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IDENTIFICATION OF A TUMOR INHIBITORY FACTOR IN RAT ASCITES FLUID

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A polypeptide which inhibits the growth of human carcinoma cells has been characterized from Novikoff rat ascites fluid. This tumor inhibitory factor co-purified with transforming growth factor activity through acid/ethanol extraction and Bio-Gel chromatography. The two activities were completely separated by reverse phase HPLC. The tumor inhibitory factor is heat stable and requires disulfide bonds for bioactivity. This factor inhibited the anchorage independent growth of the more differentiated human colon carcinoma cell lines but did not affect the less differentiated carcinoma cells. The presence of stimulatory and inhibitory activities in the same extracts suggests that the relative concentrations of these factors may be important in the control of cell growth.

Cell growth is a complex process requiring multiple factors and control points. A number of polypeptide factors have been identified from normal and neoplastic tissues and cells which affect growth in tissue culture. These include nerve, epidermal (EGF), platelet-derived, and transforming (TGF) growth factors (1-5). These factors have been chemically characterized and studies have been made on their interaction with the cell membrane and subsequent intracellular events. Less is known about endogenous growth inhibitors. Growth-inhibiting proteins from 3T3 cell plasma membranes (6,7), normal rat liver (8) and several cultured cell lines (9-11) have been described which inhibit normal but not transformed cells. We have partially purified and characterized a heat and acid stable peptide from Novikoff rat ascites fluid which will inhibit the anchorage independent growth of human carcinoma cell lines. This peptide, designated a tumor

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inhibitory factor (TIF)[1], may be important in the control of neoplastic cell growth.

MATERIALS AND METHODS

Preparation of Crude TIF

TIF was extracted from rat ascites fluid by the acid/ethanol extraction procedure of Roberts et al. (12). Ascites fluid was collected from rats 7 days after injection with Novikoff hepatoma cells and centrifuged to remove cells. Ethanol (2 vol.) and concentrated HCl (0.04 vol.) were added and the extract stirred overnight at 4°. After centrifugation at 10,000 x g for 15 minutes, the pellets were re-extracted for 4 hours in 0.5 vol of acid/ethanol/H_2O (0.04:2:1). Following a second centrifugation the two supernatants were pooled, the pH adjusted to 5.3 with concentrated ammonium hydroxide and ammonium acetate (1ml of 2M, pH 5.3 to each 80ml extract) added. Following centrifugation, absolute ethanol (2 vol.), and anhydrous diethyl ether (4 vol.) were added to the extract and the protein was precipitated at -20° for 48 hours. The precipitate was collected by centrifugation at 15,000 x g for 15 minutes, dried in vacuo, resuspended in 1M acetic acid, dialyzed (Spectropor 3, 3,500 MW cutoff) extensively versus 1% acetic acid and lyophilized.

Bio-Gel P-30 Chromatography

Crude TIF was fractionated on a 5 x 90cm column of Bio-Gel P-30 (Bio-Rad) in 1M acetic acid at a flow rate of 45ml/hr. Approximately 750mg of crude TIF in 1M acetic acid was applied in 50ml and 45ml fractions were collected. Because of the large amount of protein (4g) in the crude extract the sample was divided into 5 aliquots and run successively over the column. Aliquots were removed for determination of soft-agar growth inhibitory (TIF) activity using the previously described MOSER human colon carcinoma cell line (13). The area corresponding to TIF activity from all five runs was pooled, concentrated by lyophilization, and the activity was determined.

Reverse Phase HPLC

The pool containing TIF activity was lyophilized and dissolved in 1% TFA, (60 µl/5mg). This was diluted to 600 µl with 0.1% TFA, centrifuged to remove insoluble material, and 500 µl was injected onto a C_{18} µBondapak column (Waters) using a Varian Model 5000 high-performance liquid chromatography system. The sample was chromatographed using an acetonitrile gradient in 0.1% TFA as described by Anzano et al. (14). The effluent was monitored at 220nm with a Varian UV-50 variable wavelength detector. All reagents were HPLC grade (Baker).

Soft Agarose Assay

Underlayers of 0.4ml of 0.8% Agarose (Sea Plaque; FMC Corporation, Marine Colloids Div., Rockland, ME) growth medium were plated in 9mm culture plates and allowed to solidify at room temperature. Each pair was overlaid with a single cell suspension (0.4ml) of

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[1] Abbreviations used are: DTT, dithiothreitol; EGF, epidermal growth factor; PBS, phosphate buffered saline (0.01M sodium phosphate, 0.15M NaCl, pH 7.2); TFA, trifluoroacetic acid; TGF, transforming growth factor; TIF, tumor inhibitory factor.

2.0×10^3 NRK cells or 1×10^4 MOSER cells in 0.4% agarose in McCoy's 5A medium supplemented with 10% FBS containing lyophilized aliquots of crude extracts, column pools, or column fractions. The plates were then incubated at 37°, 5% CO_2 in a humidified incubator and examined for growth on days 14-21. Colonies (10 or more cells) were scored on an inverted microscope. Human colon tumor cells CBS, HCT 116, and HCT C (13,15,16) were plated at 50,000, 5,000 and 10,000 cells per ml., respectively and used in assays as described above.

Stability of TIF

The crude TIF extract was lyophilized to dryness and dissolved in PBS to a final concentration of 1mg/ml. Aliquots were treated with trypsin (Worthington, Type 3: 50 µg/ml, 2 hr, 37°), DTT (0.065M, 1 hr, 25°), or heat (56°, 30 min, or 100°, 3 min). The tryptic digest was stopped with soybean inhibitor (Sigma, 100 µg/ml) and the inhibitor was added before trypsin for the control experiment. After treatment, each sample was adjusted to 4ml with PBS, dialyzed extensively vs. 1% acetic acid, lyophilized and assayed for activity in the soft agarose assay using MOSER cells.

RESULTS AND DISCUSSION

Purification of TIF

Ascites fluid from Novikoff hepatoma bearing rats was extracted with acid/ethanol (12) and the resulting extract contained TGF activity (stimulation of NRK cells) and TIF activity (inhibition of MOSER anchorage independent growth). This extract was fractionated on Bio-Gel P-30 in 1M acetic acid and the TIF activity was separated from most of the protein (Fig. 1). The TIF

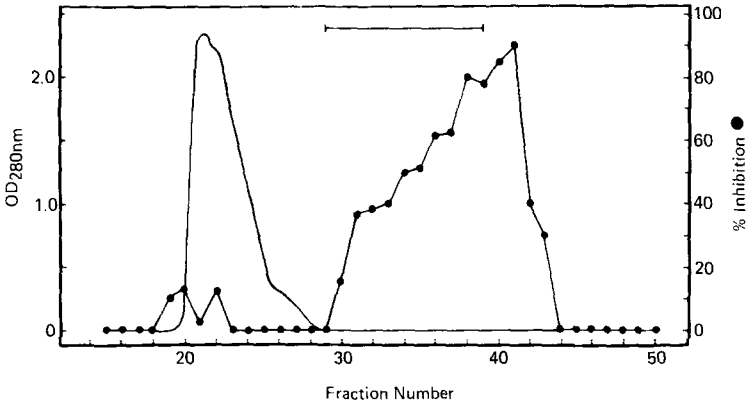

Fig. 1. Chromatography of rat ascites on Bio-Gel P-30. The acid/ethanol extract (750mg protein in 50ml) was applied to the column (5 x 90cm) equilibrated with 1M acetic acid. Aliquots (500 µl) were removed for assay of TIF activity. The bar indicates the fractions pooled from 5 identical runs for further purification.

TABLE I

EFFECT ON TIF ON ANCHORAGE INDEPENDENT GROWTH

	INDICATOR CELL LINE (COLONIES/PLATE)				
	NRK	MOSER	CBS	HCT116	HCT C
NO ADDITION	0	480	700	568	1205
CRUDE EXTRACT	82	0	89	525	1035
BIO-GEL	336	72	ND	ND	ND
TIF (HPLC)	0	20	ND	ND	ND

Samples were tested for their effect on growth of different cell types in soft agarose as described in Materials and Methods. The crude acid/ethanol extract, Bio-Gel P-30 pool, and TIF peak from HPLC were tested at 100, 10 and 10 μg/ml, respectively. ND, not determined.

activity peak (apparent molecular weight 7-10kd) was pooled and contained both TIF and TGF activity (Table I). This pool had an EC_{50} value for TIF of 0.4 μg/ml compared to 12 μg/ml for the crude extract. The TGF activity showed a similar degree of purification compared to the crude extract.

The pool from the Bio-Gel column was fractionated by reverse phase HPLC using a C_{18} μBondapak column and an acetonitrile gradient in TFA (Fig. 2A). TIF eluted at 36% acetonitrile while TGF eluted at 33% acetonitrile (Fig. 2B). This partially purified TIF represented a 2500-fold purification over the crude extract with an overall yield of 0.3%. The TIF peak did not stimulate growth of NRK cells (Table I) indicating a complete separation of TIF from TGF. These two activities can now be investigated separately. The low yield of TIF could be due to the loss of TIF activity or the separation of TIF from other factors necessary for the inhibition of MOSER cell growth.

Stability of TIF

The TIF in rat ascites crude extracts was stable to treatment at 56° and 100°. The inhibitory activity was lost upon

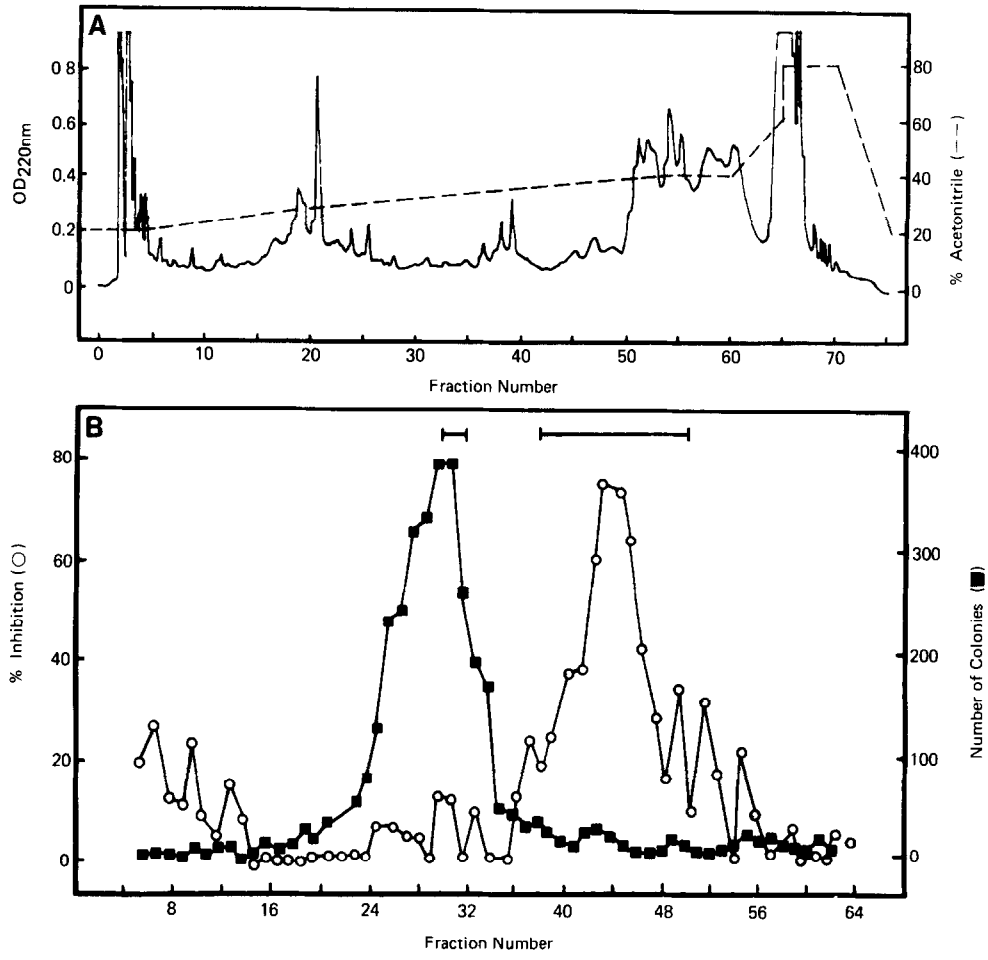

Fig. 2. Reverse phase HPLC of TIF on a Bondapak C_{18} column. One third of the Bio-Gel P-30 pool (5mg) was applied to the column (7.8mm x 30cm) in 500 µl 0.1% TFA. The gradient consisted of 20 to 40% acetonitrile as indicated at a flow rate of 0.8/min. Aliquots (100 µl) of the 1.6ml fractions were lyophilized twice and used in soft agar assays for determination of TIF and TGF activity. A. Optical density pattern at 220nm. B. Soft agar profile showing TIF (o) and TGF (■) activities. Fractions 38-50 were pooled and used for further studies of TIF.

treatment with trypsin or DTT. Rat ascites TIF is a heat stable polypeptide which requires disulfide bonds for biological activity.

Specificity of Inhibition

Partially purified TIF, as well as the crude extract, was tested for its ability to inhibit the growth of 4 human colon carcinoma cell lines in soft agarose culture (Table I). Two of the lines (HCT C and HCT 116) were judged to be undifferentiated on the basis of in vitro biological properties (13,15) which were

correlated with changes in both cell surface (17) and cytoplasmic (18) protein differentiation markers. The other two lines (MOSER and CBS) were found to be more differentiated (13) in the previously described studies. The growth of the more differentiated human colon carcinoma cell lines (MOSER and CBS) was inhibited by the crude ascites extract as well as partially purified TIF. The less differentiated colon carcinoma cell lines (HCT 116 and HCT C) were not inhibited by TIF. This specificity for cell type may reflect differences in the responsiveness of the cells to TIF or other growth factors. Normal or more differentiated tumor cells may be better able to respond to TIF.

A preliminary report of a TIF in human carcinoma cell conditioned medium has been presented (19) but the data reported was not sufficient for a comparison with the rat ascites TIF described in our study. The measurement of both stimulatory (TGF) and inhibitory (TIF) activities in extracts derived from the same cells suggests that the balance between these two peptide factors may be important in the control of cell growth. This control could be modulated by regulation of the synthesis of these factors or by specific cellular responses to TGF and TIF. The purified TIF described in this study will be useful in the examination of the role of growth inhibitors in the control of neoplastic cell growth.

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REFERENCES

1. Greene, L.A., and Shooter, E.M. (1980) Ann. Rev. Neurosci. 3, 353-402.
2. Carpenter, G., and Cohen, S. (1979) Ann. Rev. Biochem. 48, 143-216.
3. Ross, R., and Vogel, A. (1978) Cell 14, 203-210.

4. Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., Frolik, C.A., Marquardt, H., Todaro, G.J., and Sporn, M.B. (1982) Nature (Lond.) 259, 417-419.
5. Frolik, C.A., Dart, L.L., Myers, C.A., Smith, D.M. and Sporn, M.B. (1983) Proc. Nat'l. Acad. Sci. 80, 3676-3680.
6. Whittenberger, B., and Glaser, L. (1977) Proc. Nat'l. Acad. Sci. 74, 2251-2255.
7. Peterson, S.W., Lerch, V., Moynahan, M.E., Carson, M.P., and Vale, R. (1982) Exp. Cell Res. 142, 447-451.
8. McMahon, J.B., Farrelly, J.G., and Iype, P.T. (1982) Proc. Nat'l. Acad. Sci. 79, 456-460.
9. Lipkin, G. and Knecht, M.E. (1974) Proc. Nat'l. Acad. Sci. 71, 849-853.
10. Holley, R.W., Bohlen, P., Fava, R., Baldwin, J.H., Kleeman, G. and Armour, R. (1980) Proc. Nat'l. Acad. Sci. 77, 5589-5592.
11. Nilsen-Hamilton, M. and Holley, R.W. (1983) Proc. Nat'l. Acad. Sci. 80, 5636-5640.
12. Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., DeLarco, J.E. and Todaro, G.J. (1980) Proc. Nat'l. Acad. Sci. 77, 3493-3498.
13. Brattain, M.G., Brattain, D.E., Fine, W.D., Khaled, F.M., Marks, M.E., Arcolano, L.A. and Danbury, B.H. (1981) Oncodev. Biol. and Med. 2, 355-366.
14. Anzano, M.A., Roberts, A.B., Smith, J.M., Lamb, L.C. and Sporn, M.B. (1982) Anal. Biochem. 125, 217-224.
15. Brattain, M.G., Fine, W.D., Khaled, F.M., and Brattain, D.E. (1981) Cancer Res. 41, 1751-1756.
16. Brattain, M.G., Marks, M.E., McCombs, J., Finely, W. and Brattain, D.E. (1983) Br. J. Cancer 47, 373-381.
17. Marks, M.E., Danbury, B.H., Miller, C.A., and Brattain, M.G. (1983) J.N.C.I. 4, 663-671.
18. Taylor, C.W., Brattain, M.G., and Yeoman, L.C. Cancer Res. in press.
19. Iwata, K.K., Fryling, C.M., Knott, W.B., and Todaro, G.J. (1983) Fed. Proc. 42, 1833.