Identification of Endogenous Inhibitory Growth Factors from a Human Colon Carcinoma Cell Line

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ABSTRACT

A line of human colon carcinoma cells, designated MOSER, was established which synthesized tumor-inhibitory factor (TIF) and transforming growth factor (TGF) activity. Both activities were found in serum-free conditioned medium and in cell extracts. The activities coelute on Bio-Gel P-10 in acetate acid, but can be completely separated by reverse-phase high-pressure liquid chromatography. The TIF and TGF activities were acid and heat stable, and were sensitive to trypsin and dithiothreitol. MOSER cell TIF prevented the anchorage-independent growth of the more differentiated colon carcinoma cell lines tested but did not affect the less differentiated lines. Using anchorage-dependent growth conditions, the effect of TIF appeared to be noncytotoxic and partially reversible. Purified TGF stimulated the growth of normal rat kidney fibroblasts and the slow-growing CBS colon carcinoma cell line but did not stimulate MOSER cell growth. MOSER cells contain both positive (TGF) and negative (TIF) factors with relative concentrations that may be important parameters in the regulation of cell growth.

INTRODUCTION

Growth in semisolid medium by many types of malignant cells from primary tumors (including primary colon carcinomas) and cultured cell lines is dependent upon cell concentration (1-7). It has been hypothesized that the dependence of anchorage-independent growth on cell concentration reflects a requirement for autostimulatory TGF3 production by malignant cells (7). Most TGFs described to date are acidic and heat-stable polypeptides of Mr 6,000 to 30,000 (7-11). One type of TGF (TGF-α) binds to the EGF membrane receptor but does not interact with antibodies to EGF. The complete amino acid sequence of TGF-α was reported, and it showed extensive homology to mouse and human EGF (12-14). TGF-α has been purified to homogeneity and transformed growth factor activity. Both activities were identified which are autostimulatory, and they enhance the growth of differentiated colon carcinoma cell lines and 3 of 10 cultures tested.
washed 3 times in serum-free McCoy's Medium 5A, and the final cell pellet was weighed to determine the wet cell mass. For every 10 g, wet weight, of cells, a solution containing 37.5 ml absolute ethanol, 1.75 ml distilled H₂O, and 0.75 ml concentrated HCl was added. The total aqueous volume was then adjusted to 6 ml/g, wet weight, of cells, using distilled H₂O, and the mixture was stirred overnight at 4°C. After centrifugation at 10,000 × g for 15 min, the pellets were reextracted for 4 h in 0.5 volume of acid:ethanol:H₂O (0.04:2:1). Following a second centrifugation, the 2 supernatants were pooled, and the pH was adjusted to 5.3 with concentrated ammonium hydroxide. Ammonium acetate (1 ml of 2 M, pH 5.3, to each 80-ml extract), absolute ethanol (2 volumes), and anhydrous diethyl ether (4 volumes) were added to the extract, and the protein was precipitated at -20°C for 4 h. The precipitate was collected by centrifugation at 15,000 × g for 15 min, dried in a vacuum, resuspended in 1 ml acetic acid, dialyzed extensively versus 1% acetic acid, and lyophilized. This crude extract was stored at -70°C.

**TGF and TIF Assays.** Underlayers of 0.5 ml of 0.8% agarose (Sea Plaque; FMC Corporation, Marine Colloids Div., Rockland, ME) in growth medium were plated in 9.6-sq cm culture plates and allowed to solidify at room temperature. To assay for TGF, these plates were overlaid with a single-cell suspension (0.5 ml) of 2.0 × 10⁶ NRK cells in 0.4% agarose in supplemented McCoy's Medium 5A containing 10% FBS and aliquots of column fractions, column pools, or crude extracts. The plates were then incubated at 37°C for 5% CO₂ in a humidified incubator, and colonies of column fractions, column pools, or crude extracts were scored on an inverted microscope on Days 14 to 21. TIF was assayed in the same way, except that 1 × 10⁶ MOSER cells were utilized instead of NRK cells. TIF activity was expressed as percentage of reduction in MOSER colonies relative to untreated controls. One unit of activity is defined as the amount of protein (in a 1-ml assay) necessary to give 50% stimulation of NRK cells (for TGF) or 50% inhibition of MOSER cells (for TIF). Aliquots of column fractions or crude extracts were lyophilized before addition to the assay. TIF-inhibition studies of A431 cells and other colon carcinoma cell lines were performed in the same manner.

**Stability of TGF and TIF.** Extracts were tested for their sensitivity to trypsin, DTT, and heat, as described by Proper et al. (32). Crude extract (1 mg) was lyophilized to dryness and dissolved in 1 ml of phosphate-buffered saline (0.01 M sodium phosphate; 0.15 M NaCl, pH 7.2). One aliquot was treated with trypsin (type 3; Worthington Biochemicals, Freehold, NJ) at a concentration of 50 μg/ml for 2 h at 37°C, and the reaction was stopped by the addition of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) to a concentration of 100 μg/ml. For the trypsin control, trypsin (50 μg) and soybean trypsin inhibitor (100 μg/ml) were preincubated for 30 min before addition to the sample. For determination of heat sensitivity, separate aliquots were heated at 50°C for 30 min, or at 100°C for 3 min. A final aliquot was treated with 0.065 M DTT in 0.1 M NH₄ClO₄ for 1 h at room temperature. After treatment, each sample was brought to 4 ml with PBS and dialyzed for 48 h versus 1% acetic acid, lyophilized, and tested for activity by the soft agarose growth assay.

**Bio-Gel P-10 Exclusion Chromatography.** Extracts were fractionated on Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, CA; 2.5 x 72 cm) in 1 M acetic acid at a flow rate of 12 ml/h. Approximately 150 ml of dialyzed extract in 1 M acetic acid (5 ml) were applied, and 5-ml fractions were collected. Aliquots (200 to 300 μl) were removed from each fraction for determination of TIF and TGF. Peaks of activity were pooled and the dose-response activity of TIF and TGF was determined.

**Reverse-Phase HPLC.** Pool 4 (1 mg) from the Bio-Gel column was dissolved in 1% TFA (60 μl) and then diluted 10-fold with 0.1% TFA. After centrifugation to remove insoluble material, 500 μl were injected onto a C₁₈Bondapak column (Waters Associates, Milford, MA), using a Varian Model 5000 HPLC system equipped with a Varian UV-50 variable-wavelength detector. The sample was chromatographed, using an acetonitrile gradient in 0.1% TFA, as described by Anzano et al. (19), and the effluent was monitored at 220 nm. All reagents were HPLC grade (J. T. Baker Chemical Co., Phillipsburg, NJ).

**Anchorage-dependent Growth.** MOSER cells were plated at 500 cells/well in 35-mm 6-well plates (Falcon Plastics, Oxnard, CA) in McCoy's Medium 5A containing 10% FBS. Various concentrations of HPLC-purified TIF were added to the cells at the time of plating. After 1 week, the medium was changed on control plates. The medium was removed from the TIF-treated plates and replaced by medium, with or without TIF, to determine the effects of the removal of TIF. After an additional incubation of 1 week, the medium was removed and the cells were fixed and stained with Giemsa stain. The surface area occupied by colonies was determined, using a Leitz TAS image analyzer. Twenty-one fields in each well were measured, and the average surface area covered/well was determined from these measurements. Colonies were photographed before staining. The effects of TIF on GBs cells was determined in a similar manner, except that 1000 cells/well were initially plated.

**EGF Competition Assay.** A membrane fraction from human placenta was obtained, as described by Hirata and Orth (33). Membranes were washed 3 times with 10 ml Tris, pH 7.4, and resuspended to a final membrane protein concentration of 200 μg/ml in 10 ml Tris, pH 7.4, containing 1 mg bovine serum albumin/ml. The incubation mixture (0.3 ml final volume) contained 20 μg membrane protein, 3 ng 125I-labeled mouse EGF/ml, and sample or standard mouse EGF. This reaction mixture was incubated for 1 h at 4°C and centrifuged to pellet membranes, and the tubes containing membrane pellets were counted in a Beckman γ-counter. An EGF concentration of 15 ng/ml gave 50% inhibition of binding in this assay.

**RESULTS**

**Comparison of Secreted and Intracellular TGF.** Conditioned medium from MOSER cells grown in spinner culture was collected and assayed for TIF and TGF activities. Both activities were present in the conditioned medium and showed EC₅₀ values of 15 μg/ml for TGF activity. One liter of conditioned medium contained 1300 units of TGF activity, compared to 800 units from the MOSER cell recovered (3 g, wet weight) from 1 liter of medium. TheBio-Gel P-10 profiles of TGF activity from the two sources were similar. Because of the difficulty in collecting and processing large volumes of conditioned medium, the MOSER cells were chosen as the starting material for the purification described below.

**Bio-Gel Chromatography.** The MOSER cell extract was fractionated on Bio-Gel P-10 (in 1 M acetic acid), and resulted in 2 major peaks of TGF activity, as measured by stimulation of NRK cell growth (Chart 1). These peaks have apparent molecular weights of 15,000 and 7,000, respectively. TIF activity occurred as a broad peak of activity in the region of M, 5,000 to 15,000. There was substantial overlap of the TGF and TIF activities.

Three pools were made (labeled Pools 2, 3, and 4), and dose-response curves were determined for both activities. Peaks 2 and 4 (the major TGF peaks) showed EC₅₀ values for TGF of 22 and 1.8 μg/ml, respectively, a 35-fold enrichment for Pool 4. The 3 pools had IC₅₀ values for TGF activity of 3.0, 0.45, and 1.1 μg/ml, which represents a 7-fold purification (for Pool 4).

**Separation of TGF and TIF Activities.** Pool 4 (which contains TIF and TGF activities) from the Bio-Gel P-10 column was fractionated by reverse-phase HPLC on a C₁₈Bondapak column and an acetonitrile gradient (Chart 2A). Although neither
peptide is homogeneous at this stage, this procedure separated
the TIF (eluting at 35% acetonitrile) from the TGF (26% acetoni-
trile). Acetonitrile concentrations were estimated from the gra-

![Chart 1. Chromatography of MOSER cell extract on Bio-Gel P-10. The crude
extract (150 mg protein in 5 ml) was applied to the column (2.5 x 72 cm), which
was equilibrated with 1 M acetic acid. Aliquots (0.25 ml for each assay) were
removed for assay of TIF (O) and TGF (●) activities, as described in "Materials and
Methods." Bars, fractions pooled for further analysis.]

diend originally applied to the column. The TGF pool did not show
TIF activity, and the TIF pool is free of TGF activity, using NRK
target cells (Chart 2B). The amount of protein present in the TIF
pool was estimated from the A<sub>220</sub> profile. Using this value, the
TIF pool had an IC<sub>50</sub> of 2.7 μg/ml, indicating that there was no
increase in specific activity of the TIF after HPLC. The A<sub>220</sub> profile
indicated the TIF was purified away from approximately 97% of
the applied A<sub>220</sub>-absorbing material, which would result in a
calculated 33-fold purification. This suggests that the A<sub>220</sub> read-
ing may be an overestimate of the protein present or that TIF
may have been purified away from a factor which synergistically
enhances TIF action in a manner similar to the requirement of
EGF or TGF-α for the action of TGF-β (19).

Stability of TIF and TGF. The sensitivities of the TIF and TGF
activities in crude extracts to trypsin, DTT, and heat were deter-
determined. Both activities were destroyed by trypsin and DTT treat-
ment and were stable to heating to 50 or 100°C. Thus, both
activities are due to acid- and heat-stable polypeptides requiring
disulfide bonds for activity and are similar to TGF and TIF as
described previously (10, 24).

![Chart 2. Reverse-phase HPLC of Bio-Gel
Pool 4 on a C<sub>18</sub>-Bondapak column. The pool
(1 mg) was applied to the column (3.9 mm x 30
cm) in 0.1% TFA. The gradient consisted of 20
to 40% acetonitrile, as indicated in A, at a flow
rate of 0.8 ml/min. Aliquots (0.1 ml for each
assay) of the 1.6-ml fractions were lyophilized
twice and assayed for TIF (O) and TGF (●) activities, as described in "Materials and
Methods." Fractions 16 to 21 were pooled for
TGF, and Fractions 35 to 37 were pooled for
TIF.]

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Effect of Purified TIF and TGF on Cell Growth. Both TIF and TGF activities were measurable in crude MOSER cell extracts, using the appropriate indicator cell. To determine if there was any interaction between the 2 activities, HPLC-purified TIF was mixed with HPLC-purified TGF and added to NRK cells in the soft agarose assay. No inhibition by MOSER TIF of TGF-stimulated colony growth of NRK cells was observed. Therefore, TIF from MOSER cells was not antagonistic to TGF activity, using untransformed fibroblast indicator cells.

Addition of purified exogenous TGF did not stimulate the anchorage-independent growth of MOSER cells (3,000 or 10,000 cells/well). The effect of HPLC-purified TGF (Fractions 16 to 21, Chart 2B) on the anchorage-independent growth of the slower-growing CBS colon carcinoma cell line (1) was investigated. CBS cells (10,000 cells/well) were plated in soft agarose in the presence or absence of MOSER TGF. In the absence of TGF, the CBS cells showed a plating efficiency of 2%, and the growth of this cell line was stimulated 1.5- and 1.6-fold by 1 and 2.5 µg MOSER cell TGF/ml, respectively. This indicated that TGF can stimulate the anchorage-independent growth of some human carcinoma cell lines, as well as normal fibroblasts.

The HPLC-purified TGF was tested for its ability to compete with mouse EGF in a radioreceptor assay using human placenta membranes. While TGF had an EC50 value (for induction of colony formation) of approximately 1 µg/ml, no EGF receptor-competing activity was detected at 6 µg/ml. Since TGF-α is defined by its ability to compete with EGF receptor (12-14), this suggested that the MOSER TGF described was not TGF-α. Additional information will be needed to determine whether this TGF is similar to TGF activities previously described.

Effect of Endogenous Factors on Anchorage-independent Growth. In previous work, we described the classification of human colon carcinoma cell lines on the basis of their biological (26), cell surface (36), and immunological properties (5). Group I lines were undifferentiated and highly aggressive, while Group III lines were differentiated and unaggressive. Group II lines, of which MOSER is an example, had intermediate properties. Acid extracts of MOSER cell-conditioned medium inhibited the growth of MOSER cells (Table 1). These extracts also inhibited the growth of the unaggressive, differentiated Group III colon carcinoma cell lines CBS and FET (Table 1; see Ref. 26). HCT 116 (27) and HCT C (29) are highly aggressive colon carcinoma cell lines, and their growth was not inhibited by the MOSER extract. The growth of the A431 epidermoid carcinoma cell line was also inhibited. HPLC-purified TIF inhibited the anchorage-independent growth of the MOSER and CBS cell lines, but not of the HCT 116 cells. Therefore, MOSER cell TIF inhibited the anchorage-independent growth of some, but not all, carcinoma cell lines tested.

Effect of TIF on Anchorage-dependent Growth. The biological activity of HPLC-purified TIF was further investigated, using MOSER cells grown in tissue culture plates under anchorage-dependent conditions. Cells were plated at low density (500 cells/well) to minimize the effects of endogenous factors. The surface area covered by MOSER cells grown under various conditions is shown in Table 2. Treatment of MOSER cells with 0.9 µg TIF/ml reduced the surface area covered by cells by 50%. This effect was partially reversible (Table 2, Line D).

Addition of TIF to MOSER cells caused significant alterations in colony morphology (Fig. 1). The cells in the control colonies (Fig. 1A) were rounded, with less distinct intracellular morphology. The TIF-treated colonies (Fig. 1, B and C) contained fewer cells, and the cells were flatter with a larger surface area. These morphological effects of TIF were reversible. Colonies from which TIF was removed for 1 week (Fig. 1D) contain more cells which are less distinct, when compared to the cells in colonies grown in the continuous presence of TIF (Fig. 1, B and C). Similar effects were observed when TIF was added to the CBS colon carcinoma cell line. When [3H]thymidine was added to the CBS cell line, there was a 50% decrease in [3H]thymidine incorporation in the presence of TIF. This effect was reversible upon removal of TIF. Therefore, TIF appears to act upon colon carcinoma cells grown in monolayer in a noncytotoxic, reversible manner, while at the same concentrations of the polypeptide, anchorage-independent growth is completely inhibited.

DISCUSSION

We have demonstrated that a cell line derived from a human colon carcinoma secretes 2 types of activities which can modulate cell growth. These 2 activities are acid- and heat-stable polypeptides which require disulfide bonds for activity. The TIF and TGF activities were copurified through Bio-Gel P-10 chromatography but were subsequently separated by reverse-phase HPLC.

MOSER cell TGF was similar to previously described TGFs (9, 32), and 2 size classes were identified by Bio-Gel chromatography.
These include the lack of TIF receptors on these cells, or a high TIF isolated by Todaro et al. (25) are the same molecule.

The second activity (TIF) was inhibitory to some but not all human carcinoma cell lines tested. While not homogeneous, this preparation represents a purification over the crude extract, and was not contaminated with TGF activity. We have also partially purified a TIF activity from rat ascites fluid (24), which is very similar to the TIF reported in this study. Both activities show the same selectivity for the types of tumor cells inhibited, the same pattern of chemical stability, and elute in the same position on reverse-phase HPLC. Therefore, with respect to these properties, there is apparently little interspecies difference associated with rat and human TIF.

TIF acts on colon carcinoma cells in a partially reversible, noncytotoxic manner. The effects of TIF were similar to those of planar polar compounds such as N,N-dimethylformamide, diethyl sulfoxide, and butyrate on human colon carcinoma cells (37, 38). These planar polar compounds inhibited anchorage-independent growth, caused reversible morphological changes in the cells, and were not cytotoxic against cells grown on plastic (37, 38).

Todaro et al. (25) have reported a TIF activity with chemical properties similar to that of the MOSER cell TIF described in this report. There appear to be significant functional differences between the 2 TIFs. The TIF described by Todaro et al. (25) inhibited the TGFD-dependent growth of NRK fibroblasts in soft agarose. MOSER TIF did not inhibit the TGF (MOSER)-dependent stimulation of NRK fibroblast growth in soft agarose. This may be due to a lack of receptors for MOSER TIF on NRK cells. A direct comparison in the same assay system would be necessary to determine conclusively whether MOSER TIF and the TIF isolated by Todaro et al. (25) are the same molecule.

The TIF identified from MOSER cells showed specificity with respect to the types of human malignant cell lines which it was capable of inhibiting. We have described a large bank of cultured human colon carcinoma cell lines (6, 26, 36) which exhibit a wide degree of biological properties and which could be classified into 3 arbitrary groups according to tumorigenicity in athymic mice, ability to grow with anchorage independence, and differentiation (25). Group I lines were highly aggressive, Group III lines were the least aggressive, and Group II lines were intermediate. The classification of these groups, which was originally based on biological properties, has been supported by cell surface mapping of proteins and glycoproteins (36), and by immunological analyses of cytoplasmic antigens (6, 39). MOSER cells were classified as intermediately aggressive (Group II) in this system. The 2 cell lines which did not respond to MOSER TIF were highly aggressive lines from Group I, while those which were inhibited were the relatively indolent Group III lines. The TIF was also quite effective against anchorage-independent growth of MOSER cells. There are a number of possibilities which could explain the lack of response to MOSER TIF by HCT 116 and HCT C cells. These include the lack of TIF receptors on these cells, or a high net production of factors stimulating their anchorage-independent growth. The fact that extracts from MOSER human colon carcinoma cells contain both TGF and TIF activities suggests that control of cell growth may involve a balance between these positive and negative factors.

## References


COLON CARCINOMA CELL GROWTH FACTORS


Fig. 1. Effect of TIF on MOSER cell growth. MOSER cells (500 cells/well) were plated in 9.6-sq cm tissue culture plates in medium in the absence or presence of HPLC-purified TIF. Photographs were taken on an inverted microscope 14 days after plating. A, control colonies (no TIF), 14 days after plating; B, cells treated with 0.9 µg TIF/ml for 14 days; C, cells treated with 0.9 µg TIF/ml for 14 days; D, cells treated with 0.9 µg TIF/ml for 7 days, followed by replacement of medium with medium without TIF for an additional 7 days [reversal]. × 100.
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