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EVIDENCE FOR THE PRESENCE IN HUMAN SERUM OF AN ULTRAFILTRABLE FACTOR ACTIVATING SOMATOMEDINS

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SUMMARY

Human serum contains an ultrafiltrable factor (350 < M.V. < 700) which stimulates sulphation activities of native, or purified somatomedin A of either small or high molecular weight. The factor is heat stable, resists protease hydrolysis but is destroyed by strong acidic hydrolysis. It is not extractible by chloroform. It restores somatomedin activities of conserved fractions and allows good conditions of bioassays of purified fractions. This factor is not a known amino-acid, a polyamine, vitamin A, zinc, T4 or T3. It stimulates somatomedin activity equally if added together with the somatomedin, or if added before (and removed) the adding of somatomedin.

INTRODUCTION

In human serum there are several HGH dependent peptidic growth factors : IGF I and IGF II (1-2), MSA (3), somatomedins A and C (4-5).

Somatomedin A is found in the serum in two main molecular forms : a high molecular weight form (M.W. about 50,000 daltons) and a low molecular weight form (M.W. = 5,000 - 10,000 daltons) (6-7).

Our laboratory is engaged in the purification of both forms (to be published). The different stages of purification were followed by biological assays using the sulphation activity (8). However the greater the purification, the more difficult

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Abbreviations : IGF I, IGF II : insulin growth factor I and II ; MSA : multiplication stimulating activity ; MEM : minimal Eagle's medium.

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was the measurement of the biological activity and the specific activity decreased. This has been shown by several authors (9).

The purpose of this work is to show that human serum contains one (or several) ultrafiltrable factors which stimulate somatomedin A activity as measured by incorporation of $^{35}SO_4$ into pelvic cartilage of chick embryo.

MATERIALS AND METHODS

Somatomedin bioassay (HALL's method modified) (10) : The incubation medium was MEM, brought to pH 7.2 with HEPES complemented with Earle's salts, and freshly prepared glutamine (2 mmoles/l). Penicillin (200 U/ml) and streptomycin (80 µg/ml) were added. Reference serum was 20 pooled male healthy adults serums. The serums were heated during 45 minutes at 56° C before the assays. The pelvic cartilages of chick embryo (8 rudiments per bottle for one dilution) were preincubated in 7.2 ml incubation medium with reference serum (50, 100, 200, 400, 800 µl) or unknown samples (100, 400 µl) during one hour at 37° C. Then were added 100 µl of MEM containing 0.6 mg of NA_2SO_4 and 1 µCi of $NA_2{}^{35}SO_4$. The bottle were incubated for another 20 hours at 37° C in a giratory water bath (shaker model G76. New Brunswick Scientific Co). After incubation the rudiments were heated at 100° C during 10 minutes, then washed with saturated cold NA_2SO_4 (overnight) and water (2 hours). Each rudiment was placed into a scintillation vial and solubilized in 400 µl of soluene 350 (PACKARD) during 24 hours at room temperature, 4 ml instagel (PACKARD) were added to each vial and radioactivity was counted.

Statistical evaluation of assay : The level of somatomedin was calculated by SCHIMPFF method (11) and the statistical evaluation done by BLISS method (12). The results were expressed in somatomedin units per ml ; one unit beeing defined as the biological activity contained in 1 ml of the reference serum. In some assays the results were expressed in percent of the control, i.e. cartilages incubated in medium alone, defined as 100 p. cent.

Preparation of the ultrafiltrate 1000 (UF1000) : The UF1000 was prepared from a pool of normal human serums. Generally 30 ml of serum were ultrafiltrated in an Amicon 52 cell through membrane Millipore PSAC (cut-off 1000 daltons). The ultrafiltration was stopped when 10 ml of serum remained in the cell. The ultrafiltrate was stored at - 80° C until assay. Sometimes after this first ultrafiltration 20 ml of distillated water were added to retentate and a new ultrafiltration was done and the operation repeated 7 times. The ultrafiltrates were collected, freeze dried and reconstituted at initial volume with distillated water for the assays. The collected retentate was called "washed retentate".

Preparation of the ultrafiltrate 500 (UF500) : As above but the serum was ultrafiltrated through membrane Amicon Diaflo UM 05 (cut-off 500 daltons).

Chromatography : Filtration on Biogel P2 (BIORAD $\leqslant$ 400 mesh) : column 1.5 x 30 cm, loaded with 500 µl of UF1000 concentrated ten times by freeze drying, elution with distillated water (flow rate of 11 ml/h). The column was calibrated with Dextran blue, vitamin B12, reduced glutathion and potassium chromate. The eluate was pooled in 4 fractions following the molecular weight : fraction I : 900 $\rightarrow \geqslant$ 1200, fraction II : 700 - 900, fraction III : 350 - 700, fraction IV : $\leqslant$ 350 (Figure 5). Each fraction was freeze dried and dissolved in 2 ml distillated water. Filtration on Sephadex G10 (PHARMACIA) : the same conditions described for Biogel P2 were used.

Action of proteolytic enzymes : Trypsin (SERVA) : 800 µl UF1000 was brought to pH 8 with phosphate buffer 0.3 M, 30 µl of trypsin solution

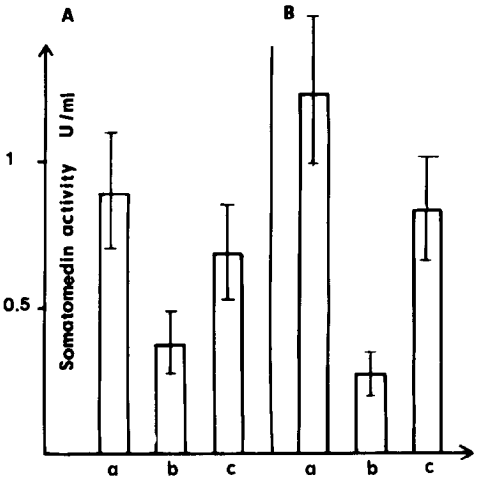


Figure 1. A : Assay with UF1000. Effect of : a) whole serum, b) washed retentate, c) reconstituted serum (1/3 retentate + 2/3 UF1000) on $^{35}SO_4$ incorporation into pelvic cartilages of chick embryo.
B : Assay with UF500 alone in the same conditions. The difference is significant $P < 0.05$ b versus a and c versus b.

(1 mg/ml in HCl 0,001 M) was added. Incubation 30 minutes at 25° C. Soluble proteinase K (BOEHRINGER) : 800 µl UF 1000 was brought to pH 8 with phosphate buffer 60 mM. 1 mg proteinase K was added. Incubation 30 minutes at 30° C. Pronase (BOEHRINGER) : 800 µl UF1000 was added to 800 µl of pronase solution (1 mg/ml) in phosphate buffer pH 8, 60 mM. Incubation 30 minutes at 40° C. Carboxypeptidase A (SERVA) : 800 µl UF1000 was added to 150 µg carboxypeptidase A in 1 ml NH_4HCO_3, 0.1 M.

Somatomedin purification : The somatomedin partially purified used in assays is of low molecular form (13) or high molecular form (14).

RESULTS

Evidence that serum contains an ultrafiltrable factor that stimulates somatomedin activity : Fig. 1 shows somatomedin activity in the whole serum, the washed retentate, and the reconstituted serum with UF1000 (A) and UF500 (B). After the ultrafiltration the serum has lost 60 % of its activity and the addition of UF1000 or UF500 restores about 80 % of starting activity. The addition of UF1000 to partially purified somatomedin of low or high molecular weight increases their biological activity from 2 to 4 times (Fig. 2). There is a relationship dose-response (Fig. 3). The UF1000 or UF500 added alone without somatomedin to incubation medium has no or very little activity (0 to 0.10 U/ml).

Physico-chemical properties of the ultrafiltrable factor : The assays were done using as somatomedin active peptides either normal serum washed reten-

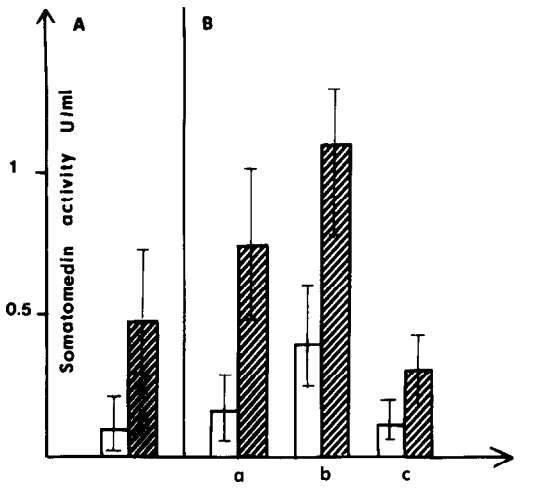


Figure 2. Effect on $^{35}SO4$ incorporation in chick embryo-cartilages.
A) Partially purified somatomedin of low molecular weight.
B) Partially purified somatomedin of high molecular weight (Curling's method). a) crude albumin fraction, b) ceruleoplasmine fraction, c) purified albumin fraction.
Somatomedin alone ▱ Somatomedin + 400 µl UF1000 ▨

tates, low mol. weight form partially purified somatomedin or high molecular weight form, since the ultrafiltrable factor was active on all forms of somatomedin. These materials were called reference somatomedin (RS) in the assays.

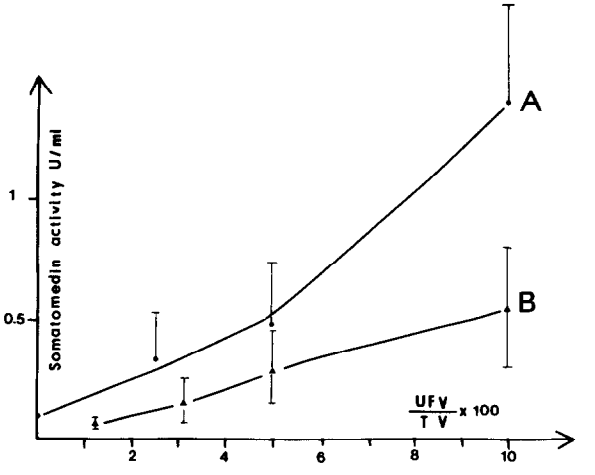
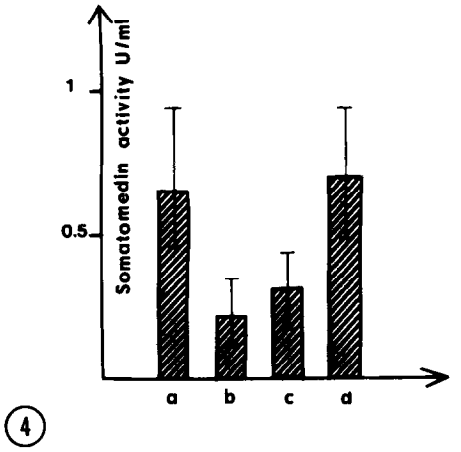


Figure 3. Relationship dose response in presence of increased percentage of UF1000.
A) Partially purified somatomedin of low molecular weight.
B) Partially purified somatomedin of high molecular weight.



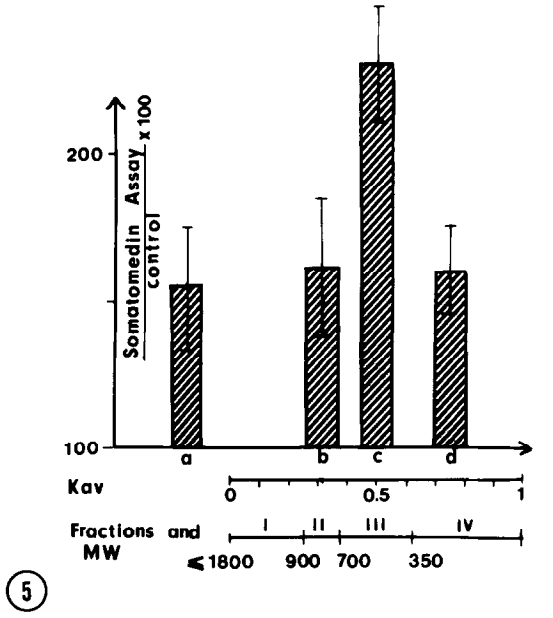


Figure 4. Fractionated ultrafiltration. Effect of : a) whole serum, b) washed retentate, c) reconstituted serum (1/3 retentate + 2/3 UF500 - 1000), d) reconstituted serum (1/3 retentate + 2/3 UF500), on $^{35}SO_4$ incorporation into chick embryo-cartilages. The difference is significant $P < 0.05$ b versus a d versus b. The difference is not significant c versus b.

Figure 5. Filtration on biogel P2. The eluate was pooled in 4 fractions. The fractions II, III, IV were tested on $^{35}SO_4$ incorporation into chick embryo-cartilage.

Determination of molecular weight : Fig. 1B already shows that UF500 is active. This fact is confirmed by an assay with fractionated ultrafiltration. A first ultrafiltration is carried out with a cut-off of 1000 daltons. The UF1000 obtained is filtered again through a membrane Amicon Diaflo uM 05 (UF500). The retentate of this second ultrafiltration is called UF500 - 1000. Fig. 4 shows that UF500 - 1000 has little activity, it increases by only 12 % the activity of washed retentate, whereas UF500 restores the starting activity. When UF1000 is filtered on Sephadex G10, there is no activity in void volume (M.W. > 700), the activity is found in a fraction with a lower molecular weight but the resolution of the column was not good. The filtration on Biogel P2 shows that activity is in fraction 3 of molecular weight between 350 and 700 (Fig. 5). Stability of the ultrafiltrable factor : The activity of UF1000 is thermostable, and is not destroyed after heating at 100° C during 1 h 30 at neutral pH, but an acidic hydrolysis destroyed nearly all the activity (Fig. 6). Extraction by chloroform : When UF1000 is extracted by chloroform, the ultrafiltrable factor activity is found in the aqueous phase (Table 1).

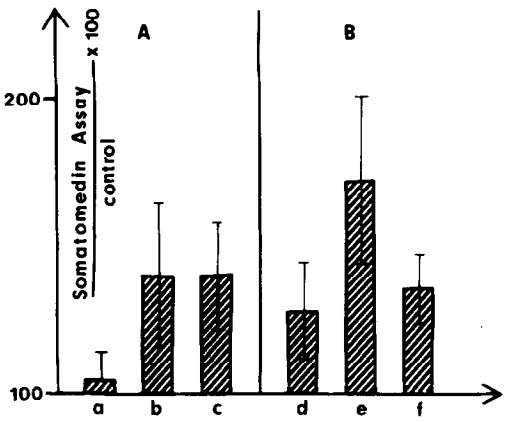


Figure 6. A. Treatment by heating. a) reference somatomedin (RS), b) RS + 400 µl UF1000, c) RF + 400 µl UF heated at 100° C during 1 h 30.
B. Treatment by HCl. a) RS, b) RS + 400 µl UF1000, c) RS + 400 µl UF1000 treated by HCl. HCl is added to UF1000 to obtain a final concentration of 6 N. UF1000 is kept 24 h at 104° C in a sealed tube, neutralized by NaOH evaporated to dryness under nitrogen pressure and dissolved in 1.2 ml incubation medium for the assay.

Action of proteolytic enzymes : The activity of UF1000 was not destroyed by proteolytic enzymes (data not shown)

Several small molecules have been shown to act on mucopolysaccharides biosynthesis or cellular growth : We checked on one hand if the UF1000 contained these molecules, and on the other hand if these molecules reproduced the action of UF1000. Vitamin A (15) : In UF1000 vitamin A, measured by fluorimetric method of HANSEN (16), is not found. 400 µl of a retinol solution (750 µg/l) was not active (data not shown). Zinc (17) : The zinc level in UF1000 is 0.1 mg/l

Table 1 : Chloroform action : UF1000 is extracted by chloroform (v/v) during 2 hours at 4° C. The chloroformic phase is evaporated to dryness and redissolved into initial volume of incubation medium to assay. The assays were done with conserved somatomedin and at different times of conservation.

Somatomedin reference (SR) U/ml	SR + 400 µl UF1000 U/ml	SR + 400 µl chloroformic phase U/ml	SR + 400 µl aqueous phase U/ml	% Recovery of aqueous phase
0.48 T = 0	1.08	0.22	0.83	77 %
0.09 T = 3 months	0.61	0.15	0.80	130 %
0.02 T = 8 months	0.49	≈ 0.01	0.37	75 %

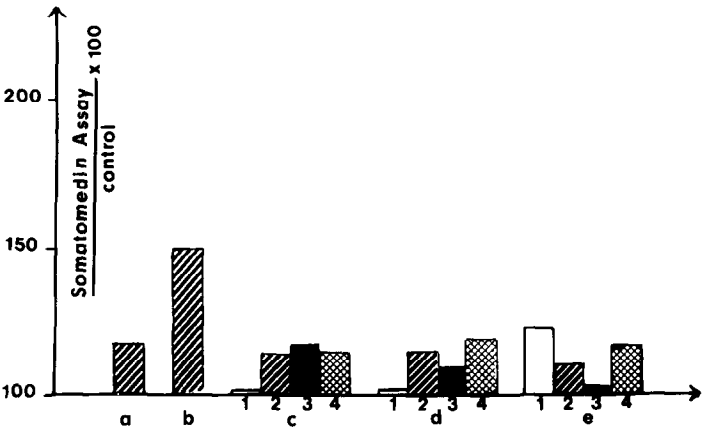


Figure 7. Measurement of $^{35}SO4$ incorporation in chick embryo-cartilage.
a : Reference somatomedin
b : RS + UF 1000
c1 : 400 µl T3 alone 15 ng/l
c2 : 400 µl T3 alone 3 µg/l
c3 : 400 µl T4 alone 30 ng/l
c4 : 400 µl T4 alone 145 µg/l
d1 : RS + 400 µl T3 15 ng/l
d2 : RS + 400 µl T3 3 µg/l
d3 : RS + 400 µl T4 30 ng/l
d4 : RS + 400 µl T4 145 µg/l
} Dilution of a solution containing 2 g/l of T3 or T4 and 20 g/l of crystallized bovine serum albumin (BSA). BSA alone has no activity.
e1 : RS + 400 µl spermine 100 ng/ml
e2 : RS + 400 µl spermidine 145 ng/ml
e3 : RS + 400 µl cadaverine 40 ng/ml
e4 : RS + 400 µl putrescine 90 ng/ml

measured by atomic absorption. The same quantity of zinc added to reference somatomedin does not increase its activity (data not shown). Thyroïd hormones (18) : They were determined by radioimmunoassay (ABBOT) and they were not detected in UF1000. T4 and T3 were added to reference somatomedin at physiological levels of free and total hormones. i.e. 400 µl of T4 solution at 30 ng/l and 145 µg/l, or 400 µl of T3 solution at 15 ng/l and 3 µg/l. In these conditions T3 and T4 were inactive (Fig. 7). Polyamines (19) : The polyamines have not been measured in the UF1000 ; in a first assay 400 µl of a solution containing 75 ng/ml of each polyamine (putrescine, spermine, spermidine and cadaverine) were added to reference somatomedin. Under these conditions, the $^{35}SO4$ incorporation in chick embryo-cartilage was not increased. In a second assay the polyamines were tested separately and at physiological levels (Fig. 7). The cadaverine was (not significantly) inhibitory, the others were inactive.

Somatomedin stimulatory factor interaction : To determine whether the stimulatory factor is a somatomedin cofactor or not, two experiments were carried out. In the first, as usual, the chick embryo-cartilages were

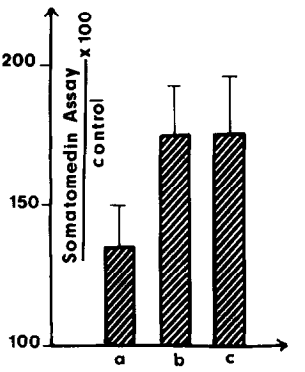


Figure 8. Somatomedin-stimulatory factor interaction. Measurement of $^{35}SO4$ incorporation in chick embryo-cartilage a) reference somatomedin, b) RS + 400 µl ultrafiltrated incubated together, c) cartilages preincubated with UF1000, washed and incubated with reference somatomedin.

incubated with the reference somatomedin and 400 µl of UF1000 ; in the second the cartilages were preincubated with 400 µl d'UF1000 washed several times with NaCl 0.15 M, then incubated with the reference somatomedin alone. Fig. 8 shows that UF1000 activity is found in both cases.

DISCUSSION

In this work we have shown the presence in human serum of an ultrafiltrable factor which stimulates the $^{35}SO4$ incorporation activities of human somatomedin A in pelvic rudiments of chick embryos. This factor is active with somatomedin activity contained in whole normal serum, as well as with partially purified low molecular weight somatomedin, or partially purified high molecular weight somatomedin. This factor increases existing somatomedin activity and also restores lost activity of purified fractions conserved several months. The factor is not destroyed by lyophilisation or by heating at 100° C during 1.30 hours, but is destroyed by strong acidic hydrolysis. Its molecular weight is between 350 and 700 and it seems not to have a peptidic structure since it is not destroyed by different proteasic enzymes.

In the SCHIMPFF method of somatomedin activity determination, each purified fraction is caracterized by a curve obtained by plotting the square-roots of the increasing amounts of protein fraction added to the medium, against the ratio : cpm of each amount/cpm of control. The BLISS statistical method applied takes into account only those curves which are not biphasic and pass by the origin. Very often during purification procedures, a purified fraction could not be bioassayable because the curve does not pass by origin with a low slope. Addition of the ultrafiltrable factor allowed us to obtain a good slope and a bioassay result.

The sulphation stimulating activity of whole serum is the result of contributions of somatomedin peptides, inhibitory factors - glucocorticoïds (21), fatty acids (22), non identified factors found in diabetic rat serum or chronically malnourished rats (23-24) - and stimulatory factors. The UF1000 containing the ultrafiltrable factor, probably also contains some free glucocorticoïds or/and fatty acids. Extraction by chloroform does not modify the stimulary activity, which remains in the aqueous phase.

Some amino-acids have been shown to be necessary to somatomedin activities : HALL (10) observed that serine and glutamine were essential to the somatomedin activity measured by uptake of $^{35}SO4$ by chick embryo-cartilage. PLET et al (26) note that dialysed serum lost part of its activity measured by ^{3}H-thymidine incorporation in fibroblastes and recovered it when a serum filtrate or serine (0.2 mM) was added. Our incubation medium contained all amino-acids including serine (10 mmoles/l) and freshly added glutamine (2 mmoles/l). In addition, the ultrafiltrable factor has a molecular weight between 350 and 700, which is too large for an amino-acid.

The stimulatory effect of UF1000 does not seem to be in relation with presence of polyamines (M.W. = 80 to 300), vitamin A or zinc.

Thyroïd hormones have a molecular weight of respectively 776.93 and 651.01 for T4 and T3. FROESH et al (18) observed that a supra-physiological level of T3 alone increases $^{35}SO4$ incorporation in chick embryo-sterna cartilages and that this increase was cumulative with NSILA somatomedin activity. We have not found any radioimmunoassayable thyroïd hormones in the UF1000. It is not excluded that the thyroïd hormones will be present at a low level - physiological free hormone levels - but at this concentration we have checked that neither T4 nor T3 is active in $^{35}SO4$ incorporation stimulating activity in presence of purified somatomedin or alone.

The ultrafiltrable factor does not seem to be a cofactor of somatomedin since it retains its stimulatory activity even if added sequentially to the medium before the somatomedin peptides.

It is well know that inhibitory factors act on whole cell metabolism and not only on the somatomedin activities (27). It is possible that the ultrafiltrable activating factor acts also on the general metabolism, preparing the cartilage for the somatomedin effect. Another hypothesis is that it could unmask receptor sites for somatomedin on plasma membranes of chondrocytes.

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