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STRUCTURE OF A NOVEL HUMAN GRANULOCYTE PEPTIDE WITH ANTI-ACTH ACTIVITY

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SUMMARY. We report the purification, structure and biological properties of a peptide of novel sequence from human granulocytes that inhibits ACTH stimulated synthesis of corticosterone in rat adrenal cell suspensions. The peptide HP-4 is homologous to a previously described human granulocyte peptide HP-1 that has no anti-ACTH activity.

We have recently reported that a group of low molecular weight peptides from rabbit neutrophils are inhibitors of the action of adrenocorticotrophic hormone (ACTH) on rat adrenal cell suspensions (1,2). The effect of these peptides which we call corticostatins is specific since they show no inhibition of angiotensin II stimulated secretion of aldosterone. We have isolated HP-1, the equivalent peptide from human neutrophils, and find that it has no corticostatic action. We continued our search in extracts of human neutrophils for similar peptides and report here the isolation of a peptide of novel sequence that inhibits ACTH stimulated secretion of corticosteroids by rat adrenal cell suspensions.

METHODS

Tissue Extraction.

Human granulocytes were obtained from patients with bacterial peritonitis, or from blood of healthy volunteers. Peritonitis fluid was centrifuged at 2000g, and the cell pellet washed in 0.9% saline twice, a cell count was performed using a hemocytometer, an aliquot of the cells stained using Wright's stain, and the remaining cells extracted by sonication in an acidic high salt medium (1 M HCl, 88% formic acid v/v, 1% TFA v/v and 1% NaCl w/v). The extract was spun at 2000g, the supernatant saved, and the pellet reextracted two more times. The crude supernatant was then desalted and the peptides concentrated using SepPak C_{18} cartridges (Waters Associates, Milford, Mass.), as previously described (3), and the eluate lyophilised. Fresh human blood was first separated into erythrocyte and leukocyte fractions by sedimentation in 1% dextran T500 (Pharmacia, Upsala, Sweden), and contaminating red blood cells removed by lysis in 0.15 M ammonium chloride. Purified granulocytes were

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obtained from blood and peritoneal exudates by Ficoll-Paque (Pharmacia) density gradient centrifugation according to the manufacturers recommended procedure. Purity of the extract was confirmed by Wright's stain.

HPLC Purification Procedures, Amino Acid Analysis and Microsequencing.

The SepPak eluate was fractionated by reverse phase HPLC using a Waters C_{18} uBondapak column eluted over a three hour period using a gradient of 0% to 80% acetonitrile in 0.1% TFA throughout. Peptides of interest were then repurified using shallow gradients of acetonitrile and 0.1% TFA as detailed in the text and figure legends. Purity was monitored by amino acid analysis of uv absorbing fractions, and the second step of HPLC was repeated until the peptide was judged pure (this usually required a total of three or four HPLC steps). For amino acid analysis the peptides were hydrolysed with constant boiling HCl in the gas phase at 105^0C for 18 hours and amino acid analyses were performed on a Beckman 6300A analyser (Paolo Alto, CA) or as previously described (4). 1.3 nanomoles of pure peptide was reduced, carboxymethylated and subjected to gas phase microsequencing as previously described (5).

Rat Adrenal Cell Bioassay.

Rat adrenal cell suspensions were prepared using previously described procedures (2). 0.5 ml aliquots of the cell suspension were incubated with 150 pg/ml ACTH, ACTH plus the test substance or incubation medium alone,and after two hours the corticosterone production was measured by radioimmunoassay (2). In a few cases the effect of the test samples on primary cell cultures was determined using a modification of the method of Ramachandran (6). Cells were dispersed as above and plated in 24 well multiwell plates in Hams F-12, 10% Fetal Calf Serum (Flow, McLean, VA) at 200,000 viable cells per well. After three days the wells were washed three times in Hams F-12 and the effect of ACTH and test substances determined in 0.5 ml Hams F-12, 0.5% FCS. Corticosterone production was measured as above, and cell viability determined by Trypan Blue exclusion.

Cell Culture and Cytotoxicity.

Cells were obtained from the American Type Tissue Collection (ATCC, Rockville, MD) and grown under the conditions recommended by ATCC. Cells were grown in 24 well multiwell plates and thymidine incorporation and cell number measurements were performed as previously described (7).

RESULTS

The Isolation and Sequence of a Human Corticostatic Peptide

The HPLC chromatographic profile of an extract of human peritoneal inflammatory cells is shown in Fig. 1. Typically the inflammatory exudate contains between 85 and 95% neutrophils as judged by Wright's staining. Peptides of the HP-1 family are characterised by three disulfide bridges, several arginine residues, no lysine or histidine, and are approximately 30 residues long. HP-1, which we have previously isolated and characterized from inflammatory exudates and human lung tumors (1), is the large u.v absorbing peak eluting at about fraction 75 in Fig. 1A. We subsequently detected a second cysteine and arginine rich peptide eluting at about fraction 80 (darkened area) which did not correspond to HP-1, or any likely artifactual modification of HP-1. This peptide was then further purified on a shallow gradient for 10 min isocratically at 0.1% TFA, going to 30% acetonitrile in 0.1% TFA in the next five minutes, followed by a 90 min linear gradient to

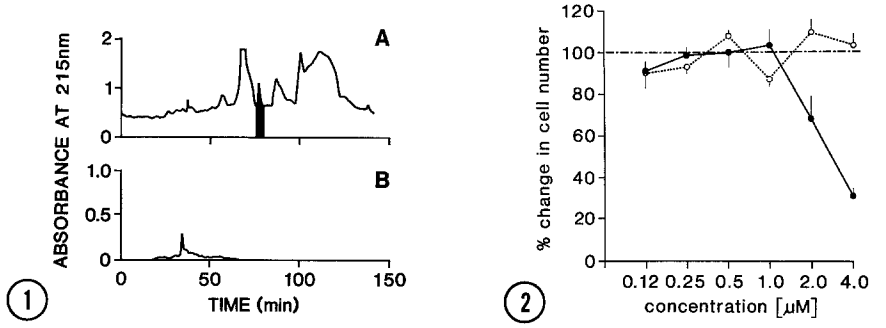

Fig. 1.
HPLC purification of a human peritoneal exudate extract. In panel A the linear gradient of 0% to 80% acetonitrile in 0.1% TFA over 3 hours was used. Fractions eluting at 80 minutes (shaded) were rerun on a shallow gradient (B), as detailed in the text.

Fig. 2.
The effect of HP-1 and HP-4 on cell growth. Cells were plated at 800,000 cells/well in 10% FCS. After 24 hours the wells were washed in serum free medium and increasing concentrations of peptide added in serum free medium. Cells were then trypsinised and the cell number determined using a Coulter Counter. Cells incubated with HP-1 are represented by closed circles and those incubated with HP-4 represented by open circles. Results are expressed as the mean ± S.E.M. (N=4).

43% acetonitrile, in 0.1% TFA, at 1.5 ml/min throughout as shown in Fig. 1B. A total of 1.35 nanomoles of the purified peptide was then submitted to gas phase microsequencing and the following sequence was obtained:

Val-Cys-Ser-Cys-Arg-Leu-Val-Phe-Cys-Arg-Arg-Thr-Glu-Leu-Arg-Val-Gly-Asn-Cys-Leu-Ile-Gly-Gly-Val-Ser-Phe-Thr-Tyr-Cys-Cys-Thr-Arg-Val

As HP-1 exists in three forms differing only at the first N-terminal residue, we decided to call this new peptide HP-4. Ficoll-paque density fractionation of human blood and peritoneal exudates shows that HP-4 is present in the granulocytes but not in the mononuclear fraction. The yield of the peptide varies but typically the ratio of HP-1 to HP-4 of 50:1 was observed.

The Effect of HP-4 on Cell Growth

The peptide HP-1 is highly cytotoxic to a variety of mammalian cell lines in vitro (1). We therefore assessed the effects of HP-4 on the growth of cells. HP-1 kills over 70% of the Chinese Hamster Ovarian cell line CHO-K1 at 4×10^{-6}M (Fig. 2). In contrast HP-4 has no cytotoxic action at these concentrations (Fig. 2). The same result was obtained whether ^{3}H-thymidine incorporation or absolute cell number was used as an index of cytotoxicity. The human epithelial-like lung carcinoma cell line SK-MES-1 was also killed by HP-1, but under the same conditions HP-4 had no cytotoxic

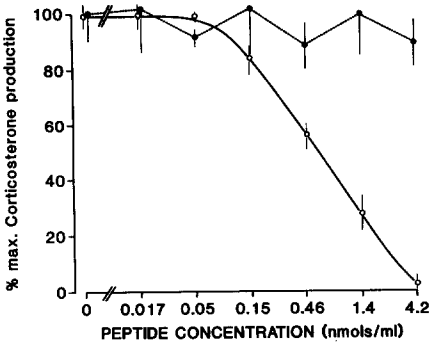

Fig. 3.
The effect of HP-1 and HP-4 on the ACTH induced release of corticosterone in the rat adrenal cell suspensions. 150pg/ml of ACTH was used to stimulate 200,000 cells/ml in the presence of increasing concentrations of HP-1 (closed circles) and HP-4 (open circles). Corticosterone production was measured by RIA (2). Values are the mean $\pm$ standard deviation of four separate experiments.

effect. The murine adrenal carcinoma cell line Y-1 was not killed by either HP-1 or HP-4 which is consistent with our previous results (1).

Inhibition of the Action of ACTH on Rat Adrenal Cell Suspensions

The peptides HP-1 and HP-4 are both homologs of the rabbit corticostatic peptides (2). We therefore tested their effects on ACTH stimulated corticosterone secretion in vitro. As is shown in Fig. 3, HP-1 does not inhibit the action of ACTH on rat adrenal cortex cell suspensions even at concentrations of $4x10^{-6}M$. In contrast HP-4 inhibits the action of ACTH with an ID_{50} of around $7.0 \times 10^{-7}M$, and completely inhibits the ACTH induced release of corticosterone at $4x10^{-6}M$. In view of the marked difference in response of adrenal cells to HP-1 and HP-4, we considered the possibility that HP-4 might be specifically cytotoxic to adrenal cells. HP-4 is not cytotoxic to the adrenal tumor cell line Y-1. Using a primary culture of rat adrenal cells HP-4 was corticostatic after 4 hours with 50% inhibition at $4X10^{-6}M$. After 48 hours 17% of the HP-4 treated cells were dead compared to 11% of the untreated cells thus a specific cytotoxic effect on adrenal cells can be ruled out.

DISCUSSION

HP-4, the first anti-ACTH peptide found in humans is a novel immune system peptide that belongs to the family of recently discovered cysteine and arginine rich peptides, some of which are antimicrobial (8) suggesting an important role in host defense mechanisms. Others however have no obvious role in host defense, and their functions if any remain unclear (2,8). We have recently

Table 1

The sequences of the corticostatin-1, HP-1 and HP-4 and their ID_{50} for the inhibition of ACTH induced corticosterone production in rat adrenal cell suspensions

Peptide	Sequence	ID_{50} (M)
CS I	G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-R-V-N-G-A-R-Y-V-R-C-C-S-R-R	2.5×10^{-8}
HP4	V-C-S-C-R-L-V-F-C-R-R-T-E-L-R-V-G-N-C-L-I-G-G-V-S-F-T-Y-C-C-T-R-V	7×10^{-7}
HP1	A-C-Y-C-R-I-P-A-C-I-A-G-E-R-R-Y-G-T-C-I-Y-Q-G-R-L-W-A-F-C-C	Not Active

shown that some of the rabbit members of this family specifically inhibit the action of ACTH, but not angiotensin II, on adrenal cell suspensions in vitro (1,2).

The human homolog of these peptides, HP-1, has diverse effects on mammalian cells, being cytotoxic to many cell lines, but stimulating cell growth in others (1). As we have shown here, (Fig. 3) HP-1 has no corticostatic activity, which led us to speculate that other undescribed corticostatic peptides might exist in the human. We find that extracts of human neutrophils contain at least one other related peptide, as defined on the basis of amino acid composition. Structurally this peptide resembles the rabbit peptides more closely than HP-1 (2,8) and inhibits the action of ACTH on adrenal cell suspensions. Like HP-1 it separates with the granulocyte fraction on density gradient centrifugation. Unlike HP-1 it shows no cytotoxic effect on two cell lines, CHO-K1, and SK-MES-1, both of which were chosen because of their susceptibility to the lethal effects of HP-1.

It is highly unlikely that the inhibition of ACTH we observe with HP-4 and the rabbit corticostatins is a nonspecific function of their positive charge as other cationic compounds are known to stimulate rather than inhibit ACTH action (9). The absence of corticostatic activity in the closely related peptide HP-1 also supports this conclusion. Table 1 compares the sequences of corticostatin-1 (CS-1), the most potent anti-ACTH peptide from rabbit neutrophils with HP-4 and HP-1. HP-1 differs from CS-1 and HP-4 in lacking a C-terminal extension after the final cysteine and it is tempting to speculate that this may determine its lack of corticostatic activity. The mechanism of action of HP-4 has not been fully elucidated, however parallel experiments with the rabbit corticostatic peptides indicate that these peptides act at the receptor level and are incapable of inhibiting secondary effectors such as cAMP (10). In view of the importance of glucocorticoids in homeostasis, the

inhibitory effects of HP-4 and related peptides, may have important practical consequences.

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REFERENCES

1. Zhu, Q., Singh, A., Bateman, A., Esch, F. and Solomon, S. (1987) J. Steroid Biochem. **27**, 1017.

2. Zhu, Q., Hu, J., Mulay, S., Esch, F., Shimasaki, S. and Solomon, s. (1988) Proc. Nat. Acad. Sci. **85**, 592.

3. Bennett, H.P.J., Browne, C.A. and Solomon, S. (1981) Biochem. **20**, 4530-4538.

4. Bohlen, P. and Schroeder R. (1982) Anal. Biochem. **126**, 144-158.

5. Esch, F. (1984) Anal. Biochem. **136**, 39-49.

6. Ramachandran, J. and Suyama, A.T. (1975) Proc. Nat. Acad. Sci. **72**, 113-117.

7. Congote, L.F. (1985) Biochem. Biophys. Res. Commun. **126**, 653-659.

8. Selsted, M.E., Brown, D.M., Delange, R.J. and Lehrer, R.I. (1983) J. Biol. Chem. **258**, 14485-14489.

9. Wolf, J. and Cook, G.H. (1971) Endocrinology **101**, 1767.

10. Zhu, Q., Bateman, A. Singh, A. Solomon, S. (1988) Endocrine Research, in press.