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A novel polypeptide from *Cervus nippon* Temminck proliferation of epidermal cells and NIH3T3 cell line

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A novel polypeptide, velvet antler polypeptide (VAPPs), having a stimulary effect on proliferation of some cell was isolated from the velvet antler of sika deer (*Cervus nippon* Temminck). This polypeptide consists of a single chain of 32 amino-acid residues VLSAT DKTNV LAAWG KVGGN APAFG AEALE RM. VAPPs showed marked stimulary effect on rat epidermal cells and NIH3T3 cell line (dose range from 10–40 mg·L^{-1} and 5–80 mg·L^{-1}, respectively).

Keywords: velvet antler, polypeptide, promoting cell proliferation

INTRODUCTION

Velvet antler (VA), especially VA of sika deer (*Cervus Nippon* Temminck) produced in the Jilin Province of China, is a precious Chinese medicine material. A characteristic biological activity of VA is its rapid growth, therefore the existence of some growth factors related to growth of cartilage, bone, epidermis, and other tissues in VA is inferred (Suttie *et al*., 1985; Ko *et al*., 1986; Garcia *et al*., 1997). We previously reported that a fraction containing several active polypeptides from VA of sika deer has been isolated and this fraction exerted a fracture-healing activity (Zhou *et al*., 1999). Our experiments also discovered a new peptide, which had been purified from VA of red deer (*Cervus elaphus* Linnaeus) (Weng *et al*., 2002). The structure of the VA polypeptide of red deer (VAPPr) was determined by a combination of MALDI-TOF MS analyses, N-terminal Edman degradation and amino acid analyses. Amino-acid sequence of this polypeptide was: VLSAA DKSNV KAAWG KVGGN APAFG AEALL RM. VAPPr at 0.4–50 and 10–50 mg·L^{-1} showed marked stimulant effects on the growth of rat epidermal cells and rabbit costal chondrocytes, respectively (Weng *et al*., 2001). In this paper, the primary structure of a new polypeptide from VA of sika deer (VAPPs) and its stimulant activity on rat epidermal cells and NIH3T3 cell line proliferation were described.

MATERIALS AND METHODS

Collection and extraction. Whole fresh velvet antlers were obtained from sika deer (*Cervus Nippon* Temminck) produced in Jilin Province of China in June 2002, stored at –80°C. The raw materials (1 kg) were cut into small pieces (approx. 1 cm^3) and washed with cold distilled water (approx. 4°C) to remove blood. The pieces were ground with 5 L of precooled acetic acid solution (pH 3.5) using a colloidal mill. The supernatant was obtained by centrifugation (8500 r.p.m. for 20 min), and further 95% ethanol was added to the supernatant to make the final ethanol concentration of 65% (v/v), then stood for 3 h at 4°C to settle the precipitate, then centrifuged again. The supernatant was evaporated under reduced pressure at 55°C, the residue was dissolved in distilled water. The water-soluble fraction was partitioned by an ultrafilter (Millipore, model 142

Abbreviations: VA, velvet antler; VAPPs, polypeptide from velvet antler of sika deer; VAPPr, polypeptide from velvet antler of red deer.

MM) to collect partially purified polypeptide with molecular mass 3–10 kDa and lyophilized.

This polypeptide (2 g dry mass) was dissolved in 200 ml of 5 mM acetic acid-sodium acetate (HAc/NaAc) buffer, pH 3.5, and applied to CM-Sepharose Fast Flow column (50 × 200 mm) pre-equilibrated with the same buffer. The first protein peak appeared when it was eluted with 5 mM HAc/NaAc, pH 3.5 at 10 mL/min flow rate. The second peak appeared after addition of 0.5 M buffer (pH 4.0) and the third one appeared at 1 M buffer (pH 4.0). The second fraction showed the highest mitogenic activity and was collected, dialysed against distilled water and lyophilized. The above freeze-dried material (200 mg dry mass) was dissolved in 5 mL of 2 mM HAc/NaAc, pH 4.0, and applied to Sephadex G-50 column (20 ×1000 mm) which had been pre-equilibrated with the same buffer. The samples were eluted with the same buffer at 1 mL/min flow rate. Fractions with mitogenic effect on epidermal cells were combined, dialysed against distilled water and lyophilized. The velvet antler polypeptide was finally purified using reverse phase HPLC (Zorbax 300SB C18 9.4 × 250 mm). Elution was performed with a linear gradient system from solvent A (0.1% trifluoroacetic acid in 15% acetonitrile) to solvent B (0.1% trifluoroacetic acid in 70% acetonitrile) at a flow rate of 2 mL/min in 30 min and absorbance was detected at 280 nm. The final yield of velvet antler polypeptide was 10.0 mg (0.010%, w/w, fresh velvet antler).

Sequence determination. Peptide samples were hydrolyzed at 105°C for 24 h with 6 M HCl containing 0.1% phenol and the hydrolysate was analyzed with a Beckman model 6300 automatic amino acid analyzer. Molecular mass was determined by MALDI-TOF mass spectrometry on an LDI-1700 apparatus. Amino-acid sequence was determined by sequential Edman degradation using a protein/peptide sequencer (PE, Inc., model ABI-PROCISE491A, Procedure: Pulse Liquid) at the Shanghai Institute of Biochemistry (China). All the processes were performed automatically.

Culture of rat epidermal cells (Baden *et al*., 2001). Newborn Wistar rats were sacrificed by decapitation within 24 h after birth. Backskins were cut into 0.5 cm^2 pieces and incubated overnight at 4°C in 0.4% dispase (Boehringer Mannheim). The epidermal and dermal layers were carefully separated. Sheets of epidermis were incubated for 15 min at 37°C in 0.25% trypsin (Gibco BRL, Life Technologies) and dispersed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (pre-heated at 56°C). The cells were counted and this cell suspension was used for further culture.

Quantitation of DNA synthesis in epidermal cells and NIH3T3 cell line. The cell suspensions of epidermal cells (2×10^5 cells·mL^{-1}, 100 μL) and NIH3T3 cells (5×10^3 cells·mL^{-1}, 100 μL) were placed into 96-well culture plates as described previously. The epidermal cells and NIH3T3 cells were incubated at 37°C in 5% CO_2 humidified atmosphere for 24 h, then the medium was replaced by FBS-free DMEM/MEM and polypeptide samples (100 μL/well) were added, while 100 μL of DMEM or MEM was added to control wells. The cells were further incubated for an additional 24 h. Three hours before the end of the culture, 10 μL of [^{3}H] thymidine solution was added to 37 kBq/well (Kato *et al*., 1983).

RESULTS AND DISCUSSION

Fractionation of the acid water extract (2 g) of whole fresh VA (1000 g) from *Cervus nippon* Temminck by a combination of acid water extraction, ethanol precipitation and ultra filtration concentrated its proliferation-promoting constituents into a peptide-enriched fraction. Final purification of this material was accomplished by column chromatography on CM-Sepharose Fast Flow, followed by chro-

Table 1. The amino-acid composition and molecular mass of the VAPPs.

Amino acid	Mol. mass of VAPPs Da	VAPPs molar ratio of amino acid	VAPPs amount of amino acid
Asp/Asn (D/N)		2.75	1D 2N
Thr (T)		1.98	2
Ser (S)		0.96	1
Glu/Gln (E/Q)		2.02	2E
Pro (P)		1.66	1
Gly (G)		4.78	4
Ala (A)		6.93	7
Cys[a] (C)			
Val (V)		3.05	3
Met (M)		1.00	1
Ile (I)		0.10	
Leu (L)		2.56	3
Tyr (Y)		0.00	
Phe (F)		1.13	1
Arg (R)		0.87	1
Lys (K)		2.37	2
His (H)		0.11	
Trp[b] (W)		1	1
No aa:s		32	
	3245.7 (calcd.)[d]		
	3262.4 (MS)[e]		

[a]Half-cystine was determined as cysteic acid with a separate sample following oxidation with performic acid. [b]Tryptophan was determined photometrically through separate measurements by utilizing the HPLC on-line spectra obtained from diode-array detection. The presence of tryptophan was then estimated from the relative intensity of UV absorbance at 280 *vs* 250 nm. [c]For VAPPs, the residues from amino acid analysis are listed to the left and the residues from sequencing to the right. [d]Calculated with average masses and with the total sum from amino-acid composition. [e]Mass was determined by MALDI-TOF MS with an experimental accuracy of ±0.1% (see Materials and Methods).

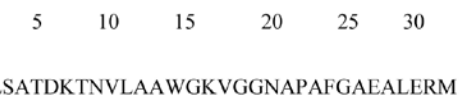

Figure 1. Amino-acid sequence of VAPPs.
The full amino-acid sequence was determined by sequential Edman degradation using a protein/peptide sequencer (PE, Inc., model ABI-PROCISE491A, Procedure: Pulse Liquid).

matography on Sephadex G-50, then by repeated reversed-phase HPLC, to provide 10.0 mg of velvet antler polypeptide (VAPPs).

VAPPs was isolated as an amorphous white solid. The mobility of VAPPs by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and its staining by Coomassie brilliant blue (a single band of molecular mass approx. 3 kDa) suggested that it contained polypeptide(s). Acid hydrolysis of this polypeptide indicated that VAPPs in composed of amino-acids. Alanine, glycine, leucine and valine were the main amino-acid residues, with no cysteine. The molecular mass calculated for the linear peptide with 32 amino acid residues (3245.7 u) was approx. 16 u lower than the molecular mass determined by MALDI-TOF MS (3262.4 u) (Table 1). From the results it is deduced that VAPPs is not a cyclic peptide, because the molecular mass of the cyclic polypeptide determined by MS should be 18 u (one H_2O), not 16 u less than the mass calculated for a linear polypeptide. An attempt to sequence the VAPPs directly *via* Edman degradation led to the conclusion that its N-terminus was open. Thus, VAPPs was established as a linear polypeptide with the amino-acid sequence illustrated in Fig. 1. The above mentioned difference between the theoretical molecular mass calculated for the linear peptide and that measured experimentally by MS might be attributed to the presence of one surplus oxygen (+ 16 u), due to the presence of oxidized methionine residue in the C-terminus of VAPPs (Goransson *et al*., 1999).

Our previous work on VA of red deer resulted in a new polypeptide (VAPPr) that was identified as VLSAA DKSNV KAAWG KVGGN APAFG AEALL RM. Compared to the sequence of VAPPr, VAPPs exhibited only four amino-acid differences, which were the 5th, 8th, 11th and 30th residues (Fig. 1).

The addition of VAPPs to medium of cultures preincubated in serum-free medium for 24 h increased the rate of [^{3}H]thymidine uptake in a dose-dependent manner in rat epidermal cells and NIH3T3 cell line. Maximal stimulation was obtained at 10–40 mg·L^{-1} in the rat epidermal cell culture and at 5–80 /mg·L^{-1} in the NIH3T3 cell line culture (Table 2).

Table 2. Effect of velvet antler polypeptide on the incorporation of [^{3}H] thymidine into DNA in rat epidermic cells and NIH3T3 cell line *in vitro*

Dose/mg · L^{-1}		[^{3}H]TdR A	Bq (per well) B
Control		44±16	23±15
VAPP	5	51±31	68±17*
	10	120±54***	189±64***
	20	300±83***	212±59***
	40	297±75***	195±23***
	80	ND	120±38**

n=4, $\bar{x} \pm S$. *P<0.05, ** P<0.01, ***P<0.001 *vs* control. A, Rat epidermic cells; B, NIH3T3 cell line (fibroblasts); ND, Not determined.

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