Autocrine Tumor Cell Growth-Inhibiting Activities from Human Malignant Melanoma

U. Bogdahn, R. Apfel, M. Hahn, M. Gerlach, C. Behl, J. Hoppe, and R. Martin

Department of Neurology, Cell Biology, and Neurochemistry Laboratories [U. B., R. A., M. G., C. B., R. M. J.], Institute of Genetics and Microbiology [C. B.], and Institute of Physiological Chemistry [J. H.], Julius-Maximilians-University, Würzburg, Josef-Schneider Strasse 11, D 8700 Federal Republic of Germany

ABSTRACT

Autocrine-secreted tumor cell growth-inhibiting activities were isolated from supernatants of a malignant melanoma cell line, HTZ 19-dM, established from a central nervous system melanoma metastasis. HTZ 19-dM was characterized by cyto- and immunocytochemistry and karyotyping; cells were propagated in defined serum-free tissue culture medium for up to 8 months. Supernatants were ultrafiltrated, dialyzed, lyophilized, and purified by Bio-Gel P-10 gel permeation chromatography, leading to three active fraction pools, MIA1 (melanoma-inhibiting activity), MIA2, and MIA3 (proteins at the cutoff of Bio-Gel P-10) inhibiting growth of 19-dM cells with 50% inhibitory concentrations of 0.79 ìg/ml (MIA1), 0.13 ìg/ml (MIA2), and 16.7 ìg/ml (MIA3). MIA1 could be further purified by reverse phase high pressure liquid chromatography; the main activity displayed a 50% inhibitory concentration of 0.33 ìg/ml. On sodium dodecyl sulfate-polycrylamide gel electrophoresis one major band (molecular weight about 14,000) and two minor bands (up to M, 17,000) were identified. Macromolecular synthesis was inhibited in 19-dM cells up to >99.5%; tumor stem cell colony formation was reduced by 99.8%; the inhibitory effect of MIA1 was irreversible, nonsaturable, and partially antagonized by a serum factor (depending on purification stage). MIA1 was heat stable (3 min at 100°C) and trypsin labile. The effect of MIA1 on allogeneic neuroectodermal tumors was also investigated; proliferation of two of three malignant melanomas and two of four glioblastomas was inhibited up to 85.2%; proliferation of a neuroblastoma cell line could be inhibited by 33.8%, whereas normal fibroblasts and low grade gliomas were not influenced in their proliferation.

INTRODUCTION

Binding of EGF2 (1) to its cell membrane receptor results in a cascade of events leading to increased cell proliferation and differentiation in nontransformed cells (2). Formerly EGF-sensitive normal rat kidney cells may lose their ability to bind EGF by transformation with the Kirsten sarcoma virus (3), which lead to the discovery of the EGF-related tumor growth factor ß (4). Since then an entire family of tumor growth and differentiation factors has been delineated (5-7). At the same time Todaro et al. have been able to purify 2 distinct tumor cell growth-inhibiting factors, TIF-1 and TIF-2, secreted by the human rhabdomyosarcoma cell line A 673 (8, 9). Both inhibit the growth of a number of allogeneic tumor cell lines but seem to promote growth of nontumorous cells. Further endogenous growth-inhibiting factors were purified by Levine et al. (TIF) (10) from a human colon carcinoma cell line, and by Yamaoka et al. (SGI) (11) from an avian sarcoma virus-transformed normal rat kidney cell line. We report here on the purification to homogeneity of a neuroectodermal derived human autocrine tumor cell growth-inhibiting activity (MIA1) and further MIA1 isolated from the human malignant melanoma cell line HTZ 19-dM. The initial biological and biochemical characterization of these melanoma-inhibiting activities will be described.

MATERIALS AND METHODS

Cells. A permanent cell line, HTZ 19-dM, was established from a malignant melanoma central nervous system metastasis and propagated in defined serum-free tissue culture medium (50% Dulbecco’s minimal essential medium, F-12 50%; Boehringer-Mannheim, Mannheim, Federal Republic of Germany), containing 0.8 ìmol l-glutamine (Gibco, United Kingdom), nonessential amino acids, 10 ìg/ml transferrin (Boehringer-Mannheim), 30 ìmol sodium selenite (Sigma Chemical Co., St. Louis, MO), and 4 ìg/ml gentamicin (Merck, Darmstadt, Federal Republic of Germany). Cells were grown as monolayer culture under standard culture conditions. Cells were characterized by cytochemistry, immunocytochemistry, and cytogenetics (data not presented here). For further screening the following cell lines were used: HTZ 4-p; [oli]dendrogloma]; HTZ 6-p6, HTZ 35-p7, HTZ 36-p4, HTB 17 [glioblastomas]; HTZ 60-p14 and HTZ 57-p14 [astrocytomas II]; HTB 69, CRL-1424, and HTZ 97-p3 [malignant melanomas]; HTB 10-p5 [neuroblastomas]; and B 34-p4 [normal fibroblast cell line] [HTB and CRL cell lines were obtained from American Tissue Type Cell Collection; HTZ cultures represent early passage tissue cultures from human brain tumor biopsies (13); B-34 was established in the Department of Human Genetics, Würzburg, Federal Republic of Germany, from a normal volunteer]. Cells for activity screening were grown in complete tissue culture medium containing in addition 10% FCS. Cell viability was tested with the trypan blue method.

Preparation of Culture Supernatant. HTZ 19-dM cells were grown in Nunc multi layer tissue culture dishes (6000 cm2 surface, 2000 ml culture medium; Nunc, Roskilde, Denmark). Supernatants were collected for further purification every 10 days; cells had been grown in serum-free medium for at least 14 days and up to 8 months without any contact to serum components. After collection supernatants were stored at -70°C until further use. Supernatants were filtered with bottle-top filters ( Falcon, 0.22 ìm; Becton Dickinson, Heidelberg, Federal Republic of Germany) and further concentrated by membrane ultrafiltration (Amicon YM 2 membrane; cutoff, M, 2,000; Amicon, Danvers, MA) to a final volume equal to 1% of starting volume (4,000 ml to 40 ml). The retained material was dialyzed for 60 h against 0.1 ìmol acetic acid (dialysis tubing; cutoff, M, 1,000; Reichelt, Heidelberg, Federal Republic of Germany) and centrifuged at 100,000 x g for 1 h at 4°C in an ultracentrifuge (Beckman, Munich, Federal Republic of Germany; model L5-65). The pellet was discarded and the supernatant was lyophilized.

Gel Permeation Chromatography. The lyophilized dialysate from two preparations was reconstituted in 1 ìmol acetic acid and further purified by gel permeation chromatography on a column (Pharmacia, Uppsala, Sweden; 2.6 x 100 cm) of Bio-Gel P-10 (507 cm2, 200-400 mesh; Bio-Rad Laboratories, Richmond, CA). The column was equilibrated with 1 ìmol acetic acid at 22°C; samples of protein (130-145 mg in 5 ml of 1 ìmol acetic acid) were applied to the column. Samples were eluted with 1 ìmol acetic acid at a flow rate of 12 ml/h with the use of a peristaltic pump (LKB, Gräfelfing, Federal Republic of Germany). A total of 150 fractions of 4 ml each were collected at 22°C and lyophilized once (further purification) or twice (screening for antitumor activity).

Reverse Phase High Pressure Liquid Chromatography. Fractions from 5 gel permeation chromatography separations containing MIA1 activity were collected and reconstituted in 11 ml 0.05% TFA. Aliquots

Received 3/9/89; revised 6/22/89; accepted 7/6/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. To whom requests for reprints should be addressed.

1 The abbreviations used are: EGF, epidermal growth factor; TIF, tumor cell growth; MIA1, melanoma-inhibiting activity; FCS, fetal calf serum; TFA, trifluoroacetic acid; RP-HPLC, reverse phase high pressure liquid chromatography; NaDodSO4, sodium dodecyl sulfate; TGF, transforming growth factor; IC50, 50% inhibitory concentration.

2 To whom requests for reprints should be addressed.
(500 μl) of the TFA solution were injected into the rp-HPLC system (Waters system including 2 MS 10 solvent delivery pumps, an automated gradient controller, a V G K sample injector, and a model 440 variable wavelength detector). All separations were performed on a reverse phase Nucleosil 120-5 C 8 (Macherey-Nagel, Düren, Federal Republic of Germany) column (250 x 4.6 mm inside diameter), protected by a guard column containing Shandon Hypersep (5 μm; Bischoff Analysetechnik, Stuttgart, Federal Republic of Germany) at room temperature and a flow rate of 1 ml/min. The column was eluted after equilibration with 0.05% TFA with a linear acetonitrile gradient separated into 3 sections, 0–25% acetonitrile in 15 min; 25–45% in 80 min; and 45–100% acetonitrile in 15 min, against 0.05% TFA (12). The column effluent was collected in 1.5-ml fractions. Aliquots were lyophilized for subsequent screening for antitumor activity and protein determination.

MIA Assay. Exponentially growing HTZ 19-dM cells were subcultured for 24 h in 96-well flat bottomed micro tissue culture plates (Costar, Zürich, Switzerland) in 100 μl serum-free medium at a density of 3 x 10^3 cell/well. Lyophilized fractions from columns were resuspended in 2 ml serum-free medium and sterilized by membrane filtration (GV 0.22 μm; Millipore, Bedford, MA). To each well 100 μl of these fractions and 50 μl of tissue culture medium were added; effects were determined in triplicate. Cells were incubated until controls were subconfluent (usually 10 days), 1 μCi [3H]thymidine (specific activity, 23 Ci/mmol; Amersham-Buchler, Braunschweig, Federal Republic of Germany) was added to each well, and DNA synthesis was determined by a liquid scintillation counting protocol (13). The effect of active fractions on tumor cells was expressed as percentage of [3H]thymidine incorporation of treated versus control cells. To determine the effect of MIA on macromolecular synthesis, cells were also incubated with 1 μCi [3H]uridine (specific activity, 53 Ci/mmol; Amersham-Buchler) to determine RNA synthesis and with 1 μCi [3H]leucine (specific activity, 120 to 190 Ci/mmol; Amersham-Buchler) to determine protein synthesis.

Human Tumor Stem Cell Assay. Exponentially growing HTZ 19-dM cells were plated into 6-well tissue culture plates (Costar) and cultured with 3 ml culture medium containing 10% FCS. After 24 hrs of incubation the medium was replaced by 3 ml tissue culture medium containing MIA-activity and 10% FCS. After 3 to 4 weeks cells were stained with Giemsa (Merck). The colony-forming efficiency was determined by calculating the ratio of formed tumor colonies (1 colony ≥50 cells) relative to plated cells. Any inhibitory effect was expressed as inhibition of colony-forming efficiency.

Stability of MIA. To determine the biochemical properties of MIA, proteolysis experiments were performed. Aliquots (0.5 ml) of 45 μM trypsin (Serva, Heidelberg, Federal Republic of Germany) were added to aliquots of MIA A (10.6 units/ml) in 0.5 ml serum-free medium and incubated at 37°C for 30 min. Then 0.5 ml 238 μM trypsin inhibitor (Serva) was added and the mixture was incubated for 20 min at 37°C. This solution was then tested in a MIA assay. Controls included tests of trypsin alone and in combination with trypsin inhibitor, MIA A alone, as well as preincubated trypsin plus trypsin inhibitor followed by MIA A. For determination of heat stability, aliquots of MIA A were preincubated for 2 h at 37°C, 3 min at 56°C, 2 h at 56°C, or 3 min at 100°C and then tested with the MIA assay (13).

NaDodSO 4 -Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in a modification of an original method (14). Samples were dissolved in a sample buffer (0.72 M 2-mercaptoethanol, 0.125 M Tris-HCl, pH 6.8-6.5, 2% NaDodSO 4) followed by heating at 100°C for 5 min prior to loading on gels; in some experiments sample buffer was used without mercaptoethanol. Bromophenol blue at a concentration of 0.025% was used as the tracking dye. The stacking gel contained 6% acrylamide (and 0.16% N,N' methylenebisacrylamide); the separating gel contained 15% acrylamide (and 0.75% N,N' methylenebisacrylamide). The stacking gel was buffered with 0.119 M Tris-HCl (pH 6.8) and 0.024% NaDodSO 4. The separating gel contained 0.025% NaDodSO 4 and 0.038 M Tris-HCl (pH 8.8). Gel slabs were 1.5 mm thick. Samples were applied to a 60-mm-wide well in the stacking gel. The running buffer contained 0.05 M Tris-HCl (pH 8.7), 0.38 M glycine, and 0.1% NaDodSO 4. The stacking gel was run at 20 mA and the separating gel at 30 mA until the tracking dye had reached the end of the gel. After electrophoresis gels were fixed overnight in a solution of 50% methanol and 12% acetic acid in water and stained with the Blum silver staining method (15).

General Methods. Total protein concentrations were determined with the Bio-Rad microassay, standardized against serum bovine albumin (Bio-Rad Laboratories). The specific activity of MIA is given in units/mg; 1 unit of activity corresponds to the amount of factor contained in 1 ml that leads to 50% inhibition of [3H]thymidine incorporation into tumor cell DNA or 50% inhibition of tumor stem cell formation.

RESULTS

Screening for Functional Activity of MIA. Following Bio-Gel P-10 gel filtration, 118 fractions (Fractions 33–149) were tested for growth-modulating activity in a MIA assay (HTZ 19-dM cells). At approximately 2 kDa 5 fractions demonstrated activity leading to >99.5% inhibition of thymidine incorporation; this activity was termed MIA A. Inconsistent moderate (up to 90% inhibition) inhibitory activity was seen between Fractions 77 and 109. Between Fractions 37 and 56 the major activity could be detected with inhibition of thymidine incorporation to >99.5%. This activity was divided into Fractions 45–56 (termed MIA B) and 37 to 44 (termed MIA C) (proteins of more than 17 kDa, at the cutoff of the Bio-Gel P10 column; Fig. 1). A similar effect of Fractions 41–150 could be observed when macromolecular synthesis (protein and RNA synthesis) were investigated in the MIA assay (Fig. 2). Protein synthesis was inhibited by MIA A-containing fractions up to 88% and by MIA A/m-containing fractions up to 87%. RNA synthesis could be suppressed by MIA A-containing fractions up to 74% and by MIA A/m-containing fractions up to 96%. Protein concentrations in these experiments were equivalent to 10.4 inhibitory units of MIA (see below). Following rp-HPLC-purification of MIA A-containing fractions 1 main inhibitory peak could be identified between Fractions 56 and 61 in the MIA assay, with more than 99.5% [3H]thymidine incorporation at 42–45% acetonitrile (Fig. 3A). Further MIAs were not available in sufficient amounts for detailed purification studies.

Purification and Characterization of MIAs. Dose-response curves (MIA assay) were performed with MIA A, MIA B, and MIA A/m, as well as MIA after rp-HPLC purification. Dose-response effects were plotted on semilogarithmic probability plots to calculate the specific inhibitory activity (Fig. 4). Protein determinations for the different purification steps led to calculation of inhibitory units for respective preparations (Table 1).
AUTOCRINE TUMOR CELL GROWTH-INHIBITING ACTIVITIES

Fig. 2. Examination of effects of MIA activities on macromolecular synthesis, using each third fraction of a Bio-Gel P-10 gel permeation chromatography of one batch of HTZ 19-dM cell supernatants. The \(^{3}H\)thymidine incorporation relative to control was determined with a MIA assay in dM (A); RNA and protein synthesis were determined with \(^{3}H\)uridine incorporation (C) and \(^{3}H\)leucine incorporation (O) assays in dM. Test cells were HTZ 19-dM cells. Arrows, molecular weight of the Bio-Gel P-10 marker proteins RNase A (M, 13,700), insulin (M, 6,000), and somatostatin (M, 2,000).

Fig. 3. A, rp-HPLC of MIA\(_{11}\), with an acetonitrile gradient (-----). Every fraction was tested for \(^{3}H\)thymidine incorporation in MIA assay (A), using HTZ 19-dM cells as test cells in dM. Protein concentrations were determined with a Bio-Rad microassay (O). B, NaDodSO\(_{4}\)-polyacrylamide gel electrophoresis (including mercaptoethanol) of biologically active (Lane C, Fraction 57) and inactive (Lane A, Fraction 53; Lane B, Fraction 55; Lane D, Fraction 62; Lane E, Fraction 65; Lane F, Fraction 67) rp-HPLC fractions. The active fraction contains a single band at approximately M, 9,000. The amount of protein loaded on Gel Lanes A to F was 0.5 \(\mu\)g.

Stability. MIA\(_{11}\) is stable at 56°C for 2-h and at 100°C for 3-min incubation times (>99.5% inhibition of \(^{3}H\)thymidine incorporation into HTZ 19-dM cells was detected). Incubation of MIA\(_{11}\) for 30 min with trypsin inactivates the inhibitory activity.

Effect of MIA\(_{11}\) on Tumor Stem Cells. The colony-forming efficiency for HTZ 19-dM cells in 10% FCS was 8.88 ± 0.57% (SD). Using 10.6 inhibitory units/ml of MIA\(_{11}\) an inhibition of -2.9 log was achieved. This is considerably more inhibition than would have been expected from the results of the \(^{3}H\) thymidine incorporation assay; 10.6 inhibitory units would equal -1.23 log inhibition (compare Fig. 4).

Effect of MIA\(_{11}\) on Growth of HTZ 19-dM Cells. MIA\(_{11}\)-treated and untreated HTZ 19-dM cells were assayed at different time points for \(^{3}H\)thymidine incorporation after the start of treatment with the factor. \(^{3}H\)Thymidine incorporation into DNA of untreated and treated cells remained low to 50 h after explanting cells into experimental dishes and treatment; at 50 h untreated cells started their exponential growth phase, whereas MIA\(_{11}\)-treated cells did not enter growth phase. This could be observed for FCS-containing medium as well as for serum-free medium. The difference was, however, that treated cells in FCS-containing medium remained vital for prolonged periods of time (>4 weeks), whereas cells in serum-free medium rounded up and died after 2 to 5 days (trypan blue incorporation). Treatment was never cytotoxic for cells, inasmuch as no increase in trypan blue incorporation could be observed up to 2 days after treatment. Treating HTZ 19-dM cells in 10% FCS medium with MIA\(_{11}\) for different time intervals and at a dose of 5 inhibitory units/ml, minimal exposure for maximum effect of MIA\(_{11}\) action was investigated. After 30 min an inhibition of 72% was achieved, which increased to 87% at 2 h maximum. Two h of exposure were necessary to achieve a maximum effect at this dosage. In a long-term experiment HTZ 19-dM cells were exposed to 20 inhibitory units/ml MIA\(_{11}\) either continuously for 4 weeks or for 5 days and then propagated for 4 weeks under standard tissue culture conditions without MIA\(_{11}\). There was no difference between either experimental set; no growth of cells was observed, indicating an irreversible effect of MIA\(_{11}\) on cell proliferation.

NaDodSO\(_{4}\)-Polyacrylamide Gel Electrophoresis. Analysis of dialysate with NaDodSO\(_{4}\)-polyacrylamide gel electrophoresis led to multiple bands from below 4 kDa to the exclusion weight of the gel at M, 45,000 (Fig. 5, Lane A). When Bio-Gel P-10 fractions containing MIA\(_{11}\) activity were subjected to NaDodSO\(_{4}\)-polyacrylamide gel electrophoresis, 2 major and at least 2 minor bands were recovered. The molecular weights range from approximately 6,500 to approximately 13,500 (molecular weights approximately 6,300, 9,000, 12,000, and 13,500; Fig. 5, Lane B). When rp-HPLC-purified MIA\(_{11}\) probes were...
subjected to NaDodSO4-polyacrylamide gel electrophoresis, 1 major band was recovered at approximately M, 9,000. In addition, 1 minor band was detected at approximately M, 12,000 (Fig. 5, Lane C). If electrophoresis was performed without mercaptoethanol, a single band is detected at M, 8,000, which demonstrates the purity of MIAH (Fig. 5, Lane D). When HPLC fractions were subjected to NaDodSO4-polyacrylamide gel electrophoresis, the major band with a molecular weight of approximately 9,000 was seen in the biologically active fraction, demonstrating one major band at approximately M, 9,000 and one minor band at approximately M, 12,000. In D rp-HPLC-purified MIAH probes (3 µg) were examined with a sample buffer without mercaptoethanol, leading to a single band with a molecular weight of approximately 8,000.

Activity of MIAH against Different Melanoma, Glioma, and Nonmalignant Cells. The antiproliferative effect of MIAH on different allogenic cells was investigated with the MIA assay. MIAH was tested at a dose of 10.6 inhibitory units/ml (Bio-Gel P-10 preparation). Three different melanomas were screened for MIA activity: 2 of 3 tumor lines demonstrated inhibition of [3H]thymidine incorporation up to 85.2%; 1 tumor line was insensitive (Table 2). Two of 4 glioblastoma multiforme tumor lines were marginally inhibited up to 28%; a neuroblastoma line could be inhibited up to 33.8%. A fibrillary astrocytoma and an oligodendroglioma were insensitive. Normal fibroblasts also did not show any inhibition upon MIAH.

Table 1. Purification and characterization of MIAs

| Purification step | Protein recovery | Specific inhibitory activity (units/mg) | Total inhibitory units (FCS) | Degree of purification | Recovery (%) |
|-------------------|------------------+--------------------------------------|-----------------------------|--------------------------|--------------|
| Cell supernatant  | 77.2 mg          | 74.6                                  | ND*                         | 1                        | 100         |
| 19-dM             | 14 liters        | 578                                   | 413                         | 11,400                   | 7.75        |
| Dialyse          | 27.9 mg          | 59.9                                  | 23.4                        | 644                      | 0.8         |
| Bio-Gel P-10      | 27 mg            | 1,270                                 | 526                         | 5.8                      | 17.0        |
| MIAH              | 0.01 mg          | 7,520                                 | 6,990                       | 11,603                   | 101.0       |
| Bio-Gel P-10      | 1.66 mg          | 1,188                                 | 3,030                       | 791                      | 16.0        |
| MIAH              | 0.26 mg          | (4 µg)                                |                             |                          |             |

* Total protein was determined using Bio-Rad microassay with bovine serum albumin as standard.

DISCUSSION

There is a considerable amount of evidence that malignant transformation of cells by oncogenic viruses or by activation of protooncogenes or retroviral oncogenes may be associated with two major steps: qualitative and/or quantitative changes in the genetic expression of endogenous growth factors may induce anchorage-independent growth and alterations of cell morphology; this is mediated by the ras gene family, the src gene family, the sis gene, the polyoma middle T-antigen, and the adenoavirus e1b gene (4, 16–18); the sensitivity or responsiveness of cells to growth factors may be modulated by a second group of genes, namely the myc gene family, myb, fos, and polyoma large T-antigen gene, as well as adenoavirus ela gene (19–21). Interaction or cooperation of gene products of both groups seems to be necessary to achieve malignant transformation. Growth factors not only act as growth promoters, e.g., the v-sis-coded B-chain of platelet-derived growth factor (4, 18) in gliomas, but far less precisely than on tumor growth factors. At this point, for tumor growth factors may be modulated by a second group of genes, namely the myc gene family, myb, fos, and polyoma large T-antigen gene, as well as adenoavirus ela gene (19–21). Interaction or cooperation of gene products of both groups seems to be necessary to achieve malignant transformation. Growth factors not only act as growth promoters, e.g., the v-sis-coded B-chain of platelet-derived growth factor (4, 18) in gliomas, but far less precisely than on tumor growth factors. At this point, for

Table 2. Effect of MIAH on different allogenic tumor cells and nonmalignous cells

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Code</th>
<th>Inhibition ± SEM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amelanotic malignant melanoma</td>
<td>HTZ 97-p3</td>
<td>3.9</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>CRL 1424</td>
<td>85.2 ± 0.9</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>HTB 69</td>
<td>52.9 ± 7.2</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>HTZ-6p6</td>
<td>28.1 ± 7.2</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>HTZ-36-p4</td>
<td>17.7</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>HTB 17</td>
<td>20.4 ± 3.9</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>HTZ-25-p7</td>
<td>14.8</td>
</tr>
<tr>
<td>Fibrillary astrocytoma</td>
<td>HTZ 57-p14</td>
<td>15.0</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>HTZ 4-p6</td>
<td>7.1</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>HTB 1-p5</td>
<td>33.8 ± 4.9</td>
</tr>
<tr>
<td>Normal fibroblast</td>
<td>AW 1-p2</td>
<td>7.8</td>
</tr>
</tbody>
</table>

* Percentage of inhibition of [3H]thymidine incorporation of treated cells related to untreated controls. Experiments were performed as quadruplicates.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1989 American Association for Cancer Research.
the first time a human tumor inhibition factor has been purified to homogeneity, and its biological activity is related to previously described TIFs.

TIF-1 \( [M, 10,000-16,000 (9)] \) and TIF-2 \( [M, 18,000-22,000 (8)] \) are 2 distinct, partially purified tumor-inhibiting polypeptides produced by a rhabdomyosarcoma cell line. TIF-1 is trypsin sensitive and heat resistant; TIF-2 is trypsin insensitive and heat labile; their action is reversible. Compared to MIA, both factors are much less active in the autologous tumor cells in an assay comparable to the MIA assay; TIF-1 led to a 59% inhibition of A 673-cells at 20–360 \( \mu g/ml \), and TIF-2 caused a 46% inhibition of A 673 cells at 130–650 \( \mu g/ml \); for comparison the IC\textsubscript{50} of MIA was 130 ng/ml in the autologous system (HTZ 19-dm cells). At 10 \( \mu g/ml \) MIA had roughly the same activity in allogenic and histologically different tumors (Table 2). With TIF-1 and TIF-2 no inhibitory activity was seen on nontumorous cells, as was the case also for MIA.

Another TIF \( [M, 5,000-15,000 (10)] \) was produced by the human colon carcinoma cell line MOSER. This is, again, a heat-stable and trypsin-sensitive crudely purified polypeptide. IC\textsubscript{50} values on autologous MOSER cells were found to be between 0.45 and 3.0 \( \mu g/ml \), using the soft agar growth assay. Inhibition of allogeneic cells was performed with conditioned medium from MOSER cells, and an inhibitory effect could be seen in 3 of 6 cell lines without further quantification. The effect of TIF\textsubscript{MOSER} was reversible in the autologous system, using a growth curve related test system. This factor was tested only in colon carcinoma and epidermoid cells and not in nontumorous cells.

The heat-labile SGI \( [M, 18,000-20,000 (11)] \) was produced by the avian sarcoma virus-transformed normal rat kidney cell line 77N1. The IC\textsubscript{50} on autologous 77N1 cells was approximately 5 \( \mu g/ml \), using the soft agar growth assay. Its inhibitory effect could be detected in 5 of 7 tumor cell lines attaining a half-maximal inhibition with less than 2 \( \mu g/ml \) SGI (DEAE-Sephadex chromatography purified) in the case of the most sensitive cell line.

In contrast to TIFs described previously (8–10) the effect of MIA\textsubscript{H} appeared to be exerted on the entire cell population. For example MIA\textsubscript{H} at 10.6 units produced a 1000-fold inhibition of tumor stem cell colony formation. Aside from SGI (11) reported TIFs (8–10) appear to exert effects on only a subpopulation of the cell lines tested. This suggests that MIA\textsubscript{H} is a more potent growth regulator than other published TIFs, at least in the system, which we have been using to study its properties. The effect of MIA\textsubscript{H} on different allogeneic tumor cells and nontumorous cells was measured thus far only with the MIA\textsubscript{H} assay, which is, however, less sensitive to its action: at 10.6 inhibitory units of MIA\textsubscript{H} \( [\text{H}^3\text{H}] \text{thymidine incorporation was reduced to } 5.9\% \) and tumor stem cell formation was reduced to 0.114\%, indicating a 50-fold increased sensitivity of the stem cell assay over the MIA assay. Taking this into account, the effect of MIA\textsubscript{H} on different tumor cells might be more pronounced than expected from our early results. It seems highly remarkable that MIA\textsubscript{H} has an irreversible effect on tumor cells but is noncytotoxic, which has not been described before to our knowledge for any other tumor-inhibiting factor.

Thus far TIFs have not shown antiviral activity (8, 9), implying that they are unrelated to interferons. We have not tested antiviral activity of MIA\textsubscript{H} in earlier experiments we have shown, however, that HTZ 19-dm cells were not sensitive to recombinant \( \alpha, \beta, \gamma \)-interferons. This may indicate that MIA\textsubscript{H} is probably unrelated to interferons.

MIA\textsubscript{H} activity was reduced by serum (10% fetal calf serum), an effect related to the degree of purification. MIA\textsubscript{H} activity could not be detected in the supernatant in the presence of serum, compared to an activity of 33% inhibition of \( [\text{H}^3\text{H}] \text{thymidine incorporation without serum. The presence of serum produced a decrease of MIA\textsubscript{H} activity of } 28\% \) in the dialysate and of 7% in Bio-Gel P-10 fractions. The increase of MIA\textsubscript{H} specific activity during purification may result in higher receptor affinity or increased receptor binding, overcoming the effect of a possible antagonistic serum factor.

We were able to demonstrate a single band (at approximately \( M, 8,000 \)) in MIA\textsubscript{H} activity containing rp-HPLC fractions by NaDodSO\textsubscript{4}-polyacrylamide gel electrophoresis, this band being present also in Bio-Gel P-10-purified material (Fig. 5, b–d). If gel electrophoresis is performed on rp-HPLC-purified material with mercaptoethanol, 2 bands at approximately \( M, 9,000 \) and 12,000 could be identified. This may indicate that the MIA\textsubscript{H} molecule contains sulfur bonds responsible for its conformation. It may be conceivable that mercaptoethanol separates a small fragment from the entire molecule (small band at \( M, 12,000 \)) and leaves the remaining molecule in a more unfolded conformation compared to the native state.

As TGF-\( \beta \) will be demonstrable at approximately \( M, 25,000 \) or 12,500 [monomer (23)], it is evident from these data that MIA\textsubscript{H} is unrelated to TGF-\( \beta \). As there is a broad band at approximately 6,500, we suspect this may reflect TGF-\( \alpha \)-activity, which we could not yet prove. Although we have been able to purify MIA\textsubscript{H} to homogeneity (Fig. 5), we have documented a loss of specific activity during rp-HPLC purification. Again the reason for this is unclear; it may be due to conformational changes or contamination of the MIA\textsubscript{H} molecule by heavy metals. Further work is required on this subject.

MIA\textsubscript{H} is produced by melanoma tumor cells, which are of neuroectodermal origin. Thus far we have been able to demonstrate inhibitory activity in different melanomas (Table 2) and also in different high grade malignant gliomas, which are also derived from neuroectodermal progenitor cells. In further studies it may be of interest to characterize the spectrum of MIA\textsubscript{H} activity in other neuroectodermal tumors and, of course, normal cells. Normal fibroblasts are not inhibited by MIA\textsubscript{H}, and low grade neuroectodermal tumor lines are also unaffected by MIA\textsubscript{H}. In a preliminary experiment we could show some activity in a neuronal derived tumor line, a neuroblastoma. Further \textit{in vitro} and \textit{in vivo} studies are needed to determine the potential clinical relevance of this novel tumor inhibition factor, which may be a potentially important growth regulator in progression, metastasis, and remission of neural crest tumors.

ACKNOWLEDGMENTS

This work is dedicated to Professor H. G. Mertens. The authors are especially grateful to Professor E. Buchner for his fruitful discussions and to Professor H. T. R. Rupniak for critical review of the manuscript. Continuous cooperation with the neurosurgical department (chairman, Professor K. A. Bushe) is highly appreciated. Thanks is also given to D. Huuk for editorial assistance and K. Bekurts for administrative support.

REFERENCES

2. Carpenter, G., King, L., Jr., and Cohen, S. Epidermal growth factor stimu-
Autocrine Tumor Cell Growth-inhibiting Activities from Human Malignant Melanoma


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/49/19/5358

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.