Isolation from fetal bovine serum of an apolipoprotein-H-like protein which inhibits thymidine incorporation in fetal calf erythroid cells

Qinggang LI,* Russell BLACHER,† Fred ESCH† and L. Fernando CONGOTE*‡

*Endocrine Laboratory, Royal Victoria Hospital, 687 Avenue des Pins Ouest, Montreal, Canada H3A 1A1 and Department of Medicine, McGill University, Montreal, Canada, and †Athena Neurosciences, 800F Gateway Boulevard, San Francisco, CA 94080, U.S.A.

A 46 kDa heparin-binding protein which inhibits thymidine incorporation in cultures of fetal calf liver erythroid cells was isolated from fetal bovine serum by affinity chromatography on heparin–Sephacrose, ion-exchange chromatography, gel filtration and reversed-phase h.p.l.c. The *N*-terminal sequence of the first 22 amino acids showed 81 % identity with the published sequence of human apolipoprotein H. The isolated protein inhibited thymidine incorporation with an ED_{50} (concn. producing 50 % of maximal effect) of 36 nM. A 100 % inhibition of thymidine incorporation and a 40 % decrease in cell numbers in cultures of fetal calf erythroid cells were observed at a protein concentration of 840 nM. No effects could be seen in cultures of 3T3 cells used as controls. Human apolipoprotein H had no inhibitory activity in any of the cell cultures tested, suggesting a species-specificity or a different structure or function for the bovine heparin-binding protein.

INTRODUCTION

Fetal bovine serum is the most widely used supplement for the growth and maintenance of cells in culture. During the purification of heparin-binding growth factors from serum, we noticed that the mixture of proteins obtained after affinity chromatography on heparin–Sephacrose and anion-exchange chromatography contained factor(s) which were able to inhibit thymidine incorporation into fetal calf erythroid cells. In this paper, we describe the isolation and properties of one of these inhibitory factors. Its *N*-terminal sequence and its molecular mass suggest that this factor is the bovine equivalent of the human apolipoprotein H (apo H, β_2 -glycoprotein I), a plasma protein of unknown function [1]. Nevertheless, several studies on the possible role of human apo H have revealed some interesting characteristics of this protein, such as its association with circulating plasma lipoproteins [2], activation of lipoprotein lipase [3], modulation of platelet adenylate cyclase [4,5], inhibition of platelet prothrombinase activity [6] and inhibition of the activation of Hageman factor [7].

MATERIALS AND METHODS

Materials

Fetal bovine serum was obtained from Flow Laboratories, Rockville, MD, U.S.A. Human apo H (β_2 -glycoprotein I) was obtained from Behringwerke, Marburg, Germany. Balb 3T3 cells were from the American Tissue Type Culture Collection, Rockville, MD, U.S.A. [*methyl*- 3H]Thymidine (specific radioactivity 50 Ci/mmol) was from ICN Biochemicals. Heparin–Sephacrose was purchased from Pharmacia, Dorval, Canada, and Zetachrom 250 QAE capsules were from Chemical Dynamics Corporation, South Plainfield, NJ, U.S.A.

Thymidine incorporation assay

We have tested the capacity of the purified serum proteins to stimulate (or inhibit) thymidine incorporation in fetal calf

erythroid cells using a serum-free culture system of fetal calf erythroid cells which has been described in detail elsewhere [8]. In summary, primary cultures of fetal calf erythroid cells are incubated with the protein to be tested in a serum-free modified F-12 medium supplemented with 30 μ g of bovine transferrin/ml (Sigma) for 20 h at 37 °C. Then the cells are incubated with [3H]thymidine for 1 h. The cells are then washed, precipitated with cold trichloroacetic acid and counted for radioactivity in a scintillation counter [8].

Isolation procedure

Heparin-binding proteins from fetal bovine serum were prepared by affinity chromatography on heparin–Sephacrose columns using a modification of the method described by Gospodarowicz *et al.* [9]. A 100 ml sample of serum was applied on to a 1.6 cm $\times$ 10 cm heparin–Sephacrose column previously equilibrated with 0.01 M-Tris/HCl buffer (pH 7.0) containing 0.1 M-NaCl at a flow rate of 30 ml/h. The column was washed with the same buffer to eliminate all unbound proteins, which were monitored by measuring the absorbance of the eluted fractions at a wavelength of 280 nm. Then the heparin-bound proteins were eluted using a linear gradient from 0.1 to 1.6 M-NaCl in 0.01 M-Tris buffer (pH 7.0) for 6.5 h at a flow rate of 30 ml/h. Portions were taken to test the thymidine-incorporation-stimulating activity of the fractions in the serum-free cell culture system indicated above [8]. The samples with thymidine-incorporation-stimulating activity were pooled, diluted with 10 vol. of 0.01 M-sodium phosphate buffer, pH 7.0, and passed through a Zetachrom 250 QAE ion-exchange capsule at a flow rate of 20 ml/min. The capsule was washed with 150 ml of 0.01 M-phosphate buffer to eliminate the non-bound proteins.

The bound proteins were eluted with a linear gradient from 0 to 0.6 M-NaCl in 5 mM-Hepes/HCl buffer (pH 6.0) at a flow rate of 15 ml/min for 40 min. Fractions of 15 ml were collected. Fractions with thymidine-incorporation-stimulating activity were applied to a 0.46 cm $\times$ 10 cm Brownlee reversed-phase column (Aquapore RP-300) with a 3 cm pre-column of the same

Abbreviations used: apo, apolipoprotein; ED_{50} , concn. causing 50 % of maximal effect.

‡ Address correspondence to this author at the Endocrine Laboratory, Royal Victoria Hospital.

material (Technical Marketing Associates, Mississauga, Canada). The proteins were eluted with a gradient prepared with solution A [1% (v/v) acetonitrile in 0.1% trifluoroacetic acid] and solution B (80% acetonitrile in 0.1% trifluoroacetic acid). The percentage of solution B was maintained at 0% for 5 min and then it was increased successively from 0 to 65% in 10 min, from 65 to 80% in 50 min and from 80 to 100% in 5 min. The flow rate was 0.3 ml/min. This separation step was repeated twice until the thymidine-incorporation-inhibiting fractions were separated from the thymidine-incorporation-stimulating fractions. Further purification was obtained by gel-permeation h.p.l.c. as described previously [10]. The final purification step employed reversed-phase h.p.l.c. using Aquapore columns as described above which gave a single peak of absorbance corresponding to the thymidine-incorporation-inhibiting activity.

Analytical methods

Acrylamide-gel electrophoresis in the presence of SDS and 2-mercaptoethanol was done by a modification of the method of Laemmli [11] using the Phorcast precast polyacrylamide gradient gel from Amersham (Oakville, Ontario, Canada). Sequence analysis from 400 pmol (measured by h.p.l.c. based on its absorbance at 214 nm) was performed using the Applied Biosystems pulsed liquid-phase sequencer [12]. The initial sequencing yield was 41%, which corresponds to the initial yield of most proteins in this type of sequencer. The average repetitive yield was 84%. Cell counts were measured using a haemocytometer, with Trypan Blue as vital stain.

RESULTS

Most heparin-binding proteins of fetal bovine serum elute from the heparin-Sepharose column at a NaCl concentration of

Fig. 1. Preliminary purification of the thymidine-incorporation-stimulating factors from fetal bovine serum

(a) Heparin-Sepharose chromatography of 100 ml of fetal bovine serum. After elution of all non-heparin-binding components, the heparin-binding proteins were eluted with a linear gradient of 0.1 to 1.6 M-NaCl. Portions were taken for the bioassay consisting of the incorporation of thymidine into calf liver erythroid cells (●). (b) Fractions 38–46 from (a) were passed through a Zetachrom 250 QAE capsule and eluted with a linear gradient of NaCl from 0 to 0.6 M. Δ , A_{280} ; —, [NaCl].

Fig. 2. H.p.l.c. purification of the 46 kDa inhibitor of thymidine incorporation

The thymidine-incorporation-stimulating fractions 25, 26 and 27 from Fig. 1(b) were separated further using a combination of reversed-phase and gel-filtration h.p.l.c. In this Figure we show some typical chromatograms obtained using these techniques. (a) Reversed-phase h.p.l.c. on Aquapore 300 columns using the gradient elution of acetonitrile and 0.1% trifluoroacetic acid indicated in the Materials and methods section. (b) Gel-filtration h.p.l.c. of the partially purified inhibitor of Fig. 2(a). (c) Final purification step of the 46 kDa inhibitor using the reversed-phase columns described in (a). ●, Thymidine incorporation into fetal calf erythroid cells; —, A_{280} . The volumes of the fractions were 0.3 ml for (a) and (c) and 0.5 ml for (b).

0.4–0.6 M-NaCl (Fig. 1a). Samples of the fractions eluted from the column were taken for the measurement of their capacity to stimulate thymidine incorporation in fetal calf erythroid cells (●). No erythroid cell inhibitory factors are evident at this step of purification. There were always two closely eluting fractions with thymidine-incorporation-stimulating activity. However, their amounts varied within different batches and therefore both were pooled (fractions 38–46) for a further purification step employing anion-exchange chromatography using Zetachrom 250 QAE capsules (Fig. 1b). If the fractions with the highest thymidine-incorporation-stimulating activity (25, 26 and 27) were pooled and applied to the Aquapore reversed-phase columns as indicated in Fig. 2(a), the presence of inhibitors of thymidine incorporation (arrow) contaminating the most prominent thymidine-incorporation-stimulating activity was evident. These inhibitory fractions were further purified by repeating this chromatographic step and then applying the samples to gel-permeation h.p.l.c. columns (Fig. 2b). The sample was finally purified and concentrated by again using the reversed-phase

	1	5	10	15	20
Fetal bovine	G	R T X P K P D	E	L P F S T V V P L K	R T Y
Adult human	G	R T C P K P D	D	L P F S T V V P L K	T F Y

Fig. 3. Comparison of the *N*-terminal sequence of the purified inhibitor and the *N*-terminal sequence of human apo H (β_2 -glycoprotein I)

The human apo H sequence was found using the program FASTP of the Protein Identification Resource of the National Biomedical Research Foundation, Georgetown University, WA, U.S.A. Boxes indicate sequence identity.

Aquapore columns (Fig. 2c). There were some variations in the base-line thymidine incorporation activity of control cell cultures. These were due to minor differences in the gestational ages of the fetuses used for the bioassay, to the inhibitory hyperosmotic effect of salts from aliquots of some chromatographic fractions (ion-exchange, heparin-Sephacrose) or to the addition of bovine serum albumin (Sigma, 150 μ g/ml) as carrier for the small samples of highly purified proteins (Fig. 2c). Commercial albumin preparations are known to contain erythroid-cell-stimulating factors [13,14] and can therefore increase the base-line levels of thymidine incorporation. The final yield of active protein isolated from 100 ml of serum was of 230 μ g. The purified fraction had an apparent molecular mass of 46 kDa in gel-permeation h.p.l.c., or 50 kDa by SDS/PAGE under reducing conditions (results not shown). The *N*-terminal sequence of the purified protein showed 81 % identity with the published sequence of human apo H (Fig. 3). The dose-response curve of the inhibition of thymidine incorporation is shown in Fig. 4 (●). No inhibition of thymidine incorporation was observed with human apo H (○). The bovine apo-H-like protein was not able to inhibit thymidine incorporation in cultures of 3T3 cells (◆). Although the highest concentration of bovine apo-H-like protein was able to completely inhibit thymidine incorporation using the standard incubation time of 20 h, this concentration decreased the actual cell number under the same conditions by 40 ± 4 % (mean $\pm$ S.E.M. of three determinations), as measured by using a haemocytometer and Trypan Blue as vital stain.

Fig. 4. Effect of the purified bovine apo-H-like protein on thymidine incorporation in calf liver cells

Fetal calf liver cells were incubated for 18 h with the protein isolated in Fig. 2(c) and for 1 h with [3 H]thymidine. The cells were washed with ice-cold Hank's balanced salt solution and the thymidine incorporated was measured as indicated in the Materials and methods section (●). Human apo H previously purified by h.p.l.c. using the same method as in Fig. 2(b) did not inhibit thymidine incorporation (○). The bovine apo-H-like protein did not inhibit thymidine incorporation in cell cultures of 3T3 fibroblasts (◆).

DISCUSSION

During the initial purification steps for heparin-binding growth factors for liver erythroid cells, it was not at all evident that inhibitory factors were present, because the very high thymidine-incorporation-stimulating activity effectively prevented their identification (Fig. 1). Only the use of reversed-phase h.p.l.c. allowed us to separate and purify these inhibitory factors. Further analysis of the nature of the fractions with thymidine-incorporation-stimulating or -inhibiting activities indicated the presence of several factors. We have recently found that the major heparin-binding erythroid-cell-stimulating factor of fetal bovine serum has similarity with erythropoietin or insulin-like growth factor II [15]. In this paper we have focused our attention on one of the major inhibiting factors, with an apparent molecular mass of 46 kDa. The *N*-terminal sequence of this factor was similar to that of human apo H which, to the best of our knowledge, has not been associated with cytostatic or cytotoxic events. Nevertheless, other heparin-binding apolipoproteins such as apolipoprotein B100 are inhibitors of lymphocytes, and the synthetic peptides based on two heparin-binding domains of apolipoproteins B and E have been shown to inhibit lymphocyte proliferation [16]. Furthermore, human apo H has a consensus sequence shared with antistasin, an inhibitor of blood coagulation and metastasis, and with many other proteins involved in malaria and viral infections, complement activation, metastasis and cell adhesion [17]. The inhibitory action of the bovine apo-H-like protein seems to be restricted to certain cell types, because it was not active in cultures of 3T3 fibroblasts. Furthermore, the inhibition is evident at concentrations which are about 100 times lower than the concentrations reported for human apo H in adult plasma [18]. Although the *N*-terminal sequences of the bovine and human proteins show a high degree of similarity, human apo H did not inhibit thymidine incorporation into calf liver cells at the same range of concentrations at which 100 % inhibition can be observed with the bovine protein. This could be due to a species-specific effect, or to differences in the sequence of other sections of the molecule. This could be further investigated by isolating large amounts of the protein in order to sequence its cyanogen bromide fragments, in particular the fragments with potential heparin-binding sites. Another possibility which cannot be excluded is that the bovine apo-H-like protein is carrying a potent low-molecular-mass inhibitor. Nevertheless, it is unlikely that a bound inhibitor would survive the very severe conditions of high acetonitrile concentrations and 0.1 % trifluoroacetic acid used during the purification procedure. Finally, a minor post-translational modification of the apo-H-like protein could confer special properties not found in a native apolipoprotein.

The inhibition of thymidine incorporation elicited by the apo-H-like protein is far more striking than the decrease in cell numbers under identical culture conditions. These results suggest that the action of this protein is mainly cytostatic rather than cytolytic. It is not known if the bovine apo-H-like protein plays a role during the fetal development of the liver. It is reasonable to assume that apo H, like many other plasma proteins, may be synthesized in hepatocytes. Therefore the development of hepatic functions during fetal life may result in an increase of the synthesis of this apo-H-like protein, which could be useful in reducing erythroid cell development in the liver in favour of a higher rate of erythropoiesis in the developing bone marrow at the end of gestation. Studies on the synthesis of this protein during fetal development could shed some light on its possible role in hepatic red cell formation. It is also possible that this protein may have cytostatic effects on other embryonic or fetal cells. It could be one of the factors present in fetal bovine serum which inhibit mouse embryonic cell proliferation *in vitro* [19].

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REFERENCES

1. Lozier, J., Takahashi, N. & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3640–3644
2. Polz, E. & Kostner, G. M. (1979) *FEBS Lett.* **102**, 183–186
3. Nakaya, Y., Schaefer, E. J. & Brewer, H. B. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1168–1172
4. Schousboe, I. (1979) *Biochim. Biophys. Acta* **579**, 396–408
5. Schousboe, I. (1980) *Thromb. Res.* **19**, 225–237
6. Nimpf, J., Bevers, E. M., Bomans, P. H. H., Till, Y., Wurm, H., Kostner, G. M. & Zwaal, R. F. A. (1986) *Biochim. Biophys. Acta* **884**, 142–149
7. Henry, M. L., Everson, B. & Ratnoff, O. D. (1988) *J. Lab. Clin. Med.* **111**, 519–523
8. Congote, L. F., Theberge, J., Mazza, L. & Li, Q. (1989) *J. Tissue Cult. Methods* **12**, 47–51
9. Gospodarowicz, D., Cheng, J., Lui, G. M., Baird, A. & Bohlen, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6963–6968
10. Congote, L. F. (1984) *Anal. Biochem.* **140**, 428–433
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
12. Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P. & Guillemin, R. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6507–6511
13. Congote, L. F. (1987) *In Vitro Cell. Dev. Biol.* **23**, 361–366
14. Congote, L. F. & Esch, F. (1987) *Biochem. Biophys. Res. Commun.* **149**, 1026–1032
15. Li, Q., Blacher, R., Esch, F. & Congote, L. F. (1990) *Biochem. Biophys. Res. Commun.* **166**, 557–561
16. Cardin, A. D., Bowlin, T. L. & Krstenansky, J. L. (1988) *Biochem. Biophys. Res. Commun.* **154**, 741–745
17. Dunwiddie, C., Thornberry, N. A., Bull, H. G., Sardana, M., Friedman, P. A., Jacobs, J. W. & Simpson, E. (1989) *J. Biol. Chem.* **264**, 16694–16699
18. Schwick, H. G. & Haupt, H. (1984) in *The Plasma Proteins*, vol. 4 (Putnam, F. W., ed.), pp. 167–220, Academic Press, New York
19. Loo, D. T., Fuquay, J. I., Rawson, C. L. & Barnes, D. W. (1987) *Science* **236**, 200–202

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