Tumor Growth Inhibitory Activity of a Lymphocyte Blastogenesis Inhibitory Factor

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ABSTRACT

A lymphocyte blastogenesis inhibitory factor (LBIF) has been characterized as an immunoregulatory molecule, especially on the T-lymphocyte proliferation. Using fast protein liquid chromatography-purified LBIF, we examined the effect of LBIF on the proliferation of various human and mouse tumor cell lines in vitro in comparison with those of IFN-α, IFN-γ, TNF-α, TGF-β1, or IL-1 α/β all of which are known as growth-inhibitory cytokines (5–11). We showed here that firstly, LBIF strongly inhibits the proliferation of tumor cell lines of various lineage. Secondly, LBIF is effective as a growth inhibitory factor on a wider spectrum of tumor cell lines than other cytokines tested here. Thirdly, LBIF sensitivity of tumor cell lines are categorized in two fashions, i.e., cytotoxic and cytostatic. Fourth, there are tumor cell lines that are resistant to growth inhibitory activity of LBIF. Thus this study has suggested that LBIF functions at crucial steps of cell cycle progression irrespective of specific cell lineage. The existence of LBIF-resistant cell lines conversely suggested a plausible involvement of LBIF receptor molecules to transduce LBIF signals. Moreover this study showed a potential activity of LBIF to inhibit the growth of tumors in vivo and to be applied to clinical investigation.

INTRODUCTION

A LBIF1 is constitutively produced from a human macrophage-like cell line, U937 (1–4). We have purified LBIF to homogeneity by FPLC (3, 4). LBIF is a single polypeptide chain and has a molecular weight of approximately 45,000 by the estimation of SDS-PAGE. LBIF shows approximately pI 4.5 upon chromatofocusing. Amino acid sequencing analysis showed that LBIF was a new immunoregulatory factor. The function of LBIF has been characterized (3, 4). (a) LBIF inhibits the proliferation of lymphocytes stimulated with lectins, IL-1, IL-2, or antigens. (b) LBIF selectively inhibits the expression of IL-2 receptor, p75, but neither the induction of IL-2 receptor, p55, nor the IL-2 production of lectin-stimulated T-lymphocytes. (c) LBIF action is cytostatic and reversible. (d) LBIF functions to both human and murine lymphocytes. (e) LBIF arrests lectin-stimulated T-lymphocytes at early G1. (f) Lectin stimulation is a prerequisite process in order for LBIF to be active on lymphocytes. These results have suggested that LBIF may play important roles in regulating cell growth.

MATERIALS AND METHODS

Cell Lines. A human T-cell leukemia, Jurkat, human Burkitt’s lymphoma, Manaca and Raji, an Epstein-Barr virus-transformed B-cell line, EBV-BLCL (obtained from Dr. de Vries, University Hospital, Leiden), a human histiocytic lymphoma, U937 (obtained from American Type Culture Collection, or provided by Dr. Fujisaki, Okayama University, Osaka), a human neuroblastoma, A375 (provided by Hayashibara Biochemical Lab., Inc., Okayama) and G-361 (provided by Japanese Cancer Research Resources Bank, JCRBB, Tokyo), a gibbon ape T-lymphoma, MLA144, murine macrophage cell lines, WEHI3 and P388D1, a murine mastocytoma cell line, P815, human adult T-cell leukemia cell lines, MT-2 and TL-Mor (kindly provided by Dr. Sugamura, Tohoku University), a human epithelial carcinoma cell line, HeLa (provided by JCRBB) were maintained in RPMI 1640 supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 0.29 mg/ml of L-glutamine (M. A. Bioproducts Inc.), 40 μM of 2-mercaptoethanol, and 7.5% heat-inactivated FBS. A human melanoma cell line, Mewo, VMRC-MELG, and C327T (provided by JCRBB) and a murine-transformed fibroblast cell line, L929 (provided by Dr. Saiki, Hokkaido University), were maintained in MEM supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 20 μl/ml of vitamin solution (100 × Boehringer Mannheim), 1 mM sodium pyruvate (Wako pure chemical, Ltd., Osaka), 20 μl/ml of nonessential amino acid (GIBCO), 0.29 mg/ml of L-glutamine, and 10% of heat-inactivated FBS.

Cytokines and LBIF. Human recombinant IL-1α (2 × 10^4 units/ml) and β (2 × 10^5 units/ml) were kindly provided by Dr. Hirai (Ohtsuka Pharmaceutical Co., Ltd., Tokyo) and Dainippon Pharmaceutical, respectively. Human natural TNF-α (0.3 mg/ml, 5 × 10^5 units/ml), human natural IFN-γ (2.1 mg/ml, 1 × 10^5 IU/ml), murine natural IFN-γ (2.8 × 10^6 IU/ml), and human natural IFN-α (3.5 × 10^5 IU/ml, 2 × 10^5 units/ml) were provided by Hayashibara Biochemical Lab., Inc. (12, 13). Human recombinant TGF-β1 was a gift by Mitsubishi Chemical Ind., Co., Ltd. (Tokyo). Lymphocyte blastogenesis inhibitory factor, LBIF, was purified from crude supernatant of a human histiocytic lymphoma U937 as described (3, 4). Briefly, U937 cells were cultured at 1 × 10^6 cells/ml in serum free RPMI 1640 medium. The
serum free supernatant is concentrated by an ultrafiltration membrane. The crude concentration is fractionated by TSK gel DEAE-5PW (21.5 mm × 15 cm, TOSOH, Tokyo) equilibrated with 20 mM Tris-HCl, pH 7.7. The separation was done by a linear gradient from 0 to 0.5 M NaCl. LBIF activity was tested as described in “LBIF assay” (3, 4). The active fractions were subsequently fractionated by Mono P chromatofocusing column (HR5/20, Pharmacia). The purity of LBIF preparation was assessed by SDS-PAGE and estimated to be about 75–80%. These results were described in previous paper (2, 4). All functional assays on LBIF were carried out by using this FPLC-purified LBIF. One unit of LBIF was defined as the amount of LBIF preparation required to induce a half-maximum response of LBIF assay as described. Approximately 10–20 ng of LBIF samples corresponds to 1 unit.

To show the purity of FPLC-purified LBIF, this sample was further resolved by reversed-phase high-performance liquid chromatography on RP-304 column (Bio-Rad) under a linear gradient from 0 to 90% acetonitrile containing 0.1% trifluoroacetic acid at flow rate of 0.5 ml/min. Fig. 1a shows the resolution pattern of this chromatography and Fig. 1b shows the result of LBIF fraction by SDS-PAGE (15% polyacrylamide gel) analysis (14). No IFN activity nor TNF activity was detected in FPLC-purified LBIF which was used in this study. IFN activity was assayed by the inhibition of cytopathic effect of sindbis virus in FL cells using NIH international IFN-α reference (Ga-23-901-531) (5, 12). TNF activity was assayed by the dye uptake microtiter method using L929 cells treated with actinomycin D as described (5, 13). Taking into consideration the minimum quantity of protein required to be detected, IFN assay (1 IU/100 pg) and TNF assay (1 unit/2 pg) have much higher sensitivities than LBIF assay has (1 unit/10 ng).

Cell Culture. Cells were cultured at 5 × 10^3 cells/well in a flat-bottomed 96-well culture plate (Falcon 3072) in the presence or absence of two- to threefold diluted cytokines. L929 cells were cultured in MEM containing 2% FBS. Three days later, cells were pulsed with 0.5 μCi/well of [3H]thymidine for the last 4 h. The radioincorporation was measured by the liquid-scintillation counter.

RESULTS

Effect of LBIF on the Proliferation of Various Tumor Cell Lines in Vitro. In order to determine whether LBIF inhibits the constitutive proliferation of various tumor cell lines in vitro, cells were cultured at 5 × 10^3 cells per well in the presence or absence of 4 units/ml of LBIF. Three days later, cell proliferation was monitored by pulsing cells with [3H]thymidine for the last 4 h. In all experiments, visual observation was done to confirm the reduction of cell number and in some cases, viable cell number was directly counted. These results primarily gave the same results as those of [3H]thymidine uptake experiments. The results were shown in Table 1 and Fig. 2. The proliferation of seven cell lines, MLA-144, Manaca, EBV-BLCL, A375, Mewo, VMRC-MELG, or C32TG was strongly inhibited at over 90% suppression. Six cell lines, Raji, U937, WEHI3, G361, SK-N-MC, and MT-2 showed sensitivity to LBIF activity at 40–70% suppression. Five cell lines, P815, P388D1, B16F1, HeLa, and TL-Mor were resistant to LBIF activity upon their proliferation.

Thus these results demonstrated that first, LBIF had the ability to control the constitutive proliferation at certain spectrum of tumor cell lines, secondly, LBIF appeared to be neither specie specific (i.e., LBIF inhibited the growth of human, ape, and murine cell lines) nor tissue specific (i.e., LBIF inhibited the proliferation of lymphomas, melanomas, or neuroblastomas). It was of note that LBIF inhibited cell proliferation by two characteristically different features. LBIF showed lethal effect on MLA144. MLA144 cells were fragmented and disappeared at day 3 in the presence of LBIF (4 units/ml). On the other hand, LBIF appeared to show cytostatic effects on other cell lines. Analyses to determine whether these cells can be arrested at any specific phase of cell cycle or that the inhibitory activity may be reversible will be required.

Dose Response of LBIF Activity. Various behaviors of tumor cells against LBIF could have resulted from their sensitivity to LBIF. To answer this question, the inhibitory activity of LBIF

![Fig. 1. a, purification of LBIF by reversed-phase HPLC on RP-304; b, SDS-PAGE analysis of LBIF. See "Materials and Methods."](image-url)
TUMOR GROWTH INHIBITORY FACTOR

Table 1 Effect of LBIF on the proliferation of various tumor cell lines

Cells were cultured at $5 \times 10^4$ cells/well in 96-well culture plate in the presence or absence of LBIF (4 units/ml). Three days later, cell proliferation was monitored by pulsing [3H]thymidine for the last 4 h.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Specie</th>
<th>[3H]Thymidine uptake (cpm ± SD)</th>
<th>None</th>
<th>LBIF (4 units/ml)</th>
<th>% inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>T-lymphoma</td>
<td>Human</td>
<td>1.2 x 10^6</td>
<td>44.664 ± 9,746</td>
<td>251 ± 108</td>
<td>99%</td>
</tr>
<tr>
<td>MLA144</td>
<td>T-lymphoma</td>
<td>Ape (gibbon)</td>
<td>22.194 ± 2,503</td>
<td>26,228 ± 1,049</td>
<td>No*</td>
<td></td>
</tr>
<tr>
<td>TL-Mor</td>
<td>Adult T-cell leukemia</td>
<td>Human</td>
<td>40,924 ± 941</td>
<td>17,334 ± 1,247</td>
<td>58%</td>
<td></td>
</tr>
<tr>
<td>MT-2</td>
<td>Adult T-cell leukemia</td>
<td>Human</td>
<td>7,617 ± 306</td>
<td>7,133 ± 239</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>B-cell lymphoma</td>
<td>Human</td>
<td>133.362 ± 9.911</td>
<td>8,001 ± 591</td>
<td>506 ± 84</td>
<td>94%</td>
</tr>
<tr>
<td>Manaca</td>
<td>B-cell lymphoma</td>
<td>Human</td>
<td>8,001 ± 591</td>
<td>2,297 ± 110</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td>EBV-BLCL</td>
<td>Epstein-Barr-virus transformed B-cell line</td>
<td>Human</td>
<td>20,370 ± 980</td>
<td>1,785 ± 369</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>L937</td>
<td>Histioytic lymphoma</td>
<td>Human</td>
<td>63,233 ± 5,233</td>
<td>23,410 ± 7,020</td>
<td>63%</td>
<td></td>
</tr>
<tr>
<td>WEHI 3</td>
<td>Macrophage cell line</td>
<td>Mouse</td>
<td>3.971 ± 967</td>
<td>2,297 ± 110</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td>P388D1</td>
<td>Macrophage cell line</td>
<td>Mouse</td>
<td>5.459 ± 149</td>
<td>4,659 ± 406</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>P815</td>
<td>Mastocytesoma</td>
<td>Mouse</td>
<td>6.287 ± 437</td>
<td>6,504 ± 437</td>
<td>No</td>
<td></td>
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<tr>
<td>Hela</td>
<td>Epidermal carcinoma</td>
<td>Human</td>
<td>43.622 ± 2,866</td>
<td>31,306 ± 2,090</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Mewo</td>
<td>Melanoma</td>
<td>Human</td>
<td>20,370 ± 980</td>
<td>1,785 ± 369</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>VMRC-MELG</td>
<td>Melanoma</td>
<td>Human</td>
<td>6,615 ± 530</td>
<td>529 ± 252</td>
<td>92%</td>
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<tr>
<td>A375</td>
<td>Melanoma</td>
<td>Human</td>
<td>79,731 ± 1,234</td>
<td>1,056 ± 208</td>
<td>99%</td>
<td></td>
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<tr>
<td>G361</td>
<td>Melanoma</td>
<td>Human</td>
<td>13,299 ± 3,578</td>
<td>3,808 ± 890</td>
<td>71%</td>
<td></td>
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<tr>
<td>C32T5</td>
<td>Melanoma</td>
<td>Human</td>
<td>8,623 ± 913</td>
<td>754 ± 224</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>B16F1</td>
<td>Melanoma</td>
<td>Mouse</td>
<td>89,156 ± 1,001</td>
<td>86,101 ± 5,130</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>Neuroblastoma</td>
<td>Human</td>
<td>20,033 ± 1,231</td>
<td>5,676 ± 227</td>
<td>72%</td>
<td></td>
</tr>
</tbody>
</table>

* % inhibition, see "Materials and Methods."

Over 90% inhibition.

No, less than 40% inhibition.

![Fig. 2. Morphological view of Manaca (A, medium; B, LBIF) and A375 (C, medium; D, LBIF). Cells were cultured for 3 days in the presence or absence of LBIF (5 units/ml). X 200.](cancerres.aacrjournals.org)
A lymphocyte blastogenesis inhibitory factor (LBIF) has been characterized as an immunoregulatory factor, especially on the T lymphocyte proliferation (1-4). As LBIF selectively inhibited the expression of IL-2 receptor, p75\(^4\), it was suggested that the functional mode of LBIF might be closely linked to a molecular mechanism involved in cell proliferation. In the present study, we have demonstrated that LBIF possesses strong anti-proliferation activity against various tumor cell lines in vitro. LBIF drastically inhibited the proliferation of a T cell lymphoma: MLA144, a Burkitt's lymphoma: Manaca, EBV-BLCL, melanomas, Mewo, VMRC-MELG, A375 or C32TG and a neuroblastoma: SK-N-MC irrespective of cell-lineage and specie-specificity.

We noticed that the behavior of tumor cell lines against LBIF was characterized into four different patterns. Firstly, it is of note that there is a group of LBIF-nonsensitive cell lines including TL-Mor, Hela, P815, P388D1, and B16f1. If LBIF directly inhibits the basic molecular mechanism for cell-cycle progression undergoing in the intracytoplasm or nucleus, LBIF should affect the proliferation of all cell lines. These results may suggest that LBIF function through putative LBIF receptors expressing on the cell surface. The same implication was also suggested by studies on T lymphocyte proliferation i.e., lectin stimulation is a prerequisite process for LBIF to be active on lymphocytes (3, 4). Secondly, in the case of a very-sensitive group, it appears that responses to LBIF begin abruptly at the concentration of about 1–2 U/ml LBIF. The molecular basis for such steep or even switchlike responses to graded signals is also an important question. Purified recombinant LBIF will be needed to investigate the existence of putative LBIF receptors and to study the relationship between specific receptor binding and biological activity. Thirdly, the difference on molecular mechanisms between a very-sensitive group (Fig. 3d) and a less-sensitive group (Fig. 3b) may raise more complicated issues. Simply a coexistence of LBIF-resistant subline in a less-sensitive group is conceivable. Alternatively, not only the number but also the structure of LBIF receptor might be related to the difference of LBIF-sensitivity.

In the case of very-sensitive group (Fig. 3d) and less sensitive group (Fig. 3b), we have not investigated whether these inhibitory activities are cytostatic/reversible or cytotoxic/lethal. It was not made clear by our observation during 3 day-culture. In contrast, the lethal effect of LBIF on MLA144, a cell of the 4th group (Fig. 3c), was remarkable. MLA144 expresses IL-2 receptor, p75 but not p55 molecules and produce a large amount of IL-2 (15). MLA144 expresses IL-2 receptor, p75 but not p55 molecules and produce a large amount of IL-2 (15). MLA144 is able to proliferate by accepting growth signal through p75 molecules with low/medium-affinity to IL-2 (Kd = 200 pM) in autocrine manner (16, 17). As LBIF selectivity inhibited the expression of p75 on lectin-stimulated T lymphocytes\(^4\), it is possible that LBIF can be lethal against the growth of MLA144.

Thus, these results suggest that LBIF may play important roles in regulating cell growth in general, not only in the regulation of immune response. LBIF may be a useful tool to...
analyze the molecular mechanism of cell cycle control in eukaryotes. Furthermore, this study has demonstrated a potential activity of LBIF to inhibit the growth of tumors in vivo and to be applied to clinical investigation.

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REFERENCES


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