Purification and Characterization of Tumor Inhibitory Factor-2: Its Identity to Interleukin 1

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ABSTRACT

The tumor inhibitory factor-2 from the conditioned medium of the human rhabdomyosarcoma cell line A673 was purified and sequenced. The 19 N-terminal amino acid residues were identical to those of human interleukin 1 (IL-1), corresponding to the residues 119–137 of the IL-1α precursor. The purified material had an apparent molecular weight similar to that of the mature secreted form of IL-1α (Mr 17,400). In addition, similarly to IL-1, it induced the production of IL-2 by T-cells. The purified protein inhibited the growth of the A673 cells from which it was derived, suggesting that it may act as an autocrine growth inhibitor. It also inhibited the growth of a human adenocarcinoma of the lung and three human mammary carcinomas, but not of two human melanoma cell lines. These biological activities, previously assigned to a putative tumor inhibitory factor molecule, are apparently due to the production by the tumor cells of IL-1α.

INTRODUCTION

The term TIF2 (1, 2) was given to acid and heat-stable growth regulators which do not demonstrate antiviral activity. They inhibited the growth of certain tumor cell lines in both soft agar and monolayer culture and stimulated the growth of normal human fibroblasts. This dual function has also been reported for the growth-inhibitor of the BSC-1 cells and TGF-β (3, 4). Likewise, TNF and IL-1, which share many biological functions (5), have been reported to be cystostatic or cytotoxic for certain tumor cells in vitro (6–9) and to stimulate the proliferation of normal human fibroblasts (6, 10).

TIFs were initially isolated and partially purified from the conditioned medium of a human rhabdomyosarcoma cell line A673. This cell line also produces a large molecular weight substance known as TGF-α (11) which antagonizes the biological effects of TIFs (1). Two TIFs (TIF-1 and TIF-2) were initially described (1, 2). They were distinguished by their apparent molecular weights, chromatographic properties, and their effects on the growth of certain target cells. Because of their potential importance in tumor development, we undertook the task to characterize TIF-2 in more detail. The amino acid sequence and biological activities of purified TIF-2 identify it as IL-1α.

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MATERIALS AND METHODS

Cell Culture. The cell lines A673, a human rhabdomyosarcoma, A375 and A2058, human melanomas, and A549, a human adenocarcinoma of the lung, have been described (11, 12). The following cell lines were obtained from American Type Culture Collection: the mink lung epithelial cell line Mv1Lu (NBL-7); the breast cancer lines MDA-MB-157, MCF-7, and SK-BR-3; the human embryonic lung cell line HEL; and mouse L929 cells. TDD cells are a normal adult skin fibroblast strain obtained from C. F. Fox (UCLA). The above cell lines were grown in DMEM purchased from Applied Biotechnologies, Inc. (Silver Spring, MD), supplemented with 10% heat inactivated (56°C; 30 min) FCS purchased from GIBCO Laboratories (Grand Island, NY). The LBRM-33-1A5 murine tumor cell line which produces IL-2 in response to PHA plus IL-1 (13) and the murine IL-2-dependent CTLL-2 line were maintained in RPMI 1640 medium (GIBCO), supplemented with 10% FCS and 5 × 10^{-4} M 2-ME.

Assay for Tumor Cell Growth Inhibition (TIF Assay). TIF activity was detected by a method previously described (1). Cells were plated at a density that allowed continuous growth throughout the time period of the assay. Indicator cells were subcultured in 50 μl DMEM supplemented with 10% FCS at 2–3 × 10^5 cells per well. Cells were cultured for 5 days, with [125I]IUDR (New England Nuclear, Boston, MA) at 0.5 μCi/ml added for the last 24 h. Inhibition or stimulation of growth was expressed as the percentage of decrease or increase of [125I]IUDR incorporated by cells treated with TIF relative to [125I]IUDR incorporation by control cells.

Preparation of Conditioned Media. Serum-free conditioned media were collected and processed from a total of 1610 roller bottles (850 cm^2; Corning, Corning, NY) seeded with the human rhabdomyosarcoma cell line A673. Each week, a batch of approximately 180 roller bottles was set up with A673 cells in DMEM supplemented with 15% FCS. When the cells reached confluency, the medium was removed and the cells were washed three times with 50 ml of DMEM (serum-free) with the last wash remaining on the cells for 6 h. Fifty ml of DMEM were added for each collection and three consecutive 24-h collections were made. Phenylmethylsulfonyl fluoride (Sigma) was added to each collection at harvest time at a final concentration of 0.5 μM/1l.

The three collections were pooled and poured through a piece of...
cheese cloth to remove cell debris. The total volume of conditioned medium was approximately 27 liters. This was concentrated approximately 10-fold on an Amicon hollow fiber cartridge system (Amicon, Lexington, MA) using a 10,000 molecular weight cutoff cartridge. At the same time it was diafiltered with 20 mM ammonium acetate (pH 4.5). The concentrate was centrifuged for 15 min at 10,000 x g and the supernatant was further concentrated fourfold by ultrafiltration through a 10,000 molecular weight cut-off filter (Amicon), centrifuged for 30 min at 10,000 x g and stored at 4°C. The concentrated and diaлизed conditioned medium from each batch was pumped onto a 21.5 x 150 mm semipreparative SP-5PW Protein Pak ion-exchange column (Waters, Milford, MA) which had been equilibrated with 20 mM ammonium acetate (pH 4.5). Depending on the amount of protein in each batch, the material was applied to the column in one or two runs. A total of 2160 mg of protein was subjected to 11 column runs. A gradient of 0.02 to 1.0 M ammonium acetate in 100 min was followed by a 15-min wash with 1.0 M ammonium acetate. A constant pH of 4.5 was kept throughout. The flow rate was 4 ml/min and 10-ml fractions were collected. Fractions containing biological activity in the TIF assay were pooled, the pH adjusted to pH 2.0 with 20% TFA, and centrifuged for 30 min at 17,400 x g.

**RESULTS**

Purification of TIF-2 Secreted from A673 Cells. In order to obtain a large amount of protein necessary for the amino acid sequence of TIF-2, a large scale purification was undertaken. Three consecutive 24-h collections of serum-free conditioned medium were gathered from a total of 1610 roller bottles (850 cm²) seeded with A673 cells. After concentration and diafiltration, the pooled conditioned medium was pumped onto 11 HPLC cation-exchange columns and eluted as described in “Materials and Methods.” Figure 1 summarizes results obtained with one of the columns. Similar results were obtained with the rest of the columns. When the fractions were tested for TIF activity against A549 cells, two peaks with inhibitory activity were detected. Fractions from peak A inhibited the growth of MVi1U cells (data not shown), indicating that the substance in peak B was TIF-1 and not TIF-2 (2).

Peak A from the cation-exchange columns was pooled and rechromatographed by a semipreparative rp-HPLC C₁₈ chromatography. This was followed by four sequential rp-HPLC analytical columns (diphenyl, CN, C₁₈, and C₄). After each chromatography step, fractions were assayed for TIF-2 activity, active fractions were pooled, diluted in order to reduce the concentration of acetonitrile, and pumped onto the next column. Figure 2 shows the chromatogram of the last purification step (rp-HPLC C₄ column which was eluted with a shallow acetonitrile gradient). The peak containing the growth inhibitory activity was analyzed by SDS-PAGE and silver staining. A single band migrating with an apparent molecular weight of 18,000 was detected.

To determine whether both tumor-inhibitory and fibroblast-stimulatory activities were mediated by the same substance, TIF-2 was isolated by elution from an SDS-polyacrylamide gel. A partially purified pool of TIF-2 from the rp-HPLC diphenyl
column was subjected to SDS-PAGE in the presence of a reducing agent. The protein from the gel slices was eluted and its biological activities on A549 cells, MDA-MB-157 cells, and the normal human fibroblast cells TOD were determined. The material from the same gel slice inhibited both tumor cell lines while not affecting the growth of others and to stimulate the growth of normal fibroblasts (3-10). Purification of the biological factor responsible for the TIF-2 activity in A673 cells and IL-1 were also found to inhibit the growth of particular cell lines while not affecting the growth of others and to stimulate the growth of normal human fibroblasts. Other growth factors were found to secrete a protein, initially described as TIF-2 (2) which inhibited the growth of certain tumor cells, but stimulated the growth of normal human fibroblasts. Other growth factors were found to share this property with TIF-2. Thus, TGF-β, TNF, and IL-1 were also found to inhibit the growth of particular cell lines while not affecting the growth of others and to stimulate the growth of normal fibroblasts (3-10). Purification of the substance responsible for the TIF-2 activity in A673 cells and determination of the amino acid sequence of its N-terminal amino acid residues led us to identify it as IL-1α. These 19 N-terminal amino acid residues were identical to the N-terminal 19 amino acids of the mature IL-1α (20), aligning with residues 119 through 137 of the IL-1α precursor sequence (21).

Biological Assays with Purified TIF-2. To determine whether the purified TIF-2 manifested a specific IL-1 activity, we tested it in a biological assay, which involves the ability of IL-1 to convert an IL-2 nonproducer murine tumor cell line, LBRM-33-1A5, to an IL-2 producer (13). This assay is more sensitive than the traditional thymocyte proliferation assay, which is based on the ability of IL-1 to augment the proliferative response of murine thymocytes to suboptimal concentrations of T-cell mitogens (13). As shown in Fig. 5, TIF-2 induced LBRM-33-1A5 cells to produce IL-2. The TIF-2 activity was comparable to that observed for recombinant IL-1α in the same assay. Similarly, both TIF-2 and rIL-1 were tested in the TIF assay against A549 cells. As in the IL-1 assay, their specific activities were very similar.

The purified TIF-2 was tested in the TIF assay against six human tumor cell lines and in a 4-day mitogen assay on serum-starved quiescent normal human embryonic lung cells. As shown in Fig. 6, the A549 cells were the most sensitive to inhibition of growth by TIF-2 with inhibition seen using picogram amounts. In addition, there was a morphological change in the appearance of these cells. This was most evident in low passages of this cell line. Two human breast cancer cell lines, MCF-7 and SK-BR-3, were also inhibited by TIF-2 as were the A673 cells which were the cells from which the TIF-2 has been derived. Two melanoma cell lines were unaffected. In contrast, the normal human embryonic lung cells HEL 299 were stimulated. No inhibition was seen on the mink lung Mv1Lu cells or on mouse L929 cells under these assay conditions (data not shown).

**DISCUSSION**

Cells of the A673 human rhabdomyosarcoma cell line were found to secrete a protein, initially described as TIF-2 (2) which inhibited the growth of certain tumor cells, but stimulated the growth of normal human fibroblasts. Other growth factors were found to share this property with TIF-2. Thus, TGF-β, TNF, and IL-1 were also found to inhibit the growth of particular cell lines while not affecting the growth of others and to stimulate the growth of normal fibroblasts (3-10). Purification of the substance responsible for the TIF-2 activity in A673 cells and determination of the amino acid sequence of its N-terminal amino acid residues led us to identify it as IL-1α. These 19 N-terminal amino acids aligned with residues 119 to 137 of the human cloned macrophage-derived IL-1α precursor (21). The first N-terminal amino acid of the mature IL-1α was initially assigned to residue 113 (21, 22). However, Cameron et al. (20) obtained evidence from the N-terminal amino acid sequence data of an anionic species of human IL-1 (pl 5.2) for the identification of its first N-terminal amino acid to residue 119 of the precursor. We have also verified the IL-1 nature of the
Identification of Tumor Inhibitory Factor-2 as IL-1

Table 1. Purification of TIF-2 from A673 cells

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)*</th>
<th>Yield (%)</th>
<th>Increase in specific activity</th>
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<tr>
<td>0</td>
<td>Culture media</td>
<td>25,599</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>1</td>
<td>Supernatant after concentration and dialysis against 20 mM NH$_2$OAc (pH 4.5)</td>
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<td>ND</td>
<td>ND</td>
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<td>2</td>
<td>Cation exchange</td>
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<td>$1.1 \times 10^4$</td>
<td>100</td>
<td>1</td>
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<td>rp-HPLC (C$_{18}$-semiprep)</td>
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<td>39</td>
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<tr>
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</tr>
</tbody>
</table>

* The number of units equals to the dilution at which half maximal inhibition of A549 cells in the TIF assay is obtained.

ND, not determined due to the possible presence of interfering growth factors (e.g., TGF-α) and growth inhibitors (e.g., TIF-1).

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Fig. 4. Alignment of N-terminal sequence of TIF-2 with the precursor of IL-1α. The top row shows a portion of the amino acid sequence of the cloned human IL-1α precursor (21). The numbers above the sequence indicate the position from the N-terminus. The bottom row shows the amino acid sequence obtained for TIF-2. Single-letter amino acid code is used.

Fig. 5. A, TIF-2-mediated induction of IL-2 production. Purified TIF-2 (○) and rIL-1α (●) were tested for their capacity to convert PHA-stimulated LBRM-33-1A5 cells to IL-2 production. The Δcpm is defined as the mean cpm of triplicate wells of the experimental group (rIL-1α/TIF-2 plus PHA) – the mean cpm (35,000) of the control (PHA alone). B, inhibition of A549 cell growth by purified TIF-2 (○) and rIL-1α (●) in the TIF assay.

Fig. 6. Biological assays with purified TIF-2. TIF-2 was tested against five human tumor cell lines in the TIF assay (---) and against normal human embryonic lung cells in the mitogen assay (-----). The following cell lines were used: A549 (●), A673, (○), SK-BR-3 (●), MCF-7 (●), A375 (○), A2058 (○), and HEL 299 (●). Control values are considered as 100% of growth.

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Material isolated from the culture medium of A673 cells by its induction of IL-2 secretion from T-cells.

IL-1α and IL-1β are products of two distinct genes with only distant homology (21). Both are synthesized as Mr, 31,000 precursors. The mature secreted form of each is a molecule with a molecular weight of 17,400 (23). IL-1β, usually associated with a neutral pI, is the predominant form in human macrophages (23). A673 cells, on the other hand, produce the mature form of IL-1α.

IL-1 has been most extensively studied in activated macrophages. It has been detected also in a variety of normal and transformed lymphoid cells (23–29) and in endothelial cells, fibroblasts, astrocytes, mesangial, dendritic, Langerhans and epidermal cells, lung tissues, glioblastomas, melanomas, and hepatomas (30–41). We have also partially purified a TIF-2/IL-1-like material from the conditioned media of a bronchogenic carcinoma of the lung and a human epidermoid carcinoma (data not shown).

IL-1 is a mediator in the immune system but has also biological activities on a variety of other cell types (reviewed in Refs. 34 and 42). Some of these overlap with those of TNF (5) which is cytotoxic and cytolytic for certain tumor cells.

TIF-2/IL-1 isolated from A673 cells inhibited the growth of certain tumor cell lines while not affecting several other cell lines and stimulated the growth of normal human embryonic lung cells in the mitogen assay (---). The following cell lines were used: A549 (●), A673, (○), SK-BR-3 (●), MCF-7 (●), A375 (○), A2058 (○), and HEL 299 (●). Control values are considered as 100% of growth.

It is interesting that TIF-2/IL-1 isolated from A673 cells had also the ability to inhibit the growth of the cells from which it was derived, indicating that it had the potential to act as an autocrine growth inhibitor. IL-1 secreted from an Epstein-Barr virus-transformed human B-cell line has been previously found to react as an autocrine growth stimulator (26). The inhibitory and stimulatory effects of IL-1 on different cells, including...
those from which it is secreted, may have an important role in the development of tumors.

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REFERENCES

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