Characterization of a Low-Molecular-Weight Growth Inhibitor Formed by Density-inhibited, Tumorigenic V79 Chinese Hamster Cells

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ABSTRACT

A novel type of low-molecular-weight growth-inhibitory factor responsible for the density inhibition of tumorigenic V79 Chinese hamster cells has been purified, if not homogenously, by a series of reverse-phase and gel filtration high-performance liquid chromatography. The factor is an acid-stable, heat-labile substance distinct from antiproliferative nucleoside analogues or polyamines and has a molecular weight of approximately 2000. The biological activity of this inhibitor was enhanced nearly 5-fold by trypsin treatment, thereby suggesting that the inhibitor may be a precursor peptide which becomes an oligopeptide with intense biological activity by proteolysis, or that trypsin treatment allows resultant small molecules to efficiently transfer across the cytoplasmic membrane. This inhibitor reversibly inhibits the growth of a broad spectrum of cell types from neoplastic and nonneoplastic cells from various species. These data suggest that this inhibitor is primarily a growth-regulatory molecule common to mammalian cells and may play an important role in regulating growth of both normal and neoplastic cells.

INTRODUCTION

Proliferation of normal cells in vitro is stringently controlled by a variety of regulatory mechanisms (1-6). Conversely, neoplastic cells are insensitive to these regulatory programs and unable to shift into the quiescent state after cell-to-cell contact, thus resulting in a high saturation density (7-9). However, even neoplastic cells decrease in their active growth potential as they become confluent (density-inhibited state). This growth characteristic of neoplastic cells in the density-inhibited state seems to be mainly due to soluble inhibitory factors, as these cells continued to proliferate, in a pile-up fashion, when the medium is frequently changed, as opposed to normal cells where frequent medium changes have little effect on proliferation of the cells at the density-inhibited state. Correspondingly we found that the medium conditioned by exposure to density-inhibited, tumorigenic V79 cells reversibly inhibits the growth of sparse, proliferating cultures of the same cell line (10, 11).

These observations have prompted us to search for the growth-inhibitory substance in the V79-conditioned medium. We found that this inhibitory activity is apparently mediated by soluble inhibitors produced endogenously and released into the medium at the time of density inhibition. An approximate Mr 2000 low-molecular-weight inhibitor has been identified by ultrafiltration and gel filtration (12). Our results suggest that growth regulation by an endogenous inhibitor is also operant in tumorigenic V79 cells. In addition, several human tumor cell lines, which secrete specific growth factors, have been shown to secrete growth-inhibitory factors (13-15). All these observations suggest that the proliferative potential of neoplastic cells may be augmented both positively and negatively and that mechanisms involved in the tumor cell growth are too complicated to be explained by the simple extrapolation of the enhanced positive growth potential. To understand such a complex nature of proliferation mechanisms inherent in tumor cells, identification and characterization of factors involved in the growth regulation of tumor cells are of paramount importance.

In this paper, we further purified and characterized a partially purified low-molecular-weight growth inhibitor derived from V79 cells, which accounts for the major growth-inhibitory activity of the V79-conditioned medium. This inhibitor appears to be an oligopeptide which reversibly inhibits growth of a wide variety of cell types of neoplastic and nonneoplastic cells, being a common regulator for growth of mammalian cells, regardless of tumorigenicity.

MATERIALS AND METHODS

Cell Culture and Preparation of Conditioned Medium. Cells were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air in Dulbecco-modified Eagle’s medium (Nissui Pharma Co., Tokyo, Japan) supplemented with 5% heat-inactivated FCS3 (Gibco Lab., Chagrin Falls, OH), with the exception of human cells which required 10% FCS. V79 cells, established from Chinese hamster lung fibroblasts, were demonstrated to be tumorigenic by s.c. inoculation of 103 cells into athymic BALB/c-nu/nu mice. BALB/c 3T3 A31-714 cells, originally isolated by T. Kakunaga (16), were recloned in our laboratory and were found to be strictly sensitive to density-dependent inhibition of cell division. This cell line was used as target cells for measurement of the inhibitory activity of fractionated materials, as described (12). HeLa is a human cervical carcinoma cell line. The cell line KSE-1, a human esophageal carcinoma, and HST-1, a human tongue squamous cell carcinoma which we established, were found to be tumorigenic by s.c. inoculation of 103 cells into athymic nude mice. The KSE-1 cell line in particular has been fully characterized (17). HSF is a human adult skin fibroblast cell line maintained in our laboratory.

For preparation of conditioned medium from density-inhibited V79 cells, V79 cells were grown to confluence in 100-mm dishes (Falcon). Since medium conditioned in the presence of 0.2% serum contained only approximately 10% of the inhibitory activity, the conditioned medium was prepared by exposing 10 ml of culture medium supplemented with 5% FCS to the confluently grown V79 cell culture for 24 h, as soon as the culture became confluent. The medium was then collected, passed through a 0.4-μm membrane filter, and stored at 4°C until use. Unconditioned medium was prepared in parallel from the same fresh medium and incubated under the same conditions, but in the absence of cells.

Reverse-Phase Chromatography. The conditioned medium was precipitated by the addition of an equal volume of acetonitrile. The supernatant, obtained after centrifugation at 4000 rpm for 15 min, was concentrated by an evaporator. Gel filtration of 30 ml of concentrated materials was carried out at 4°C on a Sephadex G-25 column (25 x 3.2 cm) equilibrated and eluted with 0.1% TFA as described (12). Low-molecular-weight growth-inhibitory fractions ranging between 500 and 2000 were combined and lyophilized. The redissolved residue with Vio volume of distilled water was applied to a reverse-phase Lobar column (Lichrosolv RP-18; Merck). Characterization of a Low-Molecular-Weight Inhibitor Formed by Density-inhibited, Tumorigenic V79 Chinese Hamster Cells

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: FCS, fetal calf serum; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; cAMP, cyclic AMP; TGF, transforming growth factor; TIF, tumor cell growth-inhibiting factor.
UV100 fluorescent stream detector (Hitachi) at 240 nm, and 2.0 ml of each fraction were lyophilized, redissolved in complete medium, and assayed for growth-inhibitory activity.

Purification of the Inhibitor by HPLC. The major inhibitory peaks (Fig. 1, Pool A) from the Lobar column were combined, lyophilized, resuspended in 0.5 ml of distilled water, and injected onto a TSK gel octadecyl silane 120A reverse-phase column (Toyosoda). Chromatography was carried out with Hitachi HPLC equipment, using an isocratic elution of distilled water containing 0.1% TFA. The column eluate was monitored simultaneously with absorbance at 210 and 280 nm. Each peak was lyophilized, resuspended in complete medium, and assayed for inhibitory activity. By repeating the rechromatography, a single fraction containing the inhibitory activity from a reverse-phase HPLC was separated from other impurities included in the fraction, and this peak fraction was lyophilized, resuspended in 100 µl of distilled water, applied to a column (7.5 x 600 mm) of TSK gel 3000 SW (Toyosoda) for gel filtration HPLC, equilibrated, and eluted with 10% acetic acid at room temperature. The column eluate was monitored with a UV detector set at 280 nm at a flow rate of 0.4 ml/min. Each peak was lyophilized, redissolved in complete medium, and assayed for growth-inhibitory activity, as described below.

Assays of Growth-Inhibitory Activity. Inhibitory activities of fractionated materials were determined by measurements of both cell growth and DNA synthesis in 3T3 cells, as described (12). To determine cell growth, replicate plates (35 mm; Falcon 3001) inoculated with 2 x 10⁴ cells were incubated for 18 h for attachment. Medium was then replaced with complete medium containing each of the lyophilized and dissolved fractions, and the incubation continued for an additional 48 h. At that time, the cell number was determined by detaching the cells with the 0.05% trypsin and 0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline and counting them in a particle data counter (Model 1D Coulter Counter). For the DNA synthesis, a double labeling assay was performed. Briefly, 3T3 cells were inoculated into replicate dishes (35 mm; Falcon) at a density of 2 x 10⁴ cells per dish and then incubated for 18 h in medium containing 0.01 µCi of [³H]thymidine (specific activity, 61 mCi/mmol) per ml. After this prelabeling, the medium was replaced with complete medium containing each lyophilized and redissolved fraction. The cells were then continuously labeled with the 1.0 µCi of [³H]thymidine (specific activity, 50 Ci/mmol) per ml for 6 h. After labeling, the radioactivities of both ³H and ¹⁴C incorporated into trichloroacetic acid-insoluble materials were determined simultaneously in a scintillation counter (Aloka Model 701). Since the radioactivity of ¹⁴C indicates the DNA content of the cycling cells at the time of the measurement of DNA synthesis, the resulting ³H/¹⁴C ratios can be considered as DNA synthesis per DNA and, therefore, the specific synthetic activity of DNA (18). In the assay of every peak in HPLC, the inhibitory coefficient, defined as a reciprocal of the percentage of cell growth, was calculated.

Stability of the Inhibitor. Aliquots from the fraction containing inhibitory activity from reverse-phase chromatography were tested for their sensitivity to trypsin and heat. Aliquots were treated with trypsin (No. T-2395; Sigma, St. Louis, MO) at a concentration of 1/8% weight of lyophilized aliquots for 2 h at 37°C, and then an equal activity of soybean trypsin inhibitor (No. T-9003, Sigma) was added. After lyophilizing and redissolving in complete medium, aliquots were assayed for cell growth. For the controls, the same amounts of trypsin and trypsin inhibitor were mixed, lyophilized, and equally assayed. For determination of heat sensitivity, lyophilized aliquots were redissolved in distilled water, heated at 95°C for 30 min, immediately refrigerated, and lyophilized for growth-inhibitory activity.

Elution Patterns on Reverse-Phase HPLC. Thymidine, thymine, dTTP, and cAMP (all from Sigma Chemical Co., St. Louis, MO) were adjusted to approximately the same concentration of the purified inhibitor by absorbance at 260 nm, and the elution patterns were compared on reverse-phase HPLC.

RESULTS

Reverse-Phase Chromatography. The low-molecular-weight inhibitor has been shown to be the major factor responsible for the density inhibition of tumorigenic V79 cells (12). To further purify this low-molecular growth inhibitor, preparations with a growth-inhibitory activity from Sephadex G-25 chromatography were fractionated over a reverse-phase Lobar column (Fig. 1). There were three major peaks containing the inhibitory activity. Fractions in the first inhibitory peak eluting at 0% acetonitrile contained enormous amounts of salts, and the inhibitory activity was toxic. The remaining two peaks eluted after the salts, and their inhibitory activities were not cytotoxic. The fractions in the second inhibitory peak eluting at approximately 5% acetonitrile were designated Pool A (Fractions 29 to 34), and the fractions in the third inhibitory peak eluting at approximately 8% acetonitrile were Pool B (Fractions 38 to 42). The inhibitory activities measured by cell proliferation largely paralleled those determined by DNA synthesis. However, in Pool B, the inhibitory activity was more evident when measured by DNA synthesis. This was expected as Pool B had been purified to apparent homogeneity and was found to be thymidine, as determined by physicochemical analyses (19). This endogenously produced cold thymidine is considered to compete with [³H]thymidine, resulting in a decrease in incorporation of [³H]thymidine. When unconditioned medium was similarly processed for gel filtration and subsequently for reverse-phase chromatography, mostly salts were isolated, and no significant peaks corresponding to Pool A appeared.

Reverse-Phase and Gel Filtration HPLC. When Pool A from the reverse-phase Lobar column was chromatographed on a TSK gel octadecyl silane 120A reverse-phase HPLC column using a 2% isocratic elution, the inhibitory activity was observed in a single small peak (Fig. 2). The potent inhibitory fractions (Peak A) were collected by repetitive reverse-phase HPLC and injected onto a gel filtration HPLC (Fig. 3). Although several small peaks appeared, these peaks contained no inhibitory activity. Inhibitory activity was demonstrated in the fraction with no discernible UV absorbance at 280 nm. The molecular weight of this inhibitor appears to be approximately 2000, according to the elution data of several markers (Fig. 3). Although there was no evidence of homogeneity, the inhibitor was considered to have been purified, as Pool B proved to be thymidine, with the same HPLC procedures (19).

Physicochemical Analyses. As shown in Table 1, Pool A was an acid-stable (pH 3) but heat-labile compound not inactivated by trypsin. However, it should be noted that the activity was enhanced nearly 5-fold by trypsin treatment (Table 1). This experiment was repeated, with reproducible results. These data...
Fig. 2. Reverse-phase HPLC of the partially purified inhibitor (Pool A). The concentrated Pool A was chromatographed using a 2% isocratic acetonitrile elution in 0.1% TFA at a flow rate of 0.8 ml/min, and the effluent was monitored simultaneously at 210 and 280 nm. Each peak was lyophilized, resuspended in fresh medium, and tested for growth-inhibitory activity on 3T3 cells. Arrows indicate the fraction containing the inhibitory activity. OD, absorbance.

Fig. 3. Gel filtration HPLC of inhibitory peak (Peak A) obtained from reverse-phase HPLC. The inhibitory peak on reverse-phase HPLC (Peak A) was concentrated by lyophilization and injected into TSK gel 3000 SW. The flow rate was 0.4 ml/min using 10% acetic acid, and the effluent was monitored at 280 nm. Each fraction eluting every 2 min (0.8 ml) was lyophilized, resuspended in fresh medium, and tested for growth-inhibitory activity on 3T3 cells. Arrows indicate the fraction containing the inhibitory activity. OD, absorbance.

Table 1 Effects of heat or trypsin on activity of the growth-inhibitory fraction (Pool A)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell no. $(\times 10^{-6})$</th>
<th>% of cell growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control medium</td>
<td>6.91 ± 0.24</td>
<td>100</td>
</tr>
<tr>
<td>Pool A (no treatment)</td>
<td>3.79 ± 0.19</td>
<td>54.8 ± 2.7</td>
</tr>
<tr>
<td>Pool A, heated (95°C, 30 min)</td>
<td>6.79 ± 0.40</td>
<td>95.4 ± 5.8</td>
</tr>
<tr>
<td>Control medium + trypsin + TI</td>
<td>7.31 ± 0.37</td>
<td>105.8 ± 5.4</td>
</tr>
<tr>
<td>Pool A, trypsin treated (37°C, 2 h)</td>
<td>0.66 ± 0.05</td>
<td>9.6 ± 0.7</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate plates.
* TI, trypsin inhibitor.

Fig. 4. Comparison of elution patterns of the purified inhibitor (Peak A) with various synthetic nucleic acid compounds including cAMP, thymidine, thymine, and dTTP. The chromatography was performed with a flow rate of 0.8 ml/min using a 2% isocratic acetonitrile elution in 0.1% TFA.

indicate that this inhibitor appears to be a small polypeptide with a trypsin-susceptible peptide bond in the primary structure. To distinguish Peak A from the inhibitory nucleic acid compounds (20, 21), Peak A was compared with cAMP, thymidine, thymine, and dTTP in elution pattern, using a 2% acetonitrile isocratic elution on reverse-phase HPLC (Fig. 4). We found that Peak A differs from these compounds in the elution pattern.

Comparison with Spermine. We also compared the inhibitor (Peak A) with spermine, the most hydrophobic among the polyamines, because polyamines have been shown to have cytotoxic effects on mammalian cells (22). Initially, we measured the concentration of polyamines in the conditioned medium, using an enzymatic method, but polyamines were not detected. Therefore, spermine was injected onto reverse-phase HPLC, using the same procedure as was used for the purification of Peak A. As shown in Fig. 5, spermine showed no UV absor-
bance but was detected by the ninhydrin reaction. The result shows that Peak A differed from spermine, as deduced by the elution rate on reverse-phase HPLC. Altogether, these results indicate that the inhibitor may be an oligopeptide distinct from antiproliferative nucleoside analogues and polyamines.

Effects of Pool A on Various Cell Lines. Pool A was tested for its effect on other normal and tumor cells from various species. Table 2 shows representative examples of the effect on a variety of cell lines from rodents to humans. Although there are considerable differences in growth suppression among these cell lines, this inhibitor is species nonspecific and effective for all cell lines tested, with either normal and malignant phenotypes. Growth inhibition became more evident when the cells were incubated for longer periods of time (Table 2). However, the inhibitory effects were reversible at least within 72 h, indicating that a toxic factor was not included in Pool A. The differential inhibitory effect of this inhibitor among various cell lines appears not to depend on the growth rate of the cells, because there was little difference in growth inhibition between rapidly growing V79 cells (doubling time, 11 h) and slowly growing human skin fibroblasts (36 h).

**DISCUSSION**

The culture medium of density-inhibited, tumorigenic V79 cells contains several molecular weight classes of growth-inhibitory factors (12). Specifically, the major factor responsible for the inhibitory activity of the conditioned medium has been demonstrated in the low-molecular-weight fractions (M, 500 to 2000). This partially purified inhibitor was subsequently separated into two distinct inhibitory fractions (Pools A and B), using reverse-phase chromatography. Using HPLC procedures, these inhibitory fractions were purified, if not homogeneously. The more hydrophobic inhibitor (Pool B) proved to be thymidine, as determined by physicochemical analyses. The partial involvement of thymidine in the inhibitory activity of conditioned medium has been ascertained (19). A similar analysis of the hydrophilic inhibitor (Pool A) revealed that this inhibitor is an acid-stable, heat-labile oligopeptide distinct from antiproliferative nucleic acid compounds or polyamines with a molecular weight of approximately 2000. This compound reversibly inhibits the growth of a broad spectrum of cell types from normal to malignant cells, suggesting that this inhibitor is primarily a growth-regulatory molecule common to all mammalian cells regardless of the tumorigenicity. The unique property of this molecule is that the inhibitory activity is markedly enhanced by trypsin treatment, thereby indicating the presence of trypsin-susceptible peptide bonds. These data also suggest that the inhibitor is a complex of the active molecular part and a carrier molecule which may mask the activity of the active part and that these complexes are linked together by trypsin-susceptible peptide bonds. The cleavage of biologically active polypeptide from the preformed and inactive precursor is a well-known process occurring in the production of insulin from proinsulin in pancreatic B-cells. Likewise, this inhibitor might be a precursor peptide similar to proinsulin and become a small peptide (oligopeptide) with intense biological activity by proteolysis. Another explanation is that it is much easier for the smaller molecule to transfer across the cytoplasmic membrane, such as via gap junctions, which limit the size of molecules to a molecular weight below 1500. It is unlikely that the crude inhibitor (Pool A) contains both growth inhibitor and trypsin-sensitive growth stimulators, because no remarkable growth-stimulating fractions were detected in the further purification steps of Pool A. Moreover, since the inhibition assay was performed under growth-stimulating conditions, including serum factors, the influence of the presence or absence of growth-stimulating factors on the assay is probably minimal. A similar activation was noted with TGF-β, a highly ubiquitous molecule produced by a variety of cell types in an inactive form that is irreversibly activated by acid treatment (24, 25).

As yet, no growth-inhibitory factors in this molecular range have been noted in neoplastic cells. In another aspect, cell-specific, endogenous growth inhibitors were isolated from cells or tissue extracts as regulators of cell growth. These inhibitors have been referred to as chalones, and many types were described (26). Recent recognition of the low-molecular nature of lymphocyte, granulocyte, and epidermal chalones has accelerated progress in their purification (27-29). Although molecular sizes of these chalones are close to the inhibitor we isolated, their biochemical and biological properties are dissimilar in both cell specificity and trypsin susceptibility.

Like chalones, polyamines are found in most tissues and inhibit the proliferation in vitro of most cell types to varying degrees, especially when cultured in fetal calf serum (30, 31). In addition, a close relationship between small chalones and polyamines has been suggested (32). Therefore, we investigated the relation of our inhibitor to the polyamines but detected no polyamines in the conditioned medium. Moreover, the elution study on reverse-phase HPLC revealed that this inhibitor is
much more hydrophobic than spermine, the most hydrophobic compound among polyamines, and thus can be readily distinguished from the polyamines.

Several growth-inhibiting soluble factors have been isolated from the conditioned medium of density-inhibited cultures of cells with a contact-mediated growth control. Wang et al. identified a growth inhibitor, a Mr 13,000 polypeptide, from Swiss 3T3 fibroblasts (4, 33, 34). Wells and Mallucci (35) isolated a growth inhibitor with a character of heat-labile, nondialyzable protein from mouse embryo fibroblasts. Sharma and Gehring (36) reported a protease-resistant, heat-stable, low-molecular-weight inhibitor (Mr 2000) from chicken embryo fibroblasts. Holley et al. have purified another class of the growth inhibitor, a Mr 24,000 glycoprotein, from African green monkey BSC-1 cells (5, 37), although this inhibitor was later found to be similar, if not identical, to TGF-β (38) and to have both stimulatory and inhibitory effects, depending on the cells and experimental conditions (25).

Biological and physicochemical comparison of these inhibitors with the inhibitor from V79 cells indicates that our inhibitor is a novel type of growth inhibitor distinct from these previously described growth inhibitors closely associated with contact-mediated growth control. Todaro and coworkers reported the partial purification of TIF-1 and TIF-2 from a human rhabdomyosarcoma cell line (13, 14). These preparations inhibit the growth of a line of human tumor cells but stimulate the proliferation of normal human cells. Therefore, TIF, TGF-β, and BSC-1 inhibitor may be a related but not identical polypeptide which may share the same receptor and thus show similar biological properties. Since it is a related but not identical polypeptide which may share the same receptor and thus show similar biological properties. Since it is a related but not identical polypeptide which may share the same receptor and thus show similar biological properties.
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