ISOLATION AND CHARACTERIZATION OF POLYPEPTIDES FROM HUMAN PLASMA ENHANCING THE GROWTH OF HUMAN NORMAL CELLS IN CULTURE

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Summary

The isolation in pure form and the chemical characterization is first described here of four polypeptides from human plasma which stimulate [^{3}H] thymidine uptake into glial cells in culture and increase the number of human embryonic lung fibroblasts. The polypeptides have a molecular weight of 5000. Although they differ in charge the amino acid compositions are essentially identical and each peptide has four disulphide bridges. The amino and carboxyl terminal residues are aspartic acid and threonine respectively. The peptides are tentively designated Somatomedin B.

Introduction

Normal cells in culture cannot proliferate in a chemically defined medium but demand the addition of so far unidentified serum factors. The source of these has generally been serum (for a review see Temin [1]). The isolation and purification of these factors is a prerequisite for the understanding of the regulation of the proliferation of cultured cells. In 1971 Salmon & Hosse reported on the stimulatory effects on human cells of a bovine serum fraction containing sulfation factor or somatomedin activity [2]. In 1973 it was found by Uthne [3] that a polypeptide fraction obtained from human serum stimulated DNA synthesis of human glial cells. It was apparent that this growth stimulating fraction was not identical to the growth hormone dependent factor, defined as somatomedin A, and which increases the uptake of labelled sulfate in proteoglycans of chicken cartilage, as determined by the method of Hall [4]. Tentatively the factor stimulating the DNA synthesis of glial cells was designated somatomedin B [3]. This report describes the first isolation in a pure form and the chemical characterization of peptides with somatomedin B activity.

Material and Methods

Fraction IV from human plasma, obtained by the Cohn technique [5] and modified according to Björling [6] was extracted with acid-ethanol as described by Uthne [3]. This extract was then gel-filtered over Sephadex G-75

(25 x 100 cm) in 1 % formic acid to remove salt and high molecular weight material [3]. The fraction corresponding to the molecular weight range 3000-8000 daltons was then gel-filtered on Sephadex G-50 (2.5 x 100 cm) in 1 % formic acid. Further purification was achieved by zone electrophoresis as described by Fryklund et al [7] on cellulose columns at pH 7.5 and pH 5.0 (column sizes 2 x 100 cm and 1 x 100 cm respectively). Final purification was obtained by gelfiltration on Sephadex G-50 fine (1.5 x 88 cm) in 0.02 N HCl.

The reduced and S-carboxymethylated peptide was prepared according to Hirs [8]. The derivative was gel-filtered on Sephadex G-50 in 0.02 N HCl to remove excess reagents.

Quantitative amino terminal analyses were determined manually by the direct PTH Edman procedure as described by Iwanaga et al [9]. Identification and spectrophotometric determinations of the PTH-amino acids were performed as previously described [7]. The molar extinction coefficient was determined essentially by the method of Karlsson et al [10]. Carboxypeptidase A digestions were performed on the RCM derivative as reported by Fryklund and Eaker [11]. Amino acid analyses were performed on a Durrum D-500 amino acid analyzer by Dr. D. Eaker, Institute of Biochemistry, Uppsala, Sweden. Prior to analysis the peptides were hydrolysed in vacuo in 6 N HCl for 24 h. The carboxypeptidase A digests were analyzed for free amino acids without prior hydrolysis.

Table I. Assay method for the detection of substances increasing the incorporation of tritiated thymidine into serumfree human glial cells.

Day 0	Cells are sparsely seeded into 50 mm Falcon Petri dishes in Eagle's minimum essential medium containing 10 % calf serum.
Day 1	Medium is changed to serumfree Ham's F-12 medium [14] without thymidine.
Day 2	Medium is changed to serumfree Ham's F-12 and test samples are added at small volume.
Day 3	Tritiated thymidine (2 Ci/mM, Radiochemical Centre, Amersham, England) is added to a final activity of 0.02 uCi/ml.
Day 4	Cultures are harvested for liquid scintillation [15].

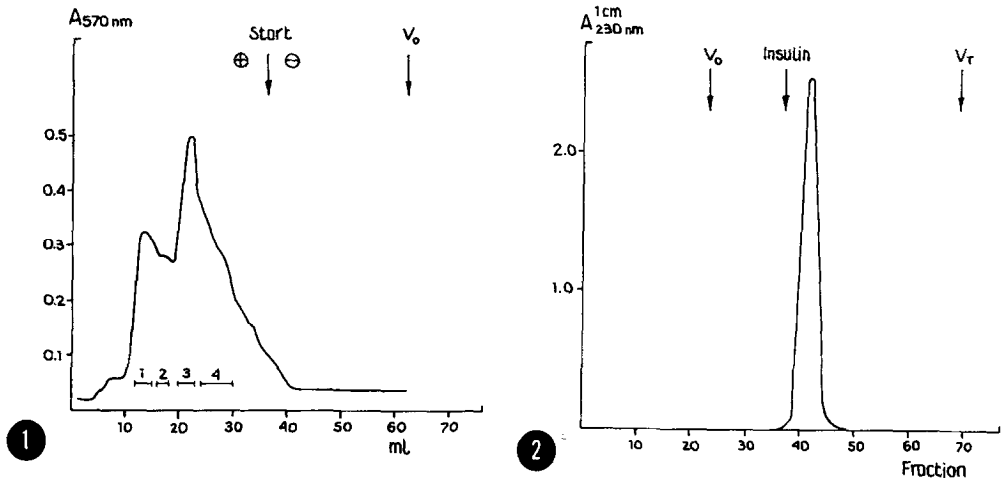

Figure 1

Zone electrophoresis on a cellulose column (1 x 98 cm) in 0.05 M pyridine acetate buffer at pH 5.0, for 29 h at 1000V and 9mA. Elution was performed in the same medium at 10 ml/h. Fraction size 1.0 ml. Aliquots of the fraction were analyzed with ninhydrin after alkaline hydrolysis. V indicates the total volume of the column. 1-4 describe the four peptide fractions obtained.

Figure 2

Gel-filtration of peptide 1, Fig 1 on Sephadex G-50 fine (1.5 x 88 cm) in 0.02 N HCl. Flow rate 10 ml/h. Fraction size 2.3 ml.

The various preparations have been tested in human glial cells as reported by Westermark et al [12]. The in vitro characteristics of these cells have been described [13]. In the assay, test samples are added to serumfree glial cultures and the incorporation of tritiated thymidine is measured by liquid scintillation of the acid precipitable material (Table I). The stimulation obtained by the test samples was compared to that obtained by a reference calf cerum pool. The somatomedin B preparations have also been tested in human embryonic lung fibroblasts cultured in glass bottles (culture surface 45 cm^2, volume 30 ml). As a standard for the stimulatory activity a basal medium was used. This basal medium contains 0.67 % calf serum; Eagle's MEM with Earle's salt solution (Flow Laboratories), benzyl penicillin 100 IU/ml, streptomycin 100 γ/ml, Tricine^R 2.25 mg/ml (Calbiochem) and 0.05 % Methocel (Dow Chemical Co.). The basal medium with 0.67 % calf serum gives 30-40 % of the optimal growth obtained in the presence of 8 % calf serum. The somatomedin B preparations tested were added to the basal medium and the increased cell growth was measured by cell counting in a counting chamber after an incubation period of 6 days.

Results and Discussion

Gel chromatography on Sephadex G-75 and G-50 yielded a somatomedin B concentrate where the main peak of somatomedin B activity was eluted somewhat later than insulin. The acidic somatomedin B active material received after column electrophoresis at pH 7.5 was further subdivided upon electrophoresis at pH 5.0 to give four anodic peptides (1-4) (Fig. 1). Gel filtration of each of the four peptides gave a symmetrical peak in the same position relative

Table II. Amino acid composition of peptide 1 (Somatomedin B)

	Native	RCM-derivative *	
Cm-Cysteine	-	8.00	(8)
Aspartic acid	5.10	5.23	(5)
Threonine	3.98	3.96	(4)
Serine	3.02	3.09	(3)
Glutamic acid	8.28	8.12	(8)
Proline	1.09	0.77	(1)
Glycine	2.25	2.07	(2)
Alanine	1.37	1.06	(1)
Valine	1.95	1.83	(2)
Methionine	-	-	(-)
Isoleucine	-	-	(-)
Leucine	1.05	0.95	(1)
Tyrosine	2.89	2.90	(3)
Phenylalanine	1.01	0.99	(1)
Histidine	-	-	(-)
Lysine	4.05	3.83	(4)
Arginine	0.99	1.27	(1)
Tryptophan	-	-	(-)
			(44)

* RCM = Reduced and S-carboxymethylated

Table III. Test for somatomedin B activity in human glial cells

Peptide[a]	Concentration	cpm[b] (^{3}H-thymidine)
1	12 µg/ml	287
2	9 "	341
3	15 "	4727
4	12 "	6129
Calf serum		
0	-	383
0.25 %	-	1385
0.5 %	-	2774
1.0 %	-	5500

a) The peptides were tested in a serum free medium.

b) Average of 2 determinations.

to insulin, indicating that each peptide has similar molecular weight. Fig.2 shows a typical elution pattern obtained with the most anodic peptide (peptide 1, Fig.1) after chromatography on Sephadex G-50 in 0.02 N HCl. Table II shows the amino acid composition of the native and RCM derivative of the same peptide. Integral values are obtained for all amino acids indicating homogeneity. As can be seen, neither methionine, histidine nor isoleucine are present in the peptide. Aspartic acid was found in quantitative yield as the only amino terminal residue. Carboxypeptidase A released one mole of threonine per mole peptide. The molar extinction coefficients at 280 nm for the native and RCM derivative are 5600 and 4700 respectively which is consistent with a content of 3 tyrosine residues and four disulphide bridges. The molecular weight is 5011 as determined from the amino acid composition. Peptides 2, 3 and 4 obtained after electrophoresis at pH 5.0 also have aspartic acid as amino terminal residue and threonine as the carboxyl. The amino acid composition of peptide 2 was identical with that of peptide 1, while the analyses of peptides 3 and 4 vary in two amino acids suggesting the presence of 1 or 2 arginine residues and 2 or 3 tyrosine residues. As seen in Fig 1. the purification on electrophoresis at pH 5.0 results in a separation according to charge, even if the peptides are not resolved into four distinct peaks. The almost identical amino acid compositions can

Table IV. Test for somatomedin B activity in human embryonic lung fibroblasts

Peptide[a)]	Concentration	Cell no. x 10^{6}[b)]
1	0.06 µg/ml	3.40
2	0.045 "	3.50
3	0.075 "	3.45
4	0.06 "	3.70
Calf serum		
0.67 %	-	2.65
8.00 %	-	7.30

a) Each peptide was added to the basal medium + 0.67 % calf serum.

b) Average of two determinations.

therefore not simply be due to an overlap between the four fractions, but could indicate various degrees of amidation.

Data on the assays of peptide 1-4 in human glial cells are shown in Table III and Table IV describes the result of the tests in human embryonic lungfibroblasts. Only the most cathodic peptides, 3 and 4 (Fig.1) increased the DNA synthesis of the glial cells, while all four peptides were about equally active in the lung fibroblast assay. The dosages required to stimulate the glial cells in serum free medium are considerably higher than those which stimulate the lungfibroblasts, where the peptides were added to a basal medium containing small amounts of calf serum (0.67 %). (The presence of calf serum was a prerequisite for the growth of the lungfibroblasts.) These assay results might be explained by these different outlines of the two assay procedures i.e. that the low serum content in the fibroblast assay makes this system more susceptible to stimulators. However, the activity obtained with only two of the peptides in the glial system could also indicate that these cells are more selectively stimulated by factors present in human serum.

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Literature

1. Temin H.M., Pierson R.W. jr and Dulak N.C. (1972) in Cristafolo C, ed Growth Nutrition and Metabolism of Cells in Culture p 49-81. Academic Press, New York.
2. Salmon W.D. jr and Hosse B.R. (1971) Proc. Soc. Exp. Biol. 136, 805-808.
3. Uthne K. (1973) Acta Endocrinol Suppl 175, 1-35.
4. Hall K. (1970) Acta Endocrinol 63, 338-350.
5. Cohn E.J., Gurd F.R.N., Surgenor D.M., Barnes B.A., Brown R.K., Derouaux G., Gillespie J.M., Kahnt F.M., Lever W.F., Liu C.H., Mittelman D., Mouton R.F., Schmid K. and Uroma E. (1950) J. Amer. Chem. Soc. 72, 465-474.
6. Björling H. (1972) Vox Sang. (Basel) 23, 18-25.
7. Fryklund L., Eaker D. and Karlsson E. (1972) Biochemistry 11, 4633-4640.
8. Hirs C.H.W. (1956) J. Biol. Chem. 219, 611-621.
9. Iwanaga S., Wallén P., Henschen A., Gröndahl N.J. and Blombäck B. (1969) Eur. J. Biochem. 8, 189-199.
10. Karlsson E., Eaker D., Fryklund L. and Kadin S. (1972) Biochemistry 11, 4628-4633.
11. Fryklund L. and Eaker D. (1973) Biochemistry 12, 661-667.
12. Westermark B., Wastesson Å. and Uthne K. (1974) Exp. Cell. Res., in press.
13. Pontén J., Westermark B. and Hugosson R. (1969) Exp. Cell. Res. 58, 393-400.
14. Ham R.G. (1965) Proc. Natl. Acad. Sci. 53, 288-293.
15. Westermark B. (1971) Exp. Cell. Res. 69, 259-264.