Vol. 152, No. 1, 1988
April 15, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 463-468

PARTIAL PURIFICATION OF A PROTEIN GROWTH INHIBITOR FROM MULTICELLULAR SPHEROIDS

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Received March 7, 1988

We report the partial purification of growth inhibitors extracted from human and mouse multicellular tumor spheroids with extensive necrosis. Sephadex G-100 column chromatography of spheroid extracts separated inhibitory fractions which eluted just after the void volume of the column. Identical chromatography of monolayer cell extracts showed no inhibitory activity. High-performance liquid chromatography of spheroid extracts separated single active peaks at apparent molecular weights of 80-89 kD. These extracts were extremely heat labile, and activity was destroyed by moderate trypsin treatment. The isolation of similar growth inhibitors from spheroids of two cell lines suggests that inhibition is important in tumor cell growth control in a three-dimensional situation.

Understanding the regulation of tumor cell proliferation in vivo will require knowledge of the interplay between stimulatory and inhibitory molecules. While considerable detail is known about positive growth factors, relatively little information is available concerning the critical inhibitory side of tumor cell growth regulation. Growth inhibitory factors have been isolated from ascites fluid (1), normal serum (2,3), normal (4,5,6,7) and malignant (8,9,10,11,12) cell lines. In general, these factors are moderately-sized polypeptides which have an effect on many types of tumor cells. Some tumor cell inhibitory factors stimulate normal cell growth, while others inhibit normal cells also. The mechanism of action of inhibitory growth factors is unknown. Crude extracts from solid tumors have also shown some toxic properties (13,14), although further identification of the responsible factor(s) has not been reported. We have recently demonstrated both cytostatic and cytotoxic activities in crude extracts from multicellular spheroids with extensive central necrosis (15); both normal diploid fibroblasts and several malignant cell types were inhibited. This growth inhibitory activity was unique in that it appeared only in spheroids with central necrosis: there was no activity in extracts from monolayer cells or from small spheroids without necrotic centers. Growth

Abbreviations used are: PBS: phosphate-buffered saline (0.14 M NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.2); HPLC: high performance liquid chromatography.

0006-291X/88 $1.50

inhibition by factors from the spheroid necrotic center can explain spheroid growth saturation (16) and may provide a crucial link between the regulation of proliferation and viability in multicellular systems. We now report the partial purification and characterization of growth inhibitory molecules from spheroids of two cell lines.

MATERIALS AND METHODS

Monolayer and Spheroid Culturing

Spheroids and monolayers were cultured from the EMT6/Ro mouse mammary carcinoma and HT1080 human adenocarcinoma cell lines as described previously (16). After selection of a uniformly-sized group of 300 spheroids at 400 μm diameter, the spheroids were cultured to a size of 1500 μm diameter in 500 ml culture flasks with daily media replenishment. The spheroid diameters increased linearly with time throughout this period at mean rates of 90 (EMT6/Ro) and 75 (HT1080) μm per day, requiring culture periods of 12 and 15 days after sorting, respectively. The 9L rat gliosarcoma cell line was selected as the growth inhibition test cell based on its high degree of sensitivity to a crude extract from spheroids (15). Monolayer cultures of 9L cells were handled as described previously (16).

Extract Preparation

Extracts from spheroids were prepared by washing a group of 300 spheroids with PBS, then suspending the intact spheroids in 5 mls of double-distilled, deionized water. This spheroid suspension was lyophilized to complete dryness, ground to a fine powder, and stored at -20° until use. Extracts from monolayers were prepared by washing several 100-mm dishes containing a confluent monolayer of cells with PBS, adding 5 mls of double-distilled, deionized water per dish, and removing the cells with a rubber policeman. These cell suspensions were then combined, lyophilized, ground, and stored as described above. The lyophilized powders were resuspended in PBS, mixed gently for 2 hours at 2°, centrifuged at 2500 rpm for 15 minutes, and the supernatant was filtered once through a 0.22 μm nylon membrane filter (Corning).

Growth Inhibition Assay

Monolayers of 9L cells were established in 60-mm dishes at 2.5×10^4 cells per dish 5-6 hours prior to adding extracts. Extracts and fractions from column separation were added directly to these dishes at a 1:10 dilution of extract:medium. A set of control dishes was harvested and counted at the time of extract addition. The remaining dishes were incubated for 60-70 hours, then the cells were removed with trypsin and the total numbers of cells per dish were counted using a Coulter counter as described in detail elsewhere (16). The counts from three separate dishes were averaged for each extract or column fraction assay. The percent growth inhibition (I) was expressed using the formula:

$$I = 100 \times \{1-[(T-C1)/(C2-C1)]\}$$

where C1 is the mean cell count at the time of adding the extracts, C2 is the mean cell count of a control set of dishes with no extract added, and T is the mean cell count for the test sample.

Sephadex Column Separation

A 50 x 1.25 cm column was prepared using Sephadex G-100 (Pharmacia) and washed extensively with degassed PBS. Extracts were prepared as described above at a concentration of 25 mg/ml of lyophilized powder. A 1 ml sample of this extract was then loaded onto the column and 2.5 ml fractions were collected at the rate of 1 ml/minute. The absorbency of the effluent was monitored at 280 nm during collection. Aliquots of 500 μl from each fraction were added to each of three 60-mm culture dishes containing cell monolayers, and these were assayed for growth inhibition as described above. As controls, 500 μl aliquots of a 0.25 dilution of the loaded extract were added to each of three culture dishes, as were 500 μl aliquots of PBS.

High-Performance Liquid Chromatography

Extracts were prepared as described above at a concentration of 100 mg/ml of lyophilized powder. After filtering, these extracts were centrifuged again at 1000 x g for 10 minutes. A 100 μl aliquot of the supernatant was then injected onto a 300 x 7.5 mm gel filtration column (BioSil TSK250, Bio-Rad) on a HPLC instrument (Waters Model 510). A buffer of degassed PBS was pumped through the column at 0.5 ml/minute, and fractions were collected every minute. The absorbency of the effluent was monitored at 280 nm during collection. Aliquots of 150 μl from each fraction were added directly to each of three 35 mm dishes containing cell monolayers, and these were assayed for growth inhibition as described above. Controls were done as described for the Sephadex column separation using the appropriate aliquots. To calibrate the column, a commercial mixture of five molecular weight standards from 670 to 1.35 kD (BioRad) was run under exactly the same conditions.

Physical/Chemical Treatments

Heat-lability of the extracts was tested by exposing unseparated extracts to 95° and 60° water baths for various lengths of time. After exposure, the extracts were placed immediately into a 0.5° water bath, then added directly to test culture dishes as described above. Trypsin sensitivity was tested by exposing unseparated extracts to 100 μg/ml of trypsin (Worthington, Type III, 11,800 U/mg) for 4 hours at 22°, incubating with 200 μg/ml soybean trypsin inhibitor (Worthington) for 30 minutes, then adding directly to test culture dishes. A control extract sample was exposed to a mixture of trypsin and trypsin inhibitor which had been preincubated together for 30 minutes; another sample of extract was incubated at the same temperature for the same total time without the addition of any trypsin or trypsin inhibitor.

RESULTS

Purification

The first purification procedure was gel filtration of crude extracts prepared in PBS on a Sephadex G-100 column as described above. Figure 1 shows the results for extracts from spheroids and monolayers of EMT6/Ro cells, while Figure 2 shows the results of a similar experiment with extracts from the HT1080

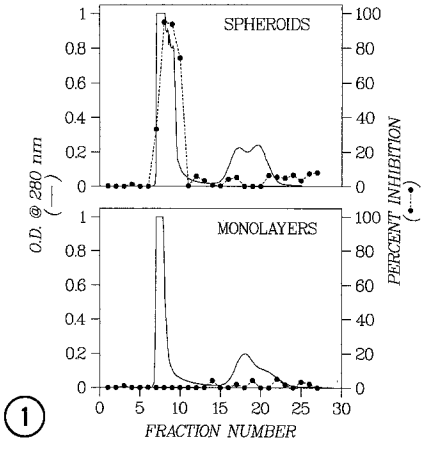

Figure 1. Sephadex G-50 column separation of extracts from EMT6/Ro spheroids (top) and monolayers (bottom). Solid line shows absorbancy at 280 nm; this is truncated above 1.0 OD units due to saturation of the detector. Circles and dotted lines show percentage inhibition of 9L cell growth.

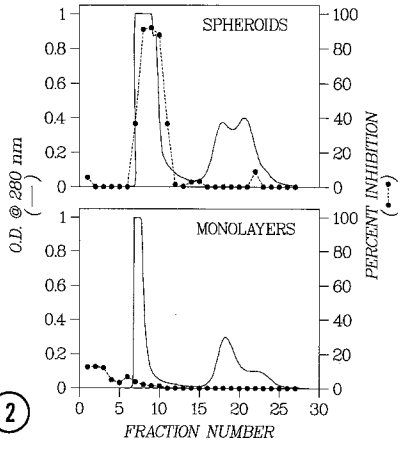

Figure 2. Sephadex G-50 column separation of HT1080 extracts, presented as described in Figure 1.

cell line. There was no significant growth inhibition from any of the eluted fractions in the extracts from monolayers. In each case, the extracts from spheroids showed marked inhibition in a single peak comprising 4-5 2.5 ml fractions eluting immediately after the void volume of the column. Comparison of the absorbencies between extracts from spheroids and monolayers demonstrated some differences. The peak representing excluded material was significantly broadened, and the amount of low-molecular-weight components was increased. Although these experiments demonstrated that the inhibitory activity was found only in the spheroid extracts, and was in the high molecular weight range, the fact that the active peaks eluted close to the void volume of the columns did not allow for an accurate estimation of size.

To get a better determination of the inhibitor size, extracts were prepared and loaded onto a BioSil TSK250 column (with a higher molecular weight cutoff than that of Sephadex G-100 as described above. Figure 3 shows the absorbency and growth inhibition patterns obtained with extracts from EMT6/Ro and HT1080 spheroids. In this case, the inhibitory activity eluted in a single peak of 3-4 0.5 ml fractions. The mean fractions for the inhibitory activity were 15.4 and 15.7 for the EMT6/Ro and HT1080 spheroid extracts, respectively. Molecular weight standards were run through the gel filtration column under precisely the same conditions, and a calibration curve calculated from these standards gave mean molecular weights for the inhibitory molecules of 80 and 89 kD for these two cell lines.

Stability

The heat lability of the EMT6/Ro spheroid growth inhibitor was tested by heating crude extracts to 60° and 90° for various time periods and then assaying for inhibitory activity as described above. Heating at 90° destroyed all activity within 1 minute. Heating at 60° gave a gradual decay in activity which was exponential in nature. A linear least squares best fit of the log of the remaining activity versus time gave a half-time for thermal decay of 4.7 minutes

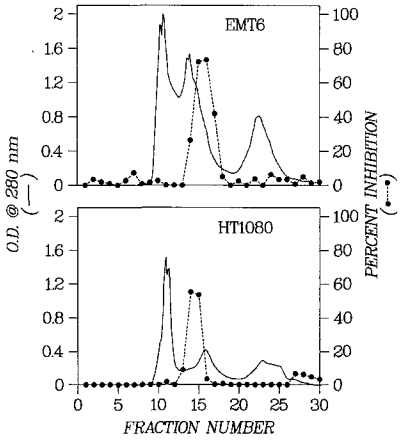

Figure 3. High-performance liquid chromatography separation of extracts from EMT6/Ro (top) and HT1080 (bottom) spheroids using a gel filtration column; data is presented as described in Figure 1.

(r^2=0.95). The HT1080 spheroid extract had a half-time for thermal decay of 4.3 minutes at 60° (r^2=0.97).

Both the EMT6/Ro and HT1080 crude spheroid extracts were exposed to trypsin as described above. After incubation of the crude extracts at 22° for 4 hours without trypsin, cell growth was inhibited 66 and 61 percent by the EMT6/Ro and HT1080 extracts, respectively. A 4 hour trypsin treatment at 22° reduced inhibitory activity to 1.9 and 1.7 percent. There was no significant loss in inhibitory activity when the extracts were incubated with a combination of trypsin and trypsin inhibitor. Exposure of monolayer cultures to trypsin and trypsin inhibitor alone had no significant effect on cell growth.

DISCUSSION

The partial purification data shown here provide several clues to the identity of these novel growth inhibitors from spheroids. The HPLC separation, heat inactivation, and trypsin sensitivity data strongly suggest that these inhibitors are large polypeptides or glycopolypeptides. An important result of these experiments was that inhibitors from these two different cell lines were virtually identical in molecular weight, heat lability and trypsin sensitivity. This suggests that these inhibitors are very similar, if not identical. We have preliminary data showing that crude extracts from large, necrotic tumors of each of these cell lines also show significant growth inhibitory activity; no purification of these tumor extracts has been done to date. The growth inhibitors described here appear to differ from tumor cell growth inhibitors previously reported (7-12): the mean molecular weights of 80-90 kD are larger and the extreme heat lability is distinctive. We are continuing further purification of these inhibitory compounds.

Another interesting result shown here is that there was no inhibitory activity in any of the fractions eluted with extracts from monolayer cells, as was shown previously with crude extracts (15). This is in contrast to other reports (5-12) of tumor cell growth inhibitors, which have generally been isolated from monolayer cultures. We have proposed (16) that growth inhibitors in spheroids are derived from the necrotic region. One possible mechanism for this would be the synthesis of a new polypeptide by cells which are deprived of nutrients and which subsequently undergo necrotic death, similar to the synthesis of glucose-regulated proteins in glucose-deprived cells (17). Precendent for a second possible mechanism is found in a recent report of a growth inhibitor produced by quiescent cells (18); it may be that the large number of quiescent cells found in spheroids of this size (19) produce growth inhibitory proteins. A third possibility is that new synthesis is not involved; rather, spheroid growth inhibitors may be produced by partial proteolysis of a non-inhibitory polypeptide into an active form. Inhibitor activation by protein digestion has been reported in other systems (2,20). The absorbency data in Figures 1 and 2 suggest that macromolecular breakdown occurs in spheroids: the

spheroid extracts showed a broader initial peak and more material in the low molecular weight region. The composition of the spheroid necrotic core is unknown, but it is reasonable to assume that it would contain proteolytic and lysosomal enzymes released from cells after lysis. Work is also underway in our laboratory to firmly establish the relationship between these growth inhibitors and necrosis, and to determine if these inhibitors are newly-synthesized proteins.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistance of Ms. Susan Carpenter and Ms. Kathy Thompson, and the support of Dr. Mudundi Raju. We thank Dr. Lawrence Gurley for the use of the HPLC instrumentation and Ms. Susan Rokop for the use of the lyophilizer. This work was supported by NIH grants CA-36535, CA-22585 and RR-02845, and by the Department of Energy.

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