GROWTH FACTOR–MEDIATED TUMOR CELL PROLIFERATION IN HAIRY CELL LEUKEMIA

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Normal human B cell proliferation is a multistep process, involving cellular activation by antigen through antigen-specific cell surface immunoglobulin receptors (sIg) (1), followed by mitogenic stimulation by the lineage-specific lymphokine growth factor, B cell growth factor (BCGF) (2). Recent studies have revealed a great deal about the biochemistry and immunobiology of BCGF, including some of its similarities and differences to T cell growth factor (TCGF; IL-2) (3). In fact, putative receptors for TCGF, as determined by the monoclonal antibody anti-Tac (4), have been recently shown to be present on a subset of normal B cells (5).

Hairy cell leukemia (HCL) is a chronic B cell leukemia, where the neoplastic cells express not only the antigens characteristic of the normal B cell lineage, but also the Tac antigen (6), suggesting that these tumor cells may represent the neoplastic counterpart of the subset of normal B cells that express this putative receptor for TCGF. We have studied the proliferative characteristics of in vitro HCL cells, which do not spontaneously grow in cell culture, when stimulated with partially purified BCGF and with cloned TCGF (IL-2), to ascertain the possible role of growth factors in the proliferation of these neoplastic B cells.

Materials and Methods

Tumor Cells. HCL cells were obtained from untreated patients with the leukemic form of the disease (white blood cells, >20,000/mm^3) after leukapheresis or venipuncture. Peripheral blood mononuclear cells (PBMC) from these patient samples were obtained after Ficoll-Hypaque (F/H) density gradient centrifugation. The PBMC were then rosetted overnight at 4°C with neuraminidase-treated sheep red blood cells (E$_n$). The E$_n$-negative cell population, which contained virtually all HCL cells by morphologic criteria in these leukemic patients, was obtained by F/H centrifugation of the rosetted cell population followed by adherent cell depletion on plastic petri dishes.

Immunophenotyping of HCL Cells. The HCL cell populations were microscopically examined for the morphologic characteristics of HCL cells on Giemsa-stained smears. Tartrate-resistant acid phosphatase (TRAP) cytochemical staining (7) was also performed. The HCL cell populations were then phenotyped by indirect immunofluorescence for a variety of cell membrane markers characteristic of HCL cells, including sIg light chain monoclonality, the monoclonal antibodies (mAb) B1 (pan B; a gift from Dr. L. Nadler) as well as the HCL-associated mAb HC2 (8) (courtesy of Dr. D. Posnett) Leu-14/M5 (9) (Becton, Dickinson & Co., Mountain View, CA). T11 (Ortho Pharmaceutical, Raritan,

NJ) was used for identifying T cells. The Tac mAb was a gift from Dr. T. Waldmann, National Institutes of Health.

In Vitro Proliferation Studies on HCL Cells. Purified populations of HCL cells were cultured in microtiter plates at 1×10^5 cells/well. Cell viability was >95% by trypan blue exclusion after the initial isolation protocol. Growth factor preparations were added to the wells at multiple dilutions. Cultures were incubated for 72 h at 37°C in 5% CO_2; 0.5 μCi [^{3}H]thymidine [^{3}H]TdR (6 Ci/mmol; New England Nuclear, Boston, MA) was added 24 h before harvest.

Growth Factor Preparations. Human BCGF (12–14 kD) free of TCGF was prepared essentially as described.[1] Briefly, 48-h lectin (0.08% PHA-P; Difco Laboratories, Inc., Detroit, MI)-stimulated, pooled peripheral blood T lymphocytes, in the presence of monocytes (20:1 T cell/monocyte ratio), were used for the production of conditioned medium. This material was concentrated 20-fold using a Pellicon (Millipore Corp., Bedford, MA) concentrator with a 10 kD membrane. A diafiltered sample was loaded and fractionated on a DEAE Sepharose column as described (10). The samples containing both TCGF and BCGF were concentrated and applied to a hydroxylapatite column in the presence of 10 mM sodium phosphate buffer. The flow-through and bound fractions from the hydroxylapatite elution were collected and assayed for growth factor activity. BCGF activity was found solely in the flow-through fractions, as tested on both anti-μ– activated B cells and long-term cultured B cell lines. These same fractions were essentially devoid of TCGF activity (<5% pre-column activity) as tested on long-term cultured T cell lines. The TCGF activity resided in the hydroxylapatite-bound proteins. The BCGF found in the hydroxylapatite flow-through fractions was used for the experiments described herein. BCGF activity was selectively enriched ~50-fold after DEAE chromatography and further enriched >100-fold after hydroxylapatite chromatography. BCGF was added to culture wells in unit (U) amounts determined on long-term cultured B cells as previously described (11). The TCGF used was purified, cloned material kindly provided by Cetus Corp., Emeryville, CA (lot No. LP222 endotoxin, 0.06 ng/2.4 $\times$ 10^6 U). TCGF was added to the culture wells in unit amounts as previously described (12).

Cell Culture Studies. HCL cells were plated in tissue culture macrowells at 0.5×10^6 cells/ml. The cells were grown in RPMI 1640 medium in the presence of 20% fetal calf serum (FCS) with or without the addition of 10% vol/vol (18 U) partially purified BCGF. The culture wells were fed at 3–4-d intervals with fresh media and 10% (vol/vol) partially purified BCGF. At varying intervals the growth factor was deleted when the culture wells were fed to evaluate growth factor dependence. Cell counts and viability by trypan blue dye exclusion were performed at regular intervals, as was the cell surface phenotype.

Results

Phenotypic Characterization of HCL Cells. Leukemic HCL cells from either leukapheresis or venipuncture were obtained from untreated patients and were assayed for cell surface markers. Table I shows that the HCL cells, which resembled hairy cells, morphologically and cytochemically (TRAP$^+$), were also phenotypically consistent with this diagnosis in terms of standard cell surface markers for B lymphocytes, including sIg, monoclonal light chain type, and expression of the pan B cell surface antigen, B1. Phenotypic markers for HCL-associated antigens such as HC2, coexpression of Leu-14/M5, and Tac were also positive on the HCL cells (see Table I for percentage distribution). It should be noted that, of the hairy cell populations examined, all but one HCL population was Tac antigen positive.

Response of HCL Cells to B Cell Growth Factor. Freshly prepared HCL cells

[1] Mehta, S., D. Conrad, R. Sandler, J. Morgan, R. Montagna, and A. Maizel. Purification of human BCGF:12 kD. Submitted for publication.

TABLE I

Immunologic Markers on Hairy Cell Leukemia Cells

Patient No.*	sIg+	κ/λ	B1	Leu-14/M5§	HC2	Tac	T11
1	94	93/2	86	91	87	73	3
2	59	60/1	38	59	ND	1.0	2
3	96	98/1	92	93	90	81	2
4	88	80/3	95	90	90	85	2
5	89	90/2	83	78	88	75	3
6	97	94/3	75	92	82	78	2
7	92	90/3	88	91	81	87	3

* HCL cells from untreated patients were phenotyped directly after double E_n rosetting, and adherence to plastic. The HCL population was morphologically consistent with HCL on Giemsa-stained preparations and contained >90 TRAP+ cells on cytospin preparations.

‡ Cell surface markers were determined using either direct or indirect immunofluorescence with polyclonal (sIg, κ/λ) or monoclonal antibody reagents. At least 300 cells were counted and the number shown reflects the percentage of positive staining cells.

§ Double staining cells with Leu-14–FITC and Leu-M5–phycoerythrin (Becton, Dickinson & Co.).

were washed three times in RPMI 1640 medium and subsequently plated in microtiter plates at 1×10^5 cells/well in RPMI 1640 and 5% FCS. Partially purified BCGF (free of TCGF activity) was added to the wells in multiple dilutions (Fig. 1A). TdR incorporation demonstrated that six of the seven HCL populations responded to BCGF whereas only background [^{3}H]TdR incorporation was observed in these control cultures without the growth factor. As noted, only one patient cell population failed to respond to the BCGF growth factor preparation.

Response of HCL Cells to Cloned TCGF.   Since the majority of HCL cells tested have been shown to be Tac antigen positive, it was of interest to determine if human TCGF could also stimulate proliferation in HCL cells. To explore this possibility, we performed parallel proliferative studies, in which HCL cells were cultured with multiple dilutions of purified, cloned human IL-2 (TCGF). In Fig. 1B, it can be seen that the cloned TCGF resulted in only minimal stimulation of [^{3}H]TdR incorporation in two of seven patients. The one patient who failed to react to BCGF also failed to respond to exogenous TCGF.

HCL Cell Growth In Vitro.   The observed proliferative response of the HCL cells to BCGF suggested that the growth factor might be able to mediate the establishment of growth factor–dependent cell lines in vitro. To test this prediction, we plated fresh HCL cells in vitro in the presence of BCGF. Fig. 2 shows that sustained HCL growth could be obtained in the presence of BCGF, but ceased when the growth factor was deleted. The phenotype of these HCL cells remained essentially constant throughout the duration of these experiments (see Fig. 2 legend). The cell population shown in Fig. 2 was also tested for Epstein-Barr nuclear antigen (EBNA) by immunofluorescence and found to be negative. Of the seven hairy cell populations tested for long-term growth, all but one demonstrated in vitro proliferation for at least 30 d in response to BCGF, with four of the seven developing into essentially continuous (>60 d) growth factor–dependent lines. In a further attempt to determine the efficacy of TCGF in mediating an HCL proliferative response, HCL cells that had been in culture for at least 10 d in the presence of BCGF were washed free of the factor and refed with fresh medium, serum, and 1–10 U of recombinant IL-2. These cell populations failed to continue to proliferate, and could not be maintained in vitro on recombinant IL-2.
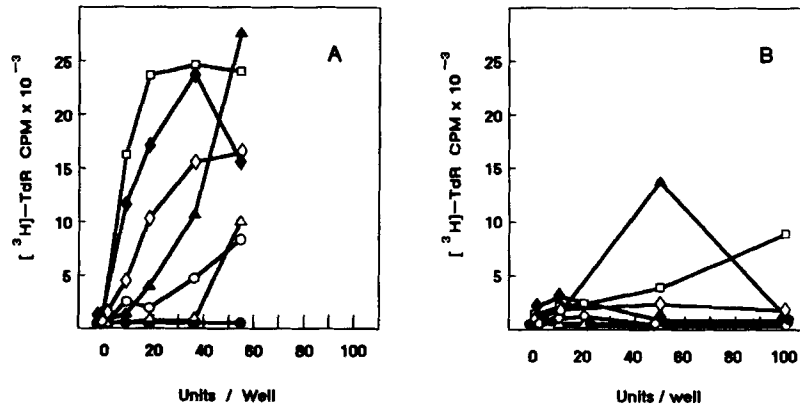
FIGURE 1.   Growth factor–mediated cell proliferation studies performed on seven untreated HCL patients leukemic cells. Microwell HCL cultures (1×10^5 cells/well) were incubated with multiple dilutions of partially purified natural product BCGF (A) or recombinant IL-2 (B) for 72 h, and labeled with 0.5 Ci of [^{3}H]TdR for the final 24 h of the culture period. Data shown represents the means of triplicate cultures. Negative control cultures without growth factor yielded <1,000 cpm. Positive control assays using cloned IL-2 on long-term T cell lines yielded: 0 U, 512 cpm; 1 U, 2,143; 5 U, 7,785; 10 U, 12,740; 50 U, 18,470; 100 U, 24,280 cpm. Symbols correspond to individual patients.
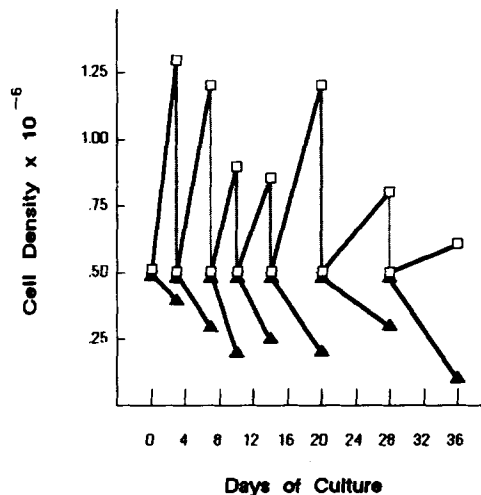

FIGURE 2.   HCL cells with the phenotype of B1 Tac HC2$^+$ were set up at a density of 0.5 × 10^6 cells/well. The cells were plated in RPMI, 20% FCS, and 18 U (10% vol/vol) of partially purified BCGF per well in 2-ml macrowell cultures. The cultures were counted at 4-d intervals adjusted to the original cell density and either refed with BCGF or media and FCS alone. After 30 d in vitro, cells from representative wells were assayed for cell surface markers. >80% of the viable cells maintained the original phenotype, while Tac and HC2 was absent on ~10% of the viable cells. (□) Viable HCL cells cultured with BCGF; (▲) viable HCL cells cultured in RPMI and FCS alone.

Discussion

The control of normal human B cell proliferation appears to be primarily a function of B cell acquisition of a receptor for BCGF and the ensuing mitogenic response to the growth factor. Recently (5), however, a number of investigators have reported that a subset of normal B cells possess the Tac antigen, and that

the antigen-positive B cells apparently respond to cloned IL-2 in vitro. The role of this apparent second growth factor in human B cell ontogeny is presently an intriguing enigma, but may represent a clue to the nature of B cell heterogeneity in man. The presence of the Tac antigen on a rather rare type of human B cell neoplasm (HCL) may indicate that the disease represents a monoclonal expansion of the normal subset of B cells expressing the Tac antigen. It is interesting, however, that the neoplastic cells retain the functional capacity to respond to the lineage-specific growth factor (BCGF), without anti-μ (Ig) or Sac activation, suggesting that the BCGF receptor is constitutively expressed. However, HCL cells appear to be only marginally responsive to TCGF, which suggests that the Tac antigen, while expressed, may be functionally inactive. Our inability to grow HCL cells in cloned IL-2 supports the contention that the Tac antigen on the tumor cells may be defective, inert, or, possibly, that it is not in fact intrinsically associated with the actual receptor for TCGF. Studies by Robb et al. (15) showed that the Tac antigen on HCL cells was associated with low affinity IL-2-binding sites, which they concluded were not involved directly in normal physiologic responses to the growth factor. Recent studies by Smith and Cantrell (13), which cast some doubt on the Tac antigen as the putative TCGF receptor, provide a possible explanation for the lack of proliferative activity exhibited by recombinant IL-2 on HCL cells in vitro. If the Tac antigen simply represents a differentiation antigen that is possibly proximal to the actual TCGF receptor and can sterically hinder the interaction of the growth factor with its receptor, it would not be expected to play a significant role in the proliferative capacity of the neoplastic cell. If this is the case, the finding of the Tac antigen on HCL may be analogous to the finding of the T cell–associated antigen T 101 (Leu-1) on chronic lymphocytic leukemia (CLL) cells of B lineage (14). The finding that the lineage-specific growth factor, BCGF, can stimulate growth in neoplastically transformed B cells such as HCL, which do not grow spontaneously in the presence of autologous or heterologous human serum, shows that the tumor cells not only maintain lineage fidelity, but also functional similarities, with their normal B cell counterparts. This may be of considerable significance in our understanding of the immunobiology of the tumor cells.

Summary

Leukemic B cells from seven patients with hairy cell leukemia (HCL), six of which contained the Tac antigen, were assayed in vitro for growth factor–mediated cell proliferation. The HCL cells showed typical phenotypic profiles by monoclonal antibody analysis. The tumor cells, which do not grow spontaneously in vitro, were found to proliferate in all but one case in response to partially purified B cell growth factor (BCGF) without anti-μ or Sac activation. Recombinant interleukin 2 however produced only a marginal response and could not support leukemic cell growth in vitro. BCGF, however, did stimulate in vitro cell growth and supported the establishment of continous (>60 d in vitro) in four of the seven HCL cases.

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