Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 339-345

A CORTICOTROPIN (ACTH) RELATED PEPTIDE WITH CYTOTOXIC ACTIVITY

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Received July 25, 1983

A commercially available preparation of corticotropin was found to have potent cytotoxic activity for several established cell lines. Neither synthetic corticotropin nor alpha melanocyte stimulating hormone demonstrated this cytotoxic activity. Gel filtration allowed separation of a 2100 dalton cytotoxic peptide from the actual corticotropin present in the commercially prepared material. The structural relatedness of the cytotoxic peptide to corticotropin was demonstrated by neutralization with antisera to alpha melanocyte stimulating hormone. These studies indicate the existence of a newly identified ACTH related peptide with cytotoxic activity.

During investigations of the lymphokine-like activities of neuroendocrine peptide hormones (1-7), we have observed and herein report that a commercially available porcine corticotropin (ACTH) preparation inhibits cell proliferation. This inhibitory effect is apparently due to a cytotoxic peptide which lacks ACTH activity but is immunologically related to and separable from authentic ACTH. In this report we describe the peptide and its action.

MATERIALS AND METHODS

Cytotoxicity Assay

ACTH from Armour Pharmaceutical, Kankakee, IL, Cat #0053-1040-01R was dissolved in phosphate buffered saline (PBS) at 20 units per ml and further diluted into Eagles Minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS) to give the indicated concentrations of ACTH. Mouse L cells were plated at 20,000/well into 24 well plates with 1.0 ml/well of EMEM + 10% FBS. The cells were allowed to attach for 24 hours before the medium was removed. 1.0 ml of fresh EMEM + 10% FBS was added containing the indicated levels of ACTH. The cultures were incubated for a further 24 hours, after which the cells were trypsinized, mixed with trypan blue and viable cells counted using a hemocytometer.

Gel Filtration

40 units of Armour ACTH were dissolved in 2.0 ml of phosphate buffered saline (PBS) and loaded onto a 2.5 x 20 cm column of Bio-Rad

0006-291X/83 $1.50

P-10 polyacrylamide beads, 4.0 ml fractions were collected as the column was eluted with PBS. The fractions were assayed for ACTH activity in cultures of Y-1 mouse adrenal tumor cells (1,11,12). The anticellular activity of each fraction was assayed on mouse L cells as described in Table 1.

Ion Exchange Chromatography

One 40 unit vial of Armour ACTH was dissolved in 10 ml of sodium acetate buffer (50 mM) at pH 6.0. Eight ml were loaded onto a 2 ml Whatman CM-52 column and eluted with 2.0 ml volumes of buffer containing the indicated concentrations of NaCl. Each fraction was assayed at 1:4, 1:12 and 1:36 dilutions on mouse L cells as described in Table 1. One cytotoxicity unit was that dilution of a fraction which gave a greater than 50% inhibition of viable cells.

Neutralization with Antiserum to αMSH

Eighty units of Armour ACTH were dissolved in 2.0 ml of PBS and loaded on a 2.5 x 35 cm column of Bio-Rad P-4. The material was eluted with PBS and 4.0 ml fractions collected. The 2100 dalton fractions which contained the majority of the cytotoxic activity were pooled. A 1:16 dilution of the pool gave greater than 90% inhibition of viable cells after 24 hours. Antibody to α-MSH (Cat. #1660/001 UCB-Bioproducts, Brussels, Belgium; distributed in the United States by Accurate Chemical & Scientific Corporation, Westbury, NY) was serially diluted in PBS and a 0.1 ml volume added to replicate culture wells immediately after the addition of the cytotoxic peptide resulting in final antiserum dilutions of 1:50 to 1:4050.

RESULTS AND DISCUSSION

Table 1 shows that a commercial preparation of porcine ACTH (ACTHAR, Armour Pharmaceuticals) caused a dose dependent inhibition of mouse L cell proliferation. This cytotoxic activity is more evident in cultures plated at lower cell densities (Table 1). This may indicate that the higher cell densities are resistant because of decreased rates of proliferation at saturation densities. In fact,

Table 1
Cytotoxic effect of an ACTH preparation on mouse L cells

ACTH* units/ml	Cultures plated at 20,000/well viable cells/well $x10^3$ (% inhibition)	Cultures plated at 80,000/well viable cells/well $x10^3$ (% inhibition)
1.0	3±3 (94)	48±24 (65)
0.5	18±6 (65)	96±24 (30)
0.25	45±16 (13)	116±24 (13)
Control (PBS)	52±15	136±24

*U.S.P. Units of Corticotropin

more defined studies with various cell numbers have shown that the cytotoxic activity is cell density dependent (data not shown). We have defined the activity in terms of percent inhibition compared to controls since this would include both inhibition of proliferation as well as cell killing. This activity has been consistently observed in three different lots of Armour ACTH. It was not observed (data not shown) in a more purified pharmaceutical porcine ACTH preparation (Parke-Davis), a highly purified natural porcine ACTH (100 units/mg, Sigma, St. Lous, MO) or a synthetic (1 thru 24) ACTH (Cortrosyn, Organon Inc., W. Orange, NJ). Synthetic αMSH (ACTH acetyl 1-13 amide) also did not demonstrate a cytotoxic effect. Taken together, these results suggested that the anticellular activity was attributable to a factor in the Armour preparation other than ACTH. Gel filtration of the ACTH preparation on a Bio-gel P-10 column showed this to be the case since the cytotoxic factor could be separated from the majority of the ACTH activity (Figure 1). The elution pattern of the two activities indicates that the anticellular activity is due to material having a smaller molecular weight than ACTH. The apparent molecular weight of the cytotoxic factor is approximately 2100 daltons as determined by gel filtration on Bio-Rad P-4 (data not shown). When the ACTH preparation was treated with pronase (Sigma Catalog #5147) at 1 mg/ml for 1 hour at 37°C, and the pronase removed by gel filtration

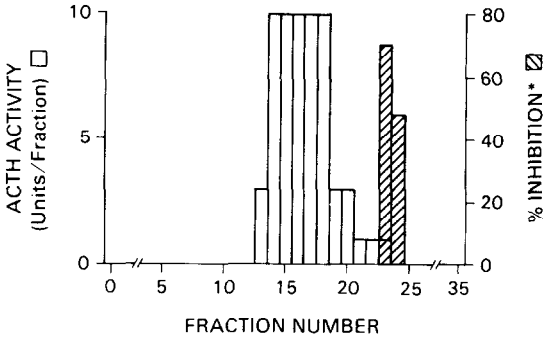

Figure 1. Separation of cytotoxic activity from ACTH activity by gel filtration.

Table 2
Carboxymethyl-Cellulose Chromatographic Purification of the Cytotoxic Peptide

NaCl (mM)	O.D. (280)	Cytotoxicity Units
0	1.16	0
50	0.184	0
100	0.162	0
150	0.380	0
200	0.196	0
250	0.044	8
300	0.020	8
400	0.026	24
500	0.012	0

on the P-10 column, the cell inhibitory activity was destroyed. Thus the anticellular factor is pronase sensitive and is probably a peptide. When the Armour ACTH was fractionated by ion-exchange chromatography on a Whatman CM-52 column (Table 2), we found that the cytotoxic peptide was separable from the majority of the protein. Further, the high salt content required to elute the cytotoxic activity from the column indicates that the cytotoxic peptide is strongly basic. When the 400 mM NaCl fraction from the CM-cellulose column was assayed for cytotoxicity on mouse L cells, 0.6 µg (O.D. 280) of protein would cause a 50% reduction in the number of mouse L cells. Preliminary reverse phase high pressure liquid chromatographic (HPLC) analysis (C-18 column) of the cytotoxic peptide following gel filtration and ion-exchange chromatography demonstrated a single predominant UV absorbing peak with a 6.67 minute retention time (data not shown). Thus the peptide was highly purified and the retention time is consistent with the basic charge of the peptide which was indicated by the CM cellulose column. Given the 2100 dalton molecular weight of the active peptide and assuming 100% purity, the 50% effective dose (0.6 µg) is at least 3.0×10^{-7} M.

When other cell types were plated at 20,000/well and assayed for sensitivity to ACTH inhibition, a great deal of similarity was noted in the dose response curves generated for most of the cell lines

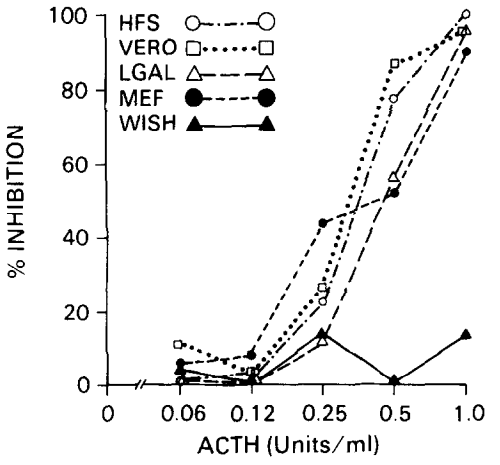

Figure 2. Sensitivity of different cells to the cytotoxic activity. The indicated cell types were plated at 10, 20, 40 and 80 thousand cells per well. The cells were incubated for 24 hours, the media removed and fresh media with the indicated levels of Armour ACTH were added. After an additional 24 hours, the cells were counted. Shown are the results obtained using cells plated at 20,000 per well. HFS, secondary human foreskin fibroblasts; VERO, African green monkey kidney cells; LGAL, mouse L cell fibroblasts; MEF, primary mouse embryo fibroblasts; WISH, human amnion.

(Figure 2). A notable exception is the WISH cell line which was apparently resistant to the inhibiting factor. The sensitive cells include normal (human foreskin and mouse embryo fibroblasts) and transformed (monkey VERO, mouse L929 and hamster BHK) cells. Thus the inhibitory factor is not acting through a mechanism that is linked with only normal or transformed cell lines although cell proliferation may be required for sensitivity. These results also demonstrate that the cytotoxicity of this porcine ACTH associated factor can be demonstrated across species lines. While the reason for the apparent resistance of the human WISH cells is presently unknown these cells may provide a means for determining elements of the mechanism of action, such as the presence or absence of a receptor. In addition, this resistance rules out any relatively nonspecific mechanism of killing such as a detergent-like effect on the cell membrane.

Given the possible derivation of the cytotoxic peptide from ACTH, we determined if the anticellular peptide was immunologically related to ACTH. This seemed to be the case since a monospecific rabbit

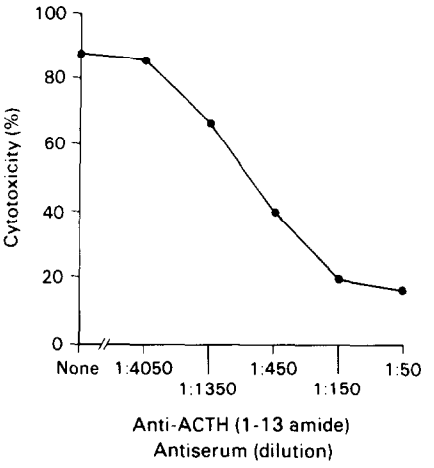

Figure 3. Neutralization of the cytotoxic peptide by αMSH antiserum.

antiserum to synthetic αMSH (ACTH 1-13 amide) (UCB Biochemicals Cat. #1660, distributed in the United States by Accurate Chemical and Scientific Corporation, Westbury, NY) was able to block the cytotoxic activity of the peptide at a 1:1350 dilution of the antiserum (Figure 3). It is important to note that, as mentioned above, synthetic αMSH did not demonstrate the cytotoxic activity even at 10 μg/ml (data not shown). Thus the data suggest that the cytotoxic peptide is immunologically similar to the amino terminus of ACTH but it may contain additional amino acid sequences beyond those of αMSH (m.w. 1665) since the molecular weight of the peptide is 2100 daltons. ACTH and αMSH are known to be the products of extensive processing of the much larger pro-opiomelanocortin protein (8,9). It seems that the newly identified cytotoxic peptide may represent a previously unreported cleavage product of pro-opiomelanocortin. Alternatively the peptide may represent a new ACTH related peptide which was generated during the commercial processing of the pituitary glands.

Our assay for cytotoxicity seems to require sparsely plated, rapidly dividing cultures of cells and for this reason may have escaped detection by previous investigators, reliant upon in vivo assays or in vitro assays of dense cell cultures. Recently, Redding

and Schally have reported the identification of a hypothalamic peptide having antimitogenic but not cytotoxic activity on normal and transformed cell lines in vitro (10). While we have yet to determine the role of the ACTH related peptide in the normal physiology of the animal, it is tempting to speculate that these pituitary and hypothalamic peptides may exert direct inhibitory effects on rapidly proliferating cells such as those which occur in neoplasias. In addition, we have preliminary evidence that this peptide may be a potent immune modulator (manuscript in preparation). Thus, like other neuroendocrine hormones (1-7) this peptide seems to have lymphokine activity.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Research Grant AM 30046 and a James W. McLaughlin Postdoctoral Fellowship to L.D.D.

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