Leukemia-derived growth factor (non-interleukin-2) produced by murine lymphoma T-cell lines

(autocrine/conditioned medium)

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Communicated by William N. Valentine, August 15, 1984

ABSTRACT

Autocrine growth factor activity was found in supernatants of AKR T-cell lymphoma lines cultured in serum-free medium. This factor was designated leukemia-derived growth factor (LDGF). Active supernatants stimulated the growth of the AKR murine T-cell lymphoma line SL 12, its own derivatives, and all other murine T-cell lymphoma lines tested. Growth factor activity in conditioned medium was found to be different from interleukin 2 (IL-2) and several other known growth factors. LDGF was able to stimulate growth of the human leukemia T-cells MOLT 4, and the LDGF from MOLT 4 cells stimulated the mouse cells. Because mouse T-cell lymphoma lines produce and respond to this factor, it may support the continued proliferation of these cells and could be responsible for their malignant in vivo properties.

In the T-cell lymphoma of AKR mice, factors responsible for the malignant transformation of thymocyte progenitors and maintenance of the malignant phenotype have not been determined. This disease regularly occurs in old mice and is associated in its development with expression of oncogenic retroviruses in the preleukemic period (1). Inoculation into newborn mice of oncogenic retroviruses accelerates the onset of this T-cell lymphoma in all mice to age 60–90 days. Recent studies have shown that one of the AKR-derived oncogenic retroviruses, SL 3–2v, has an ecotropic host range and is highly lymphomagenic (2, 3). It also has changes in the direct repeat region of the long terminal repeat of the provirus, which represent the major difference between this virus and the nononcogenic endogenous retrovirus of AKR, Akve (4). Because the direct repeat regions contain enhancer elements (nontranslated portions of DNA that can regulate transcription of cellular and viral DNA), specific viral integration near a cellular gene resulting in the enhanced transcription of its product might be responsible for the malignant phenotypic properties of these lymphoma T cells.

The human T-cell leukemia line MOLT 4 has been shown to produce a growth factor in serum-free medium that is not interleukin 2 (IL-2) (5). This finding has led us to investigate if AKR lymphoma T cells produce a similar growth factor. In the case of the mouse, it might represent the gene product stimulated by the specific integration of the lymphomagenic virus as described above. The following studies report our findings demonstrating that AKR lymphoma T cells produce a factor that stimulates their own growth, which was designated leukemia-derived growth factor (LDGF).

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study were SL 12 and its cloned derivatives, SL 12.1, SL 12.3, SL 12.4 (6), and SL 3 (2)—all AKR T-cell lymphoma lines derived in this labora-
tory. An additional AKR T-cell lymphoma line, SAK 8, and the BALB/c T-cell lymphoma line W-7 were obtained from S. Bourgeois. The murine myelomonocytic leukemia cell line WEHI 3 was obtained from J. Greenberger. The human leukemia cell lines included T-cell leukemia lines MOLT 4 and CCRF-CEM, the B-cell line BJAB, and the myeloid leukemia line COLE 21 (obtained from P. Koeffler). Studies of cell-surface markers showed all of the mouse T-cell lines to be thy-1+ and to represent an intermediate stage of T lymphocyte differentiation (6). The mouse cell lines originally were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and later were adapted to growth in serum-free medium as described (7); the medium consisted of Iscove’s modified Dulbecco’s medium with added delipidated serum albumin at 400 µg/ml and transferrin at 1 µg/ml (AT-IMD medium). The human cell lines were maintained in the same serum-free medium. The cell lines were tested for mycoplasma contamination by using the fluorescent Hoechst stain (8). All lines were mycoplasma negative.

Collection of Conditioned Medium (CM). CM was collected from the mouse cell lines growing in AT-IMD medium twice a week when the cells reached a concentration of about 1 × 10^6 viable cells per ml. The cells were centrifuged at 1000 rpm for 10 min. The CM was removed, filtered, and stored at 4 or −20°C, and the cells were suspended in fresh serum-free medium at cell concentrations of 2–3 × 10^5 per ml.

Evaluation of Proliferation of Cell Lines in Response to CM. Growth curves for cell lines were obtained with cells that had been passed at least two times in serum-free medium. The cells were washed in phosphate-buffered saline and resuspended at given cell concentrations in AT-IMD medium in 96-well plates; the cultures contained 0–50% concentrations of CM from the cell lines grown in AT-IMD medium. Each culture was done in triplicate in humified 5% CO₂/95% air. Growth was followed daily for 8 days (without changing the medium) by counting viable cells (trypan blue dye exclusion method) in a hemocytometer.

Soft Agar Assay. Agar plates were prepared in 35-mm Petri dishes (Falcon) by first applying a 1-ml base layer of 0.5% agar (Sea Plaque agarose; FMC, Rockland, ME) in AT-IMD medium with appropriate concentrations of growth factor containing CM. Over this basal layer, an additional 0.5-ml layer of 0.4% agar in the same medium with appropriate concentrations of CM and 400 test lymphoma cells were added. The cells were incubated at 37°C in a humified atmosphere of 5% CO₂/95% air for 4–7 days. Colonies of 5–30 cells were counted by using the phase-contrast microscope. In control plates addition of growth factor-containing CM was omitted; 400 cells in a 35-mm dish were used because this sparse in-
oculum would minimize, if not completely avoid, spontaneous aggregation.

\[^{3}H\]Thymidine Uptake. The method used for these experiments is described in ref. 5. CM heated at 80°C for 5 min was used in these studies.

Sensitivity of Growth Factor Activity to Treatment. One-milliliter amounts of SL 12.4 cell-conditioned medium with growth-promoting activity were incubated with 100 µg of chymotrypsin (Sigma) at 37°C for 30 min. The chymotrypsin was inactivated by addition of 10 µM phenylmethylsulfonyl fluoride (Sigma) (8). The treated CM was tested for activity. Supernatant pools with growth-promoting activity were also heated at 96°C for 10 min, 80°C for 5 min, or 56°C for 30 min and tested for growth factor activity.

Assays for IL-2 and Epidermal Growth Factor (EGF). IL-2 assay was done with CTL-2 cells as described (9). Controls for cell growth in this assay utilized a standard source of human IL-2 that stimulated the CTL-2 cells. Assays for EGF binding as described by Kawamoto et al. (10) were performed by C. MacLeod (University of California, San Diego).

Burst-Promoting Activity (BPA) Assay. Bone marrow cells were obtained by flushing the femurs of 6-wk-old BDF1 mice with α medium. Cells (10^7) were plated in 24-well tissue culture plates in 0.25 ml of the medium containing 1% delipidated bovine serum albumin, 10% fetal bovine serum, 2 units of erythropoietin (Toyobo, Osaka, Japan) per ml, 0.88% methylcellulose (Dow), and various concentrations of the test samples. The culture was incubated at 37°C in humidified 5% CO2/95% air. At day 8, erythroid bursts were evaluated by the use of an inverted microscope. All assays were done in triplicate.

Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF) Assay. Bone marrow cells from BDF1 mice (5 x 10^6) were plated in 35-mm culture dishes in 1.0 ml of McCoy’s 5A medium containing 20% horse serum, 0.88% methylcellulose, and various concentrations of the test samples. In control dishes, L cell conditioned medium of known activity was used as a standard of GM-CSF. At the end of 7 days of incubation at 37°C in humidified 5% CO2/95% air, colonies of 50 or more cells were counted with an inverted microscope. All assays were done in triplicate.

RESULTS

Evidence for Growth Factor Activity. Seven T-cell lymphoma lines (6 AKR and 1 BALB/c) were studied. They all could be maintained in serum-free AT-IMD medium. The cells grew in suspension culture and were composed of large lymphoid blast cells when examined microscopically. All AKR-derived cell lines produced progressively growing tumors when inoculated intradermally into syngeneic mice. When AKR T-cell lymphoma cell lines passed two times in AT-IMD medium from serum-containing medium were seeded at 1 x 10^4 cells per ml in AT-IMD medium, they did not grow. The addition of CM from SL 12, SL 12.1, SL 12.3, SL 12.4, SAC 8, and SL 3 cells stimulated their growth in an exponential manner. Fig. 1 represents growth of SL 12 in SL 12.4 CM and is representative of all growth curves obtained with the above cells. All supernatants cross-reacted—i.e., all AKR T-cell lymphoma line supernatants tested demonstrated growth-stimulatory activity for itself or other AKR T-cell lymphomas. Autologous growth stimulatory activity also was found in CM from W-7, a BALB/c-derived T-cell lymphoma line. W-7 CM also stimulated the AKR line SL 12.4. When cells were seeded in numbers greater than 10^6 per ml, they grew exponentially without added CM, and the addition of CM did not enhance their growth (data not shown). Upon continued passage in AT-IMD medium (cultures split twice a week), the numbers of cells that were able to grow without added CM decreased so that, in some instances, growth occurred when as few as 6 x 10^4 cells were seeded in the culture wells. Fig. 2 demonstrates the phenomenon of the growth at lower cell numbers in lines grown continuously in AT-IMD medium for 1 month. These cells when seeded at 5 x 10^4 or 1 x 10^5 did not grow (data not shown).

CM from the myelomonocytic cell line WEHI 3, which had been adapted to AT-IMD medium, did not stimulate T-cell lymphoma growth and was, in fact, inhibitory to cell growth; this inhibitory activity was demonstrated with lymphoma cells cultured at a threshold level of 10^6 cells per ml (data not shown) and greater than threshold levels (Fig. 3).

SL 12.4 CM Stimulates Production of Lymphoma Cell Colonies in Soft Agar. SL 12.1 and SL 12.4 cells formed colonies in soft agar, after 7 days in serum-free conditions, when CM from SL 12.4 cells was added to the cultures. In contrast, cells cultured under these conditions without added CM re-
mained as single cells after 7 days. Colony growth was dose dependent, thus showing that single cells could be stimulated to replicate by addition of medium containing growth factor activity. The presence of inhibitory factors was suggested when CM in higher concentration was used in the soft agar assay (Fig. 4A). Heating of CM at 80°C for 5 min restored maximal growth-promoting activity in a dose-dependent manner. The colony number reached plateau levels at 20% CM (Fig. 4B). The quality of the supernatant (LDGF activity) varied from batch to batch. This depended on the time of harvest. CM harvested from cells at higher concentrations had greater inhibitory activity.

**[3H]Thymidine Incorporation.** This method was used as another assessment of growth stimulatory activity of LDGF. The data in Table 1 show that CM from three cell lines stimulated [3H]thymidine incorporation in a dose-dependent manner.

**Cross-Reaction of Murine Growth Factor Activity and Human LDGF.** The human T-cell line MOLT4f has been shown to have LDGF activity (5). Therefore, the SL 12-derived supernatants were tested on the human cells, and the human cell-derived factor was tested on the mouse cell lines. Fig. 5 illustrates the cross-stimulatory activity of CM from human and murine lymphoma T cells. The mouse factor showed growth stimulation not only of MOLT4 but also of the T-cell

**Table 1.** The effect of LDGF on [3H]thymidine incorporation by SL 12.4 cells

<table>
<thead>
<tr>
<th>CM, %</th>
<th>CM-stimulated [3H]dT incorporation, cpm ± SD</th>
</tr>
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<tbody>
<tr>
<td>SL 3.2</td>
<td>SL 12.3</td>
</tr>
<tr>
<td>0</td>
<td>175 ± 41</td>
</tr>
<tr>
<td>1</td>
<td>139 ± 22</td>
</tr>
<tr>
<td>5</td>
<td>178 ± 12</td>
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<td>1,968 ± 45</td>
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<td>66,410 ± 3,930</td>
</tr>
<tr>
<td>50</td>
<td>187,223 ± 28,420</td>
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</table>

*The CM was obtained from growth-phase SL 12.4 cells in AT-IMD medium heated at 80°C for 5 min.

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**Fig. 3.** Inhibitory activity in WEHI 3 CM, SL 12.4 cells were seeded at the initial cell concentration of 5 x 10⁴ cells per ml, which grew without added CM (>). Growth inhibitory effect can be seen when 50% WEHI 3 CM was added (c).

**Fig. 4.** Growth stimulating effect in soft agar. The soft agar assay using SL 12.4 cells and SL 12.4 CM without any treatment suggested the presence of inhibitory factors at 20% CM (A). After heating at 80°C for 5 min, maximum growth-promoting activity was detected, and a dose dependency was seen (B).

**Fig. 5.** Cross-stimulatory activity of CM from human and murine growth-phase lymphoma T cells. Both SL 12.1 CM (50%) (c) and SL 12.4 CM (50%) (>) stimulated growth of MOLT4f cells (A). Growth-stimulatory effect of MOLT4f CM (>) was also shown. Tests were performed at the initial cell concentration of 5 x 10⁴ cells per ml, which did not grow (......) without addition of CM. Cross-reactivity tests were performed by using SL 12.1 cells as recipients (B). An initial cell concentration of 1 x 10⁴ cells per ml was used. Symbols have the same meaning in A. The lack of a logarithmic phase of growth reflects the quality of CM used.
line CCRF-CEM. BJAB, a human B-cell line, and COLE-21, a human myeloid leukemia cell line, were not stimulated by mouse CM. The human LDGF stimulated the growth of the mouse cells.

Characterization of Growth Factor. Heating the CM to 96°C for 10 min destroyed growth factor activity. Activity was present after heating at 56°C for 30 min. Treatment with chymotrypsin resulted in the removal of activity.

Tests for Other Growth Factors in Supernatants. Active growth-promoting CM (serum-free) from SL 12, SL 12.1, and SL 12.4 were tested for other known growth factors. There was no IL-2 activity (11) in these conditioned media. Also there was no 125I-labeled EGF (12) (mouse origin) binding to the cells. This was shown using 0, 2, 20, and 50 μM EGF. The same experiments with human cell lines HT 180 and A 431 showed characteristic binding. Tests for BPA gave the following results: 0%, 5%, 10%, or 20% CM was added to the marrow cells. Fig. 6 presents the results with SL 12 CM, which show a dose-dependent increase in marrow bursts over baseline (AT-IMD medium control). SL 12.1, SL 12.3, and SL 12.4 CM did not demonstrate enhancement of BPA (data not shown). Colonies with AT-IMD medium were the same as colonies with active CM. The same WEHI 3 supernatants that were inhibitory to the lymphoma T cellS in serum-free medium were also found to be inhibitory to BPA activity in normal bone marrow cultures (data not shown). Granulocyte–macrophage colony-stimulating activity (GM-CSA) was found in SL 12 CM and at a much lower level (vol/vol) than a standard source of CSAs (L cell-conditioned medium). 10% L cell-conditioned medium produced 126 ± 33 colonies. SL 12 CM produced no colonies with 10% CM, 8 colonies with 20% CM, and 43 colonies with 40% CM. No GM-CSA activity was found in any concentration of SL 12.3, 12.1, and 12.4 CM.

DISCUSSION

In this study, we have shown that murine lymphoma T cells produce a factor that we propose to call LDGF. This factor stimulates the growth of the cells that produce it. It was demonstrated by culturing cells in serum-free medium at concentrations where growth in the absence of lymphoma cell-conditioned medium does not occur. LDGF activity was confirmed by demonstrating colony formation from single lymphoma cells in soft agar when CM-containing LDGF was added to the cultures. The activity of LDGF was shown to be dose-dependent in the semisolid medium. This dose dependency was best demonstrated when CM was heated at 80°C for 5 min. This treatment, we postulate, removes nonspecific inhibitory factors of large molecular size, whereas the putative small protein with LDGF activity remains. We also have shown that the lymphoma T cells adapt to serum-free conditions with time by decreasing the threshold number of cells necessary for growth, suggesting that cells adapt to the serum-free conditions by increasing their production of LDGF and/or receptors for this factor.

Our demonstration of cross-reactivity of LDGF from human and murine T cells indicates that the molecule is highly conserved. Its lack of stimulation of the lymphoma B cells, BJAB, show that it has the same T-cell specificity demonstrated previously with human LDGF (5).

Other examples of growth factors that may be involved in the proliferation of tumor cells have been described. They are: the transforming growth factors, isolated from solid tumor cell lines (13), the many growth factors for hematopoietic cells, the T-cell growth factor (IL-2) produced by the human T-cell leukemia virus-positive cell lines (14), and LDGF from the human leukemia T-cell line MOLT4 (5).

We assayed several of these factors in LDGF SL 12-containing CM. We found that transforming growth factors do not play a role in our system. They are known to act via EGF or the EGF receptor (13, 15), and our cells demonstrated no 125I-labeled EGF binding. The hematopoietic growth factor BPA was found in the SL 12 cell supernatants but not in SL 12.1, 12.3, and 12.4 supernatants. Also, GM-CSA activity was present in SL 12 cell CM and not in CM of SL 12.1, SL 12.3, and SL 12.4. These results suggest that BPA and GM-CSA are not responsible for the growth stimulation observed. These findings, however, are the first description of BPA activity production by a murine T-cell line.

The inhibitory activity of the WEHI 3 CM for BPA as well as for the growth stimulation of lymphoma T cells suggests that this cell line, when grown in serum-free medium, produces a nonspecific inhibitor. Such inhibitors have been described when cells are cultured in serum-free conditions (16).

It recently has been shown (17) that some human T-cell leukemia virus-containing T-cell leukemia lines do not maintain their continuous growth through IL-2 stimulation (17). Therefore, IL-2 production is not the only mechanism for growth stimulation in these cells. Since all human T-cell leukemia virus-transformed cells have IL-2 receptors, a virus-encoded induction of IL-2 receptors could be the mechanism needed to maintain abnormal growth in this system (18). IL-2 is not a factor in our studies because such activity was absent from CM-containing LDGF activity. Many malignant lymphoid T-cell lines do not produce (5, 15, 19) or respond (5, 19) to IL-2. These cell lines may produce and utilize different (non-IL-2) growth factors. An example of such a factor is the human LDGF (5). It should be noted here that LDGF differs from IL-2 in its species cross-reactivity—i.e., human IL-2 "drives" murine T-cell growth but murine IL-2 has no effect on human T cells (20).

The inactivation of LDGF-SL 12 activity by 96°C heat for 10 min and chymotrypsin attests to the protein nature of the factor. When the CM was treated at 56°C for 30 min to inactivate retroviruses, the growth factor activity persisted. This suggests that stimulation of cell proliferation via viral interaction with viral receptors, as has been proposed (21), is not the mechanism of the cell proliferation observed here.

Thus, we show production of an autocrine growth factor, LDGF, from mouse lymphoma T-cell lines. It is present in all T-cell lymphomas tested. These findings suggest that continued production of this autostimulatory factor may be responsible for the malignant phenotype of these cells; in the case of AKR mouse lymphoma, as mentioned in the introduction, factor production may be induced by a specific integration of the lymphomagenic retroviruses in the genome of these cells. Since our cells have the phenotype of relatively

FIG. 6. BPA activity of SL 12 CM. BPA activity was measured as bursts per 1 × 10^8 bone marrow cells. •, BPA activity of SL 12 CM; □, control medium. The culture was performed in the presence of 10% fetal bovine serum and 2 units of erythropoietin per ml.
immature thymocytes (6), we postulate that the normal counterpart of LDGF is produced by certain thymic progenitor cells to stimulate the active but highly controlled cell division that normally occurs in the thymic cortex.

We acknowledge Ms. Connie Streifinger and Mr. Stephen Weinroth for their technical assistance and Ms. Donna Richiuti for the help and preparation of the manuscript. This research was supported by Department of Energy Contract DE-AM03-76SF00012, National Institutes of Health Grant CA 12386, and The Margaret E. Early Trust Grant PC831212.