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Isolation of a Low-Molecular-Weight Growth Inhibitory Factor from Hybridoma Cell Cultures

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A low-molecular-weight growth inhibitory factor was produced by hybridoma cells. The number of viable cells in hybridoma cell cultures reached a maximum of about 5×10^5 cells/ml when the inhibitory factor had accumulated to a critical level, after which the number of viable cells declined with a concomitant increase in the number of dead cells. The growth inhibitory factor was purified to apparent homogeneity by ultrafiltration, reverse-phase chromatography, passage through cation exchangers, and gel filtration. Analysis by reverse-phase chromatography and micellar electrokinetic chromatography using a capillary electrophoresis system indicated that the final inhibitory fraction was pure. The factor had a molecular weight of 500 or less, as judged by ultrafiltration, and its behavior upon ion-exchange chromatography indicated that it was uncharged. Its absorbance maximum at 263 nm indicated that it was not a peptide, but that it may have a conjugated system of carbon–carbon double bonds. © 1999 Academic Press

Myeloma and hybridoma cells secrete a growth inhibitory toxic factor (1). The inhibitory factor is not produced by other cell types (human lymphoma and mouse embryo fibroblasts) that have been studied (1). The factor prevents growth of hybridoma cells to high cell densities and thus limits the amount of monoclonal antibodies that may be obtained from batch cultures of hybridoma cells (1).

The molecular weight of the inhibitory factor has been estimated by gel filtration to be not more than 5,000, and it is not a common waste product such as lactate or ammonia (1). Otherwise, little is known about the factor. Isolation and a more thorough characterization of the factor is clearly necessary in order to identify it more closely and determine its biological role. Its identification is of interest, since the factor might function as a growth regulatory metabolite and it has a potential as a cytostatic drug. Moreover, its identification may enable development of methods for its removal from hybridoma cell cultures, thus enabling growth of hybridoma cells to higher cell densities with increased production of monoclonal antibodies. In this study we report the purification to apparent homogeneity and partial characterization of the inhibitory factor.

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MATERIALS AND METHODS

Cells, Culture Conditions, and Production of the Inhibitory Factor

The hybridoma cells used in this study (cell line 6D11) produce monoclonal antibodies to platelet-derived growth factor and they were initially formed by fusing mouse spleen-derived lymphocytes with mouse myeloma Sp2/0 cells (2). The cells were maintained at 37°C as stationary cultures in 75-cm^2 tissue culture flasks (Costar) with Dulbecco's modified Eagles medium containing 4.5 g/ml glucose (Bio-Whittaker), supplemented with 25 mM Hepes buffer (Gibco/BRL), 2 mM L-glutamine (Bio-Whittaker), 0.1 mg/ml kanamycin (Gibco/BRL) and 10% (v/v) heat inactivated fetal calf serum (Gibco/BRL). The cells were diluted in fresh medium to a density of about 5×10^3 cells per ml once or twice every week, after they had reached a density of about 5×10^5 cells per milliliter. The cell number and viability were determined by dye exclusion with 0.2% (v/v) trypan blue using a hemocytometer chamber. To produce supernatants containing the inhibitor, hybridoma 6D11 cells were cultured for an additional two days after having reached a maximum cell density of $0.5–1.0 \times 10^6$ cells per milliliter, and the cells were then removed by centrifugation at 1000*g* for 10 min. The cell-free supernatant containing the inhibitory factor was stored at −20°C until use.

Assay for Inhibitory Activity

The hybridoma 6D11 cells were used as target cells. Varying amounts (1–50 μl) of fractions to be assayed for inhibitory activity were added to 6-mm tissue culture wells (type 3595; Costar) containing 2×10^3 hybridoma cells in a final volume of 200 μl Dulbecco's modified Eagles medium containing 4.5 g/ml glucose, supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, 0.1 mg/ml kanamycin and 10% (v/v) heat inactivated fetal calf serum. After incubation at 37°C for 48 h, 1 μCi of [methyl-^{3}H]thymidine (specific activity: 5 Ci/mmol; New England Nuclear) was added, and the cultures were harvested with a Skatron cell harvester after 4 h of [methyl-^{3}H]thymidine incorporation (3). The results were calculated as percent inhibition of target cell [methyl-^{3}H]thymidine incorporation (%TI), as follows:

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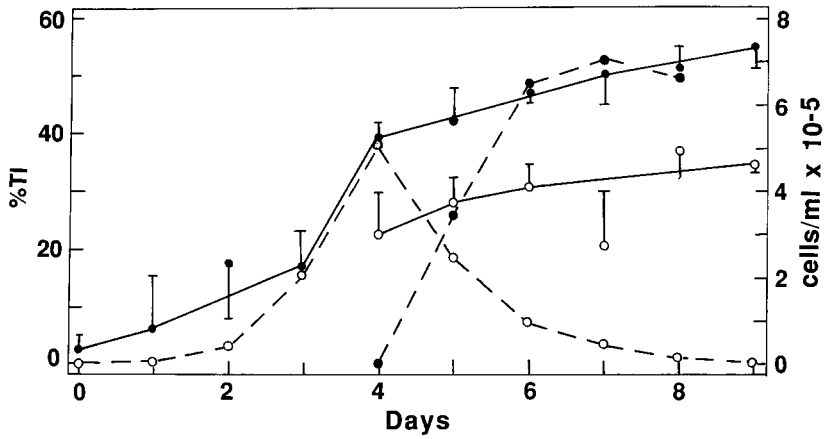

FIG. 1. Amount of inhibitory activity, number of viable cells and number of dead cells all as a function of the time after passage of hybridoma cells to fresh medium. After having reached a cell density of about 5×10^5, hybridoma 6D11 cells were on day 0 diluted in fresh medium to a density of about 5×10^3 cells per milliliter and cultured for 9 days. Culture samples were taken at the indicated days after passage and the inhibitory activity was determined, adding either 50 (●) or 13 (○) μl to the 6-mm tissue culture assay wells. The number of viable (–○–) and dead (–●–) cells was also determined at each time point.

$$\%TI = 100 - \frac{\text{cpm in cells exposed to fractions assayed for inhibitor}}{\text{cpm in control cultures without inhibitor}} \times 100.$$

The fractions were assayed in triplicates or quadruplicates, and the standard deviations obtained for %TI were on average 5–6.

Purification of the Inhibitory Factor

The purification of the inhibitor was carried out on a "micro-scale" basis using the SMART chromatography system (Pharmacia Biotech) for micropreparative purification, and protein concentrations were determined by the method of Bradford (4), using the Bio-Rad Laboratories Protein Assay Kit and lyophilized bovine gamma globulin as the standard protein.

Step I: Filtration of cell-free supernatant. Before column chromatography using the SMART-System, cell-free-supernatants containing the inhibitor were filtered using Centricon centrifugal concentrators (Amicon) with a molecular weight cut-off of 3000. The inhibitory factor passed through the filter.

Step II: Reverse-phase chromatography. After filtration, 1 ml of the supernatant was concentrated to 200 μl using a Hetovac VR-1 vacuum centrifuge and then applied to a Sephasil C18 (SC 2.1/10; Pharmacia Biotech) reverse phase column connected to the SMART chromatography system. The column had been equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. The inhibitory factor was eluted from the column with a linear gradient ranging from 0 to 100% (v/v) 2-propanol containing 0.1% TFA. The flow rate was 100 μl per min and 200 μl fractions were collected. Propanol and TFA in the column fractions were removed by concentrating the fractions to 100 μl using the Hetovac VR-1 vacuum centrifuge, and all the column fractions were subsequently assayed for inhibitory activity.

Step III: Ion-exchange chromatography. The reverse phase chromatography fraction which contained inhibitory activity was passed through a Mono S (PC 1.6/5; Pharmacia Biotech) cation exchange column equilibrated to pH 3 with 20 mM sodium citrate buffer. The column was connected to the SMART chromatography system. The inhibitory activity was recovered in the flow through fraction (i.e., it did not bind to ion exchanger), but contaminating substances bound to the exchanger and were thus removed.

Step IV: Gel filtration. The "flow-through" fraction which contained inhibitory activity after ion exchange chromatography was concentrated to about half the volume using a Hetovac VR-1 vacuum centrifuge and then applied in aliquots of 20 μl to a Superdex Peptide (PC 3.2/30; Pharmacia Biotech) gel-filtration column connected to the SMART chromatography system. The column had been equilibrated with 0.1 M NaCl in 10 mM sodium phosphate, pH 7. The inhibitory substance was eluted from the column with the same buffer at a flow rate of 50 μl per minute and 100-μl fractions were collected, and all the column fractions were assayed for inhibitory activity.

Step V: Reverse-phase chromatography. Gel-filtration fractions containing inhibitory activity were concentrated to half their volume using a Hetovac VR-1 vacuum centrifuge and aliquots of 100 μl were applied to the same reverse phase column as described above, equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. The inhibitory factor was eluted from the column with a shallow linear gradient of 2-propanol as indicated in the Fig. 4. The flow rate was 100 μl per minute and 50-μl fractions were collected. Propanol and TFA in the column fractions were removed by concentrating the fractions to half their volumes using the Hetovac VR-1 vacuum centrifuge, and the column fractions were subsequently assayed for inhibitory activity.

Capillary Electrophoresis

After the final purification step (step V), the purity of the inhibitory factor was analyzed by micellar electrokinetic chromatography (5) using a HP ^{3D}Capillary Electrophoresis System (Hewlett–Packard) with a diode-array detector and HP ^{3D}ChemStation (Hewlett–Packard) as a controller. A 25-μl fraction containing the inhibitory substance was diluted to 100 μl such that the fraction contained 8 mM sodium dodecyl sulfate and 50 mM sodium phosphate, pH 9.3. The sample was applied with pressure injection (50 mbar for 3 s) and electrophoresis was carried out at 25°C and with a voltage of 30 kV, using quartz capillaries with a length of 1 m, internal diameter of 50 μm and a "bubble-cell" (detection site) 8.5 cm from the cathode. The diode-array detector determined the spectrum between 190 and 600 nm throughout the absorbance peak as it passed the "bubble-cell."

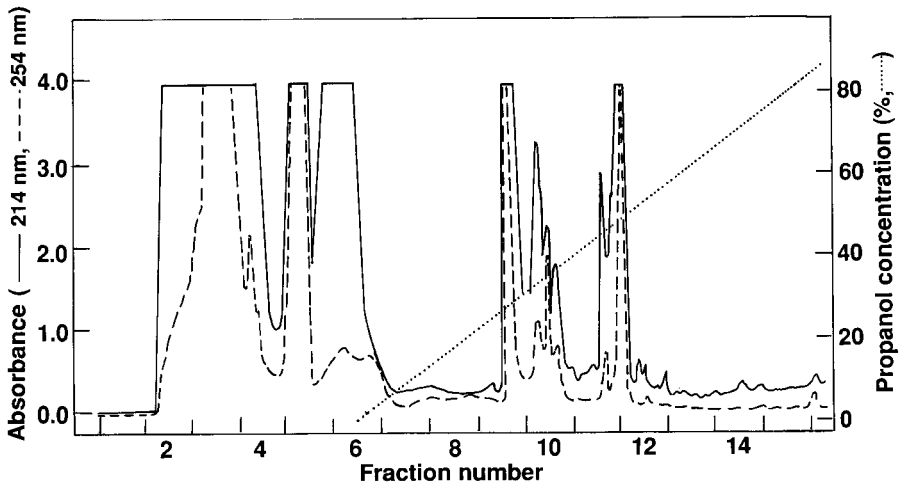

FIG. 2. Purification of the inhibitory factor by reverse-phase chromatography after first having passed the cell free supernatant containing the inhibitor through a filter with a molecular weight cut-off of 3000. One milliliter of the filtered supernatant was concentrated to 200 μl by vacuum centrifugation and applied to the reverse phase column. Column fractions of 200 μl were collected, concentrated to 100 μl (thus removing TFA and propanol) and 4 μl of each concentrated fraction were added to 6-mm tissue culture assay wells to determine the inhibitory activity. An inhibitory activity of 65 %TI was detected in fraction 9. The activity in the other fractions was less than 10 %TI.

RESULTS AND DISCUSSION

Production of the Inhibitory Factor

The amount of inhibitory activity which accumulated extracellularly as a function of the time after passage of hybridoma cells to fresh medium was determined (Fig. 1). The number of viable cells reached a maximum value (about 5×10^5 cells/ml) at day 4 after passage, when the inhibitory activity had accumulated to a significant and critical level (Fig. 1). After day 4, the number of viable cells declined markedly, with a concomitant increase in the number of dead cells and a slight further increase in inhibitory activity (Fig. 1). The increase in inhibitory activity was clearly due to the accumulation of a substance in the supernatant rather than the depletion of essential nutrients, since 10 μl of the supernatant resulted in 30 to 50%TI, whereas no significant inhibition was obtained with 10 μl water (results not shown). The increase in inhibitory activity was, however, not likely due to the production of the common waste products, lactate or ammonia, as judged from the concentration and toxicity of these waste products (1).

The dose–response curve, showing %TI as a function of the amount of inhibitory substance, had a hyperbolic shape (results not shown). At inhibitor concentrations where %TI was less than 40, there was approximately a linear relationship between %TI and the amount of inhibitor added to the assay cultures. At higher inhibitor concentrations (when %TI was higher than 40), the increase in %TI obtained with increasing amounts of inhibitor was less than that a linear relationship would predict.

Purification and Characterization of the Inhibitory Substance

For purification of the inhibitory substance, culture supernatants were collected on day 6 after passage (i.e., two days after reaching maximum cell density). The inhibitory activity in the supernatants passed through filters with a molecular weight cut-off of 500,

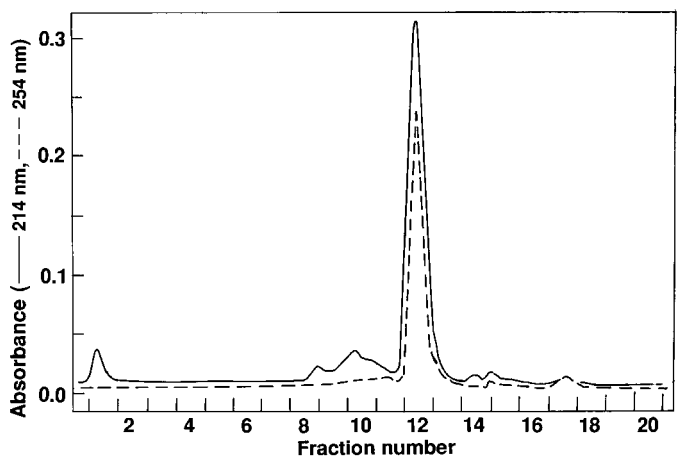

FIG. 3. Purification of the inhibitory factor by gel filtration on a Superdex Peptide column, after first having purified the factor by filtration, reverse-phase chromatography and passage through a Mono S cation exchanger. Twenty μl, which contain inhibitory factor purified from about 200 μl cell culture supernatant, was applied to the column. Column fractions of 100 μl were collected, concentrated to 50 μl by vacuum centrifugation and 10 μl of each concentrated fraction were added to the 6-mm tissue culture assay wells to determine the inhibitory activity. An inhibitory activity of 40 %TI was detected in fraction 12.

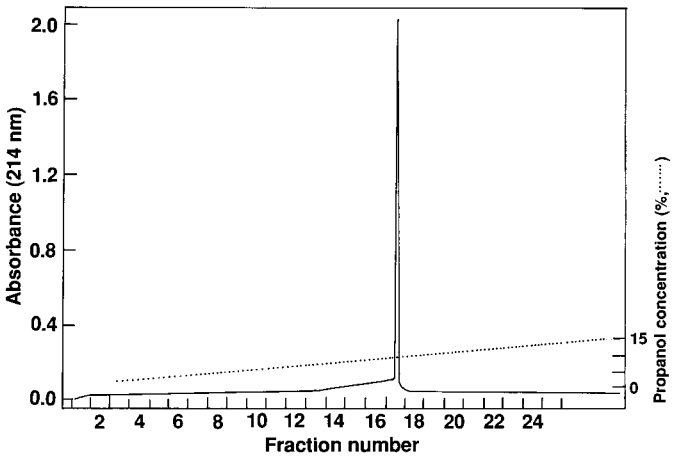

FIG. 4. Reverse phase chromatography of the inhibitory factor obtained after the gel-filtration purification step. The amount of inhibitory factor applied to the column corresponds to the amount purified from about 1 ml of cell culture supernatant. Column fractions of 50 μl were collected, concentrated to 25 μl by vacuum centrifugation (thus removing TFA and propanol) and 2 μl of each concentrated fraction were added to the 6-mm tissue culture wells to determine the inhibitory activity. An inhibitory activity of 35% was detected in fraction 17.

indicating that the molecular mass of the inhibitory factor was not more than about 500 daltons. This is consistent with the observation that the inhibitory activity elutes with a molecular weight less than 1,000 upon gel filtration of culture supernatants on a Sephacryl S-100 (Pharmacia Biotech) column (results not shown), and its consistent with earlier results showing that the molecular weight was less than 5000 (1). Before application on a reverse phase column, the culture supernatant was always filtered using Centricon centrifugal concentrators (Amicon) with a molecular weight cut-off of 3,000. The inhibitory factor was thereby separated from macromolecules present in the cell culture medium, the protein concentration being reduced from 1 to 0.003 mg/ml with about a 70% recovery of the inhibitory activity.

Subsequent reverse phase chromatography of the filtrate containing inhibitory activity was also a very efficient purification step. Most of the contaminating material passed through the column, whereas the inhibitory factor was retarded, being eluted from the column at a propanol concentration of 25–30% (fraction 9, Fig. 2). After reverse phase chromatography, the protein concentration in the fraction containing inhibitory activity was less than could be detected by the method of Bradford (4), the amount of protein being less than one-tenth of that present before chromatography. Moreover, the absorbance at 214 nm was reduced by more than 98%. There was little loss of inhibitory activity.

When carrying out this reverse phase chromatography step, it was important not to apply more than 0.2 ml of the concentrated filtrate to the 0.35-ml reverse-phase column. If larger sample volumes were applied, the inhibitory activity would start to elute when the column was washed (in fractions 4–6, Fig. 2) after sample application, but before the propanol gradient was applied. The activity consequently became distributed in several column fractions. This was not a problem in the last reverse-phase chromatography step, when the inhibitory factor appeared to be essentially pure (see below).

Upon ion exchange chromatography, the inhibitory factor behaved as if it was uncharged. The inhibitory activity passed directly through the ion exchange columns, irrespective of whether an anion or cation exchanger was used, or whether the chromatography was carried out at neutral pH or at the more extreme pH of 3 or 11 (results not shown). However, contaminating substances bound to the exchangers and were thus removed. Consequently, as the third step in the purification procedure, the inhibitory activity obtained after reverse phase chromatography was passed through a Mono S cation exchanger at about pH 3, as described under Materials and Methods. In this step the total absorbance at 214 nm was reduced to one-third of what it was before passage through the ion exchanger. About

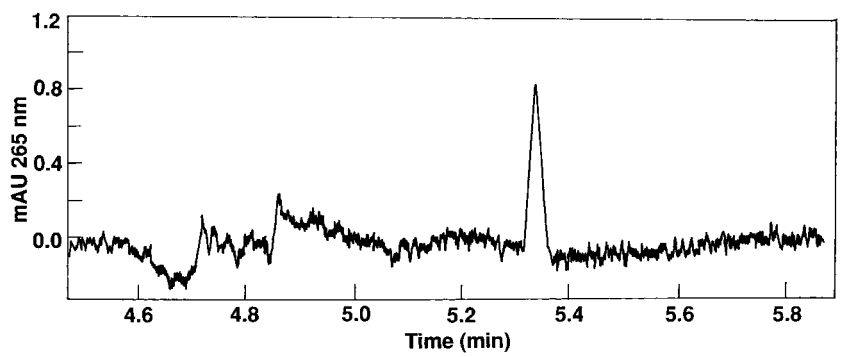

FIG. 5. Capillary electrophoretic analysis by micellar electrokinetic chromatography of the inhibitory fraction obtained after the final reverse phase chromatography step.

50% of the inhibitory activity in the cell culture supernatant had been recovered at this stage.

Upon gel filtration (step IV) on a column with a molecular weight separation range (for peptides) of 100 to 7000, the inhibitory activity eluted in the latter half of the separation range, together with the only major absorbance peak (Fig. 3). Coelution of the inhibitory activity with the absorbance peak suggests that the factor was relatively pure at this stage of the purification procedure. This is consistent with the results from the subsequent reverse phase chromatography step (step V), where the inhibitory activity also co-eluted with the only absorbance peak, which was narrow and symmetrical (Fig. 4). At this stage, the total absorbance at 214 nm was about 0.04% of that one started with in step 1, and the recovery of inhibitory activity was about 20%. The inhibitory activity eluted from the reverse phase column at a relatively low propanol concentration (Fig. 4), suggesting that the inhibitory factor is relatively hydrophilic/polar, despite the fact that it appears to be uncharged. This is consistent with the observation that as much as about 80% of the activity was obtained in the water phase upon extraction with butanol (results not shown).

Capillary Electrophoresis, Absorbance Spectrum, and Stability

Consistent with the results from the last purification steps (gel filtration and reverse phase chromatography), capillary electrophoretic analysis of the inhibitory factor by micellar electrokinetic chromatography indicated that the factor was purified to homogeneity. Only one homogenous absorbance peak was obtained upon micellar electrokinetic chromatography (Fig. 5), the spectrum between 190 and 600 nm being similar throughout the absorbance peak, as determined by the diode-array detector.

In addition to the high absorbance between 200 and 220 nm common to many organic molecules, the inhibitory factor had an absorbance maximum at about 263 nm (Fig. 6). An absorbance maximum above 250 nm suggests the presence of a substance with a conjugated system of carbon-carbon double bonds—possibly an aromatic substance. The factor is apparently not a peptide, since no combination of aromatic amino acids is likely to result in an absorbance maximum at about 263 nm. Moreover, the factor behaved as an uncharged molecule upon ion exchange chromatography, contrary to what would be expected of a peptide. The factor was stable, as there was no apparent loss of activity upon drying, freeze-thawing, or long-time (several months) storage at either 4 or −20°C.

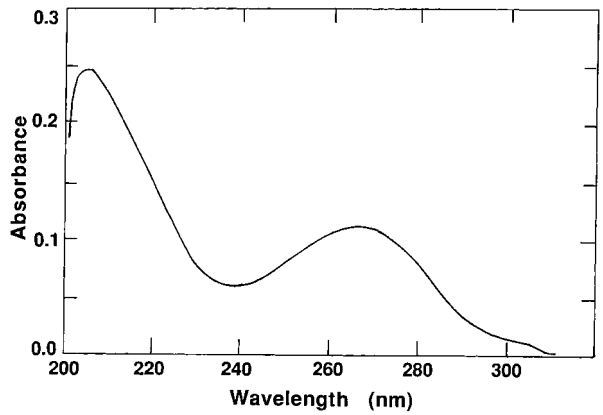

FIG. 6. The UV-absorbance spectrum of the inhibitory factor after the final reverse phase chromatography step. Reference wave length was at 360 nm.

A "scaled-up" version of this purification procedure should enable the purification of enough of the inhibitory factor for the determination of its structure—and thereby its identity—by nuclear resonance spectroscopy. This might in turn enable the development of a protocol for the synthesis of large amounts of the inhibitor. It will be of interest to determine the sensitivity of various cell types to the inhibitor, and thereby determine its potential as a cytostatic drug. A rough estimate suggests that about 1 μg/ml of the factor will inhibit growth of the hybridoma cells by about 50% (%TI of 50), assuming that the factor has an extinction coefficient of about 10^4 cm^{-1} M^{-1} at 260 nm and a molecular weight of about 500. It will also be of interest to determine which other cell types produce this factor. This, combined with structural determination, may clarify the biological role of the inhibitor.

ACKNOWLEDGMENTS

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