assays. B-cell proliferation was determined by [^{3}H]thymidine incorporation of B cells that had been activated with a 1:25,000 dilution of *Staphyloccocus aureus* Cowen I (Sac; Immunoprecipitin, Bethesda Research Laboratories) for 72 hr and then cultured with cytokines for an additional 72 hr, as described (34). Immunoglobulin secretion by mononuclear cells cultured in the presence and absence of pokeweed mitogen (1:100; Bethesda Research Laboratories) and cytokines for 10 days was determined by ELISA, as described (34).

RNA Preparation and Analysis. Cells were lysed with guanidine isothiocyanate, and total cytoplasmic RNA was isolated by centrifugation over cesium chloride cushions (43). RNA was then size-fractionated by electrophoresis in 1% agarose-formaldehyde gels, transferred to GeneScreen hybridization membranes (DuPont/New England Nuclear), and hybridized to the appropriate ^{32}P-labeled cDNA probe. The β-actin probe was provided by M. Thomas (Washington University). After hybridization, the filters were washed and exposed to Kodak XAR-5 film for 4–24 hr.

Immunoblot Assay. Proteins were size-fractionated in SDS/10% PAGE gels and transblotted to Immobilon-P membranes (Millipore). Filters were blocked in 1% casein and then incubated sequentially with anti-HMW-BCGF antibodies and ^{125}I-labeled protein G (DuPont/New England Nuclear). HMW-BCGF-containing bands were identified by autoradiography. The assay has been described (32).

Production of Recombinant HMW-BCGF Using λZAP Phage. λZAP phage containing the HMW-BCGF or control cDNAs were grown in top agar containing XL-1 blue *Escherichia coli* with or without 0.4 mM isopropyl β-D-thiogalactoside (IPTG) for 12 hr at 37°C. Proteins were then eluted from the top agar with phosphate-buffered saline containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 1 mM phenylmethylsulfonyl fluoride. Proteins for use in functional assays were dialyzed against RPMI 1640 medium and absorbed with De-Toxigel (Pierce).

Production of HMW-BCGF Using SFFV.neo. A published protocol for expression of CR2 in K-562 cells using SFFV.neo

FIG. 1. cDNA sequence from the pI 7.8-HMW-BCGF clone 210B. The signal peptide is underlined. Amino acid sequence that is consistent with sequence from native HMW-BCGF in brackets; amino acid sequence that is consistent with sequence from recombinant HMW-BCGF is in parentheses. In both cases, deduced amino acids that do not agree with amino acids from sequencing HMW-BCGF protein are underlined twice. Potential N-linked glycosylation sites are indicated with arrowheads.

was used (44). In brief, K-562 cells were electroporated with fusion plasmid and then selected in medium containing G418. Supernatants containing HMW-BCGF or control proteins were harvested and used directly in assays.

RESULTS

Screening of a λZAP cDNA library made from PHA-stimulated Namalva cells (31) with the monoclonal antibody BCGF/1/C2 (32) and a polyclonal antisera to HMW-BCGF resulted in the identification of a 1.8-kb cDNA clone that is shown in Fig. 1. The cDNA has a short 5′ untranslated region followed by a start site that does not contain an ideal Kozak consensus sequence (45). The coding sequence is 1492 bases and is followed by a 3′-untranslated region lacking a polyadenylylation signal and poly(A) tail. Additional cDNAs are currently being sought that contain the missing 3′ untranslated bases. The DNA sequence predicts a mature protein of 483 amino acids that is 53.1 kDa. A 15-amino acid signal peptide is present, as would be predicted for a secreted protein (46). N-terminal amino acid sequence obtained from native HMW-BCGF follows immediately after the signal peptide. Amino acid sequences of additional peptides obtained from native HMW-BCGF and predicted from the HMW-BCGF cDNA are also indicated in Fig. 1. Three potential N-linked glycosylation sites are predicted, consistent with the observation that native HMW-BCGF is glycosylated (31). And, multiple cysteines are predicted that could potentially participate in the formation of disulfide bonds. The predicted HMW-BCGF amino acid sequence has no significant overall sequence homology to any known cytokines. HMW-BCGF does have 8% overall sequence homology with the complement protein Bb, consistent with antigenic and limited functional similarities between these two proteins (47, 48).

Expression of recombinant HMW-BCGF using a λZAP phage resulted in a 60-kDa β-galactosidase fusion protein (Fig. 2). Proof that the band designated 210B protein is recombinant HMW-BCGF was obtained by subjecting it to amino acid sequencing by Edman degradation after digestion with cyanogen bromide. Peptides sequenced from recombinant 210B protein are shown in parentheses in Fig. 1. Furthermore, recombinant 210B protein was recognized by anti-HMW-BCGF antibodies in immunoblot assays (data not shown).

We examined the ability of recombinant HMW-BCGF to induce B-cell proliferation and inhibit immunoglobulin secretion. The recombinant HMW-BCGF produced from λZAP induced 5-fold enhancement in [^{3}H]thymidine incorporation of Sac-activated B cells above the background produced by Sac activation alone (Fig. 3*A*). The increase in [^{3}H]thymidine incorporation induced by recombinant HMW-BCGF was dose dependent and specifically inhibited by anti-HMW-BCGF or anti-HMW-BCGF receptor antibodies (Fig. 3*B*). However, this recombinant HMW-BCGF did not induce as much [^{3}H]thymidine incorporation as native HMW-BCGF (Fig. 3*A*). We also produced recombinant HMW-BCGF in K-562 cells by using the expression plasmid SFFV.neo (44). The recombinant HMW-BCGF produced in this way was similar in molecular weight to native HMW-BCGF and recognized by HMW-BCGF antibodies (Fig. 4*A*). This recombinant HMW-BCGF induced equivalent [^{3}H]thymidine incorporation by Sac-activated B cells to optimal doses of native HMW-BCGF (Fig. 4*B*). Quantitative analysis of native and recombinant HMW-BCGF awaits a more stable and efficient expression system.

Recombinant HMW-BCGF produced with λZAP inhibited immunoglobulin secretion as well as native HMW-BCGF (Fig. 5). Thus, recombinant HMW-BCGF induced the same activities as native HMW-BCGF, although induction of proliferation was more sensitive than inhibition of immunoglob-

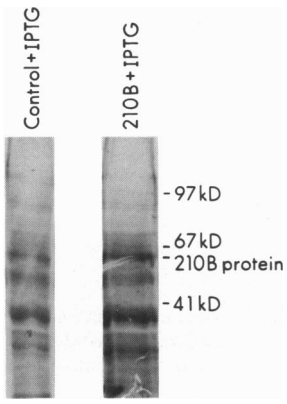

FIG. 2. Protein was produced from λZAP phage containing either the cDNA shown in Fig. 1 or an unrelated cDNA using XL-1 blue *E. coli* as described. Proteins were separated by SDS/10% PAGE and silver stained. Migration of molecular size standards is indicated in kDa.

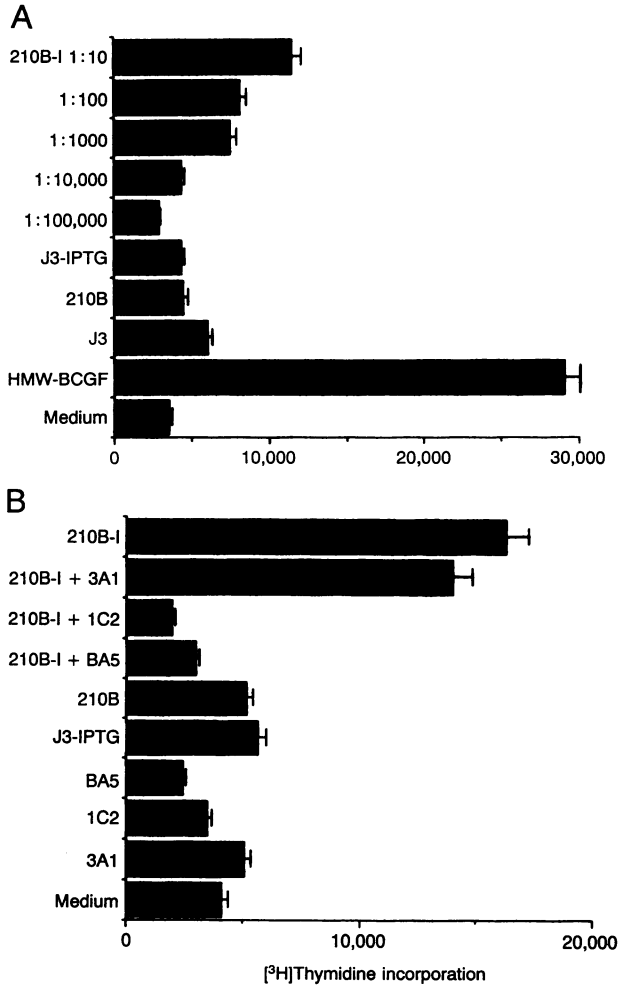

FIG. 3. (*A*) Proteins produced as in Fig. 2 were used in the Sac proliferation assay as described (34). Protein obtained from the bacterial plates was diluted as indicated. 210B-I is protein produced from the pI-7.8 HMW-BCGF cDNA after IPTG induction, whereas 210B is protein produced from pI-7.8 HMW-BCGF cDNA without IPTG induction. J3 is an unrelated cDNA. J3-IPTG, 210B, and J3 are shown at 1:10 dilutions. HMW-BCGF is native HMW-BCGF used at 10 units per ml. (*B*) Protein was produced from the λZAP phage containing pI-7.8 HMW-BCGF (210B) induced with IPTG (210B-I), as described in *A* and was used as its optimal concentration for the induction of proliferation (1:10 dilution, ≈100 ng/ml) in the presence or absence of 10 μg (per ml) of the IgG1 κ monoclonal antibodies BCGF/1/C2 (1C2; anti-HMW-BCGF), BA5 (anti-HMW-BCGF receptor), or 3A1 (recognizes a poorly characterized thymocyte antigen). Controls include the 210B not induced with IPTG and a phage containing an irrelevant cDNA induced with IPTG (J3-IPTG) used at the same total protein concentration as 210B-I.

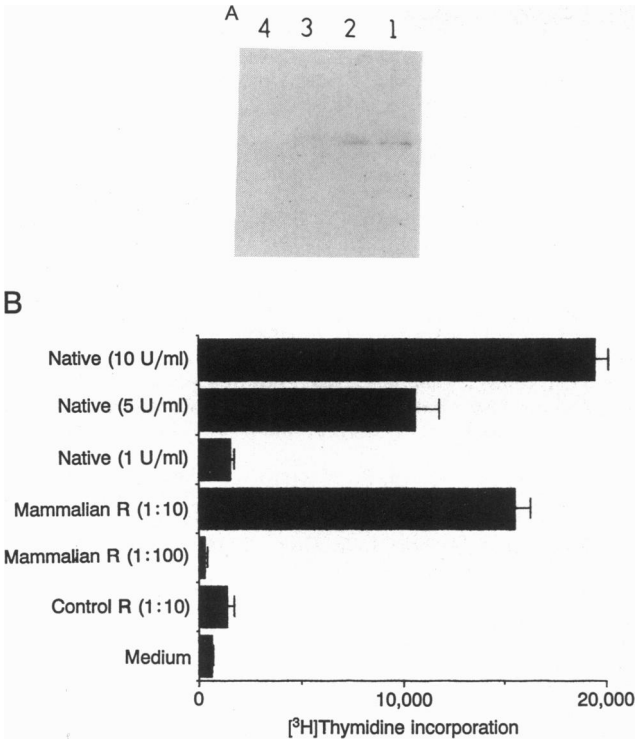

FIG. 4. (*A*) pI 7.8 HMW-BCGF or CR2 cDNAs were ligated into SFFV.neo and electroporated into K-562 cells as described (44). Supernatants from G418-selected cells were concentrated 10 times and evaluated by immunoblot analysis as described. Lanes: 1, native HMW-BCGF-containing supernatant; 2, supernatant of K-562 cells transfected with SFFV.neo-containing 210B (pI 7.8-HMW-BCGF cDNA); 3, supernatant of K-562 cells transfected with SFFV.neo containing a CR2 cDNA; 4, fetal calf serum. (*B*) Proteins were produced as in *A*. The proliferation was performed identically to that shown in Fig. 3. Native is native HMW-BCGF produced from Namalva cells, Mammalian R is the supernatant of K-562 cells transfected with SFFV.neo containing pI 7.8-HMW-BCGF cDNA (210), and Control R is the supernatant of K-562 cells transfected with SFFV.neo containing a CR2 cDNA (44).

ulin secretion to the differences in the molecule and/or the contaminating proteins present when it is made in bacteria.

Previous experiments showed that production of HMW-BCGF by Namalva cells is increased after stimulation with PHA (31). Furthermore, HMW-BCGF production by normal T cells was identified 8–12 hr after PHA stimulation and was generally absent 36–48 hr after PHA stimulation (49). Fig. 6 demonstrates that mRNA that hybridizes to the HMW-BCGF cDNA is upregulated in Namalva cells after 8 hr of PHA stimulation. And, HMW-BCGF mRNA is expressed 8–12 hr after PHA stimulation and gone by 48 hr after PHA stimulation in normal T cells. The T cells of some donors expressed more HMW-BCGF mRNA at 8 hr after PHA stimulation than the T cells of the donor shown, but all ceased to express HMW-BCGF mRNA by 36–48 hr after PHA stimulation. Thus, the mRNA expression of HMW-BCGF is consistent with the previous data on HMW-BCGF protein production (31, 49).

DISCUSSION

The cDNA described in this communication meets several criteria for encoding the HMW-BCGF we have described (31, 32, 34): the predicted amino acid sequence contains peptides obtained from native HMW-BCGF; the protein produced from the cDNA is recognized by antibodies to native HMW-BCGF; the protein produced from the cDNA induces proliferation of Sac-activated B cells and inhibits immunoglobulin

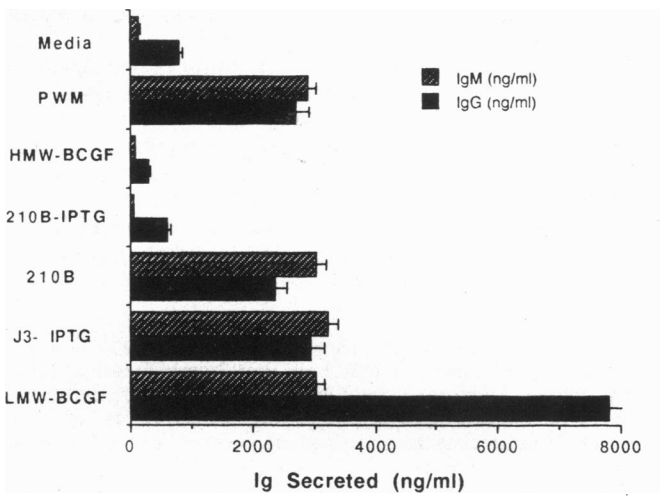

FIG. 5. Mononuclear cells were stimulated with medium alone, pokeweed mitogen (PWM) (1:100), or PWM plus proteins as indicated. The same proteins were used in this figure as in Fig. 3. HMW-BCGF was used at 10 units per ml; 210B-IPTG, 210B, and J3-IPTG were used at a 1:10 dilution; and LMW-BCGF was used at 10 units per ml. Concentration of IgG and IgM in cell supernatants was determined by ELISA, as described (34).

secretion by pokeweed mitogen-stimulated mononuclear cells like native HMW-BCGF; and the cDNA hybridizes to mRNA expressed in activated Namalva and normal T cells at time points consistent with the pattern of HMW-BCGF production noted previously. This cDNA demonstrates that HMW-BCGF is a cytokine distinct from IL-4 and IL-5 (50–52). The sequence homology with complement protein Bb, furthermore, is consistent with the antigenic and functional similarities and differences between these two molecules (47, 48). Further study will be necessary to identify precisely which regions of the HMW-BCGF molecule are critical for induction of its different activities.

The availability of recombinant HMW-BCGF and tools to examine its production will allow analysis of its predicted role in generation/expansion of normal memory B cells (30, 34) as well as in proliferation of various B-cell tumors (35, 36). In addition, it will be important to understand why HMW-BCGF shares various functional activities with IL-4, such as long-term growth of B cells *in vitro* (16), induction of proliferation of anti-μ activated B cells, and inhibition of immunoglobulin secretion (53–55). IL-4 may also enhance immunoglobulin secretion (56–58), and is necessary for normal IgE production (59). cAMP has been postulated to be responsible for some of the inhibitory effects of IL-4 (60). Interestingly, both HMW-BCGF and human IL-4 (but not murine IL-4) induce similar increases in cAMP in Sac-activated B cells,

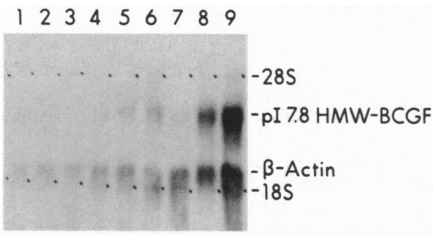

FIG. 6. pI 7.8-HMW-BCGF cDNA and a β-actin probe obtained from M. Thomas were labeled by nick translation, and RNA blots were performed as described (65). The different lanes contain mRNA from 1–7 normal T cells stimulated with PHA for 0 hr (lane 1), 2 hr (lane 2), 4 hr (lane 3), 8 hr (lane 4), 12 hr (lane 5), 24 hr (lane 6) and 48 hr (lane 7); unstimulated Namalva cells (lane 8) and Namalva cells plus PHA (8 hr) (lane 9) also furnished RNA.

although HMW-BCGF induces increases in cAMP at much lower doses than IL-4 (10 units per ml for HMW-BCGF vs. 500 units per ml for IL-4) (48, 61). Intracellular signaling resulting from IL-4 or HMW-BCGF stimulation is otherwise quite different (46, 62, 63).

In conclusion, this communication establishes HMW-BCGF as a separate cytokine. Use of recombinant HMW-BCGF and probes based on its cDNA will determine more precisely its physiologic roles, as well as the reasons for its shared activities with other cytokines, including IL-4 and the recently cloned CD40 ligand (64). We propose to call it interleukin 14.

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