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ISOLATION OF TWO BIOLOGICALLY ACTIVE PEPTIDES, ERYTHROTROPIN I AND ERYTHROTROPIN II FROM FETAL CALF INTESTINE

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A bioassay based on the measurement of thymidine incorporation into trichloroacetic acid-insoluble materials in erythroid cell suspensions from fetal calf liver was used as the assay for purification of two small peptides (erythrotropins I and II) from fetal calf intestine. The peptides were purified using reversed-phase and gel permeation high performance liquid chromatography (HPLC). The two peptides have very similar amino acid compositions and a molecular weight of about 3500 daltons. Erythrotropin II stimulated thymidine incorporation and potentiated the action of erythropoietin in cultures of erythroid cells from fetal rat liver.

Erythropoietin, the most important of the factors regulating red cell formation, has been purified extensively from human urine (1). No attempt has been made to purify this glycoprotein or other factors acting on erythroid cells from fetal tissues. We have increased the sensitivity of our previously developed serum-free culture system of fetal calf liver cells (2) by using the incorporation of ^{3}H-thymidine (3) as a parameter of the erythropoietic response. This improved in vitro bioassay combined with the method of Bennett et al (4,5) for the extraction and purification of peptides has been used for the isolation of two factors from fetal calf intestine which strongly potentiate the action of erythropoietin in fetal liver cells.

MATERIALS AND METHODS

Thymidine incorporation assay. The livers from calf fetuses of 90 to 120 days of gestation were cut in small pieces and suspended in Hank's balanced salt solution. The pieces were forced several times through the hole of a syringe (3 mm diameter), the through a 18G needle and finally through a 20G needle. The cell suspension was centrifuged at 700xg for 5 min. The upper layer of the pellet containing a yellow ring of non-erythroid cells was removed with a Pasteur pipete and the remaining cells were suspended in a modified F12 medium

Abbreviations used: HPLC, high performance liquid chromatography; ODS-silica, octadecylsilyl-silica; TCA, Trichloroacetic acid; TFA, trifluoroacetic acid; U, Unit of activity as defined by the thymidine incorporation assay using step III sheep plasma erythropoietin as standard.

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supplemented with albumin, transferrin (2) and 0.1 mM ∝-thioglycerol (6). The few hepatocytes left were all dead as determined by the trypan blue technique. The cells were incubated in an humidified incubator with 5% CO_2 at 37°C. Each tube contained 10^7 cells in 0.5 ml medium containing the peptides to be tested or sheep plasma step III erythropoietin (Connaught Laboratories) at concentrations from 2 to 200 mU/ml. After a 20 h incubation 0.8 µCi (Methyl-^{3}H) thymidine was added per tube (New England Nuclear; final specific activity in F-12, 0.53 Ci/mmol). 1 h later the incubation was stopped by adding 10 ml ice cold Hank's balanced salt solution and the cells were centrifuged at 700 x g for 5 min. The cells were washed again in 5 ml Hank's solution, and then treated with 1 ml ice cold 0.75M TCA. The cold acid-insoluble materials were separated by centrifugation, hydrolyzed in 0.75 M TCA at 75°C and aliquots of the hydrolyzate were taken for counting. Erythropoietin concentrations were calculated from a standard curve using the non linear regression program of Duggleby (7). Erythropoietin (200 mU/ml) caused in average a 70 fold increase in thymidine incorporation over control cell cultures.

Thymidine incorporation studies with erythroid cells of fetal Sprague-Dawley rats (16 days of gestation; Charles River) were carried out as described above, using 10^6 cells per tube.

Isolation procedure. Whole intestines from calf fetuses (90 to 120 days of gestation) were frozen and stored in liquid nitrogen. Batches of 50 g were mixed with dry ice and cut in small pieces. The dry ice-intestine mixture was pulverized and mixed with 250 ml of the NaCl-HCl-HCOOH-TFA extraction medium of Bennett et al (4). The mixture was warmed up to about 4°C with continuous stirring and centrifuged at 10000 x g for 20 min. The supernatant was subjected to reversed-phase extraction with ODS-silica cartridges (Waters) using one cartridge for 10-12 ml solution. Each cartridge was washed with 20 ml ice-cold 0.1% (v/v) TFA and the adsorbed material was eluted with 3 ml acetonitrile: water: TFA (800:200:1). The volume of this ODS-silica extract containing 10 mg protein (measured by the method of Bradford (8)) was reduced to 33% with a stream of nitrogen. Two volumes of 0.1% TFA were added and the sample pumped directly onto two µBondapak C18 columns connected in series as described for globin chain separation (9). This time the fractions were eluted from the column at room temperature and at a flow rate of 1.5 ml/min using a linear gradient for 40 min starting with a mixture of acetonitrile: water: TFA (280:720:1) and finishing with 420:680:1. Fractions of 1.5 ml were collected. Each fraction was mixed with 0.5 ml acetonitrile containing 2-mercaptoethanol and TFA in order to obtain a final concentration of 3mM mercaptoethanol and 0.1% TFA. Aliquots were taken for the bioassay and the fractions were stored at -40°C. The fractions eluted from these columns which stimulated ^{3}H-thymidine incorporation into acid insoluble materials were evaporated under a stream of nitrogen until a volume of 100 µl was obtained. Then the sample was injected onto a Waters I-125 gel permeation HPLC column previouly equilibrated with acetonitrile: water: TFA (400:600:1) as indicated by Bennett et al (5). The column was eluted at a flow rate of 1 ml/min. Fractions of 0.3 ml were collected and mixed with 0.1 ml acetonitrile containing 2-mercaptoethanol-TCA as indicated above. Protein determinations after this chromatographic step were done by planimetry of the absorbance profiles at 214 nmn using bovine serum albumin as standard. The fractions containing stimulating activity as judged by thymidine-incorporation were purified using the 2 µBondapak C18 columns indicated above. This time we used a linear gradient for 40 min between acetonitrile: water: (TFA 310:690:1) and finishing with 390:610:1. The eluted fractions were mixed again with the mercaptoethanol-TFA mixture and stored frozen at -40°C. Amino acid analysis of the purified fractions was done after acid hydrolysis in constant boiling point HCl (Pierce) at 110°C for 20h.

RESULTS AND DISCUSSION

In vitro bioassays similar to the one described in the Methods section are ideal for the rapid and sensitive screening of factors acting on erythroid cells. In preliminary experiments we extracted different tissues using the ODS-silica cartridges indicated in Methods and we tested solutions of these extracts in F12 medium (10 µg protein/ml) for their capacity to stimulate ^{3}H-thymidine incoporation into acid-insoluble cell fractions. Of the tissues studied the intestinal extracts had the largest effect on thymidine incorporation and this tissue was used as a source for purification of erythropoietin-like factors.

The combination of reversed-phase extraction (4) and high performance liquid chromatography (4,5) was used for the purification of two substances (Fig. 1) which are similar to erythropoietin in at least one property, namely the stimulation of thymidine incorporation in liver erythroid cells. Fig. 1A shows the separation of 10 mg of ODS-silica extract from fetal calf intestine by reversed-phase HPLC as indicated in Materials and Methods. Only two major fractions (labeled 1 and 2) significantly stimulated the incorporation of thymidine in fetal calf liver cells. A total of 100% of the activity originally present in the extract is recovered in the fractions 1 and 2. The proportions of the fractions 1 and 2 were different in different batches of intestine extracts. The retention time of both substances is completely different from that found for erythropoietin under similar chromatographic conditions (Congote, submitted for publication). Three HPLC runs similar to that shown in Fig. 1A were necessary to process the complete extract of 50 g tissue. Fractions 1 and 2 were then further purified by gel permeation HPLC columns and eluted as indicated in Fig.1B and 1C. Their molecular weights (measured by the method of Bennett et al (5) using these columns were approximately 3500. These molecular weights are quite different from that of erythropoietin (1). The third chromatographic step shown in Figs. 1D and E gave purified preparations with activities of 4500 U/mg for fraction 1 and 12000 U/mg for fraction 2. It is too early to conclude that fraction 2 is more

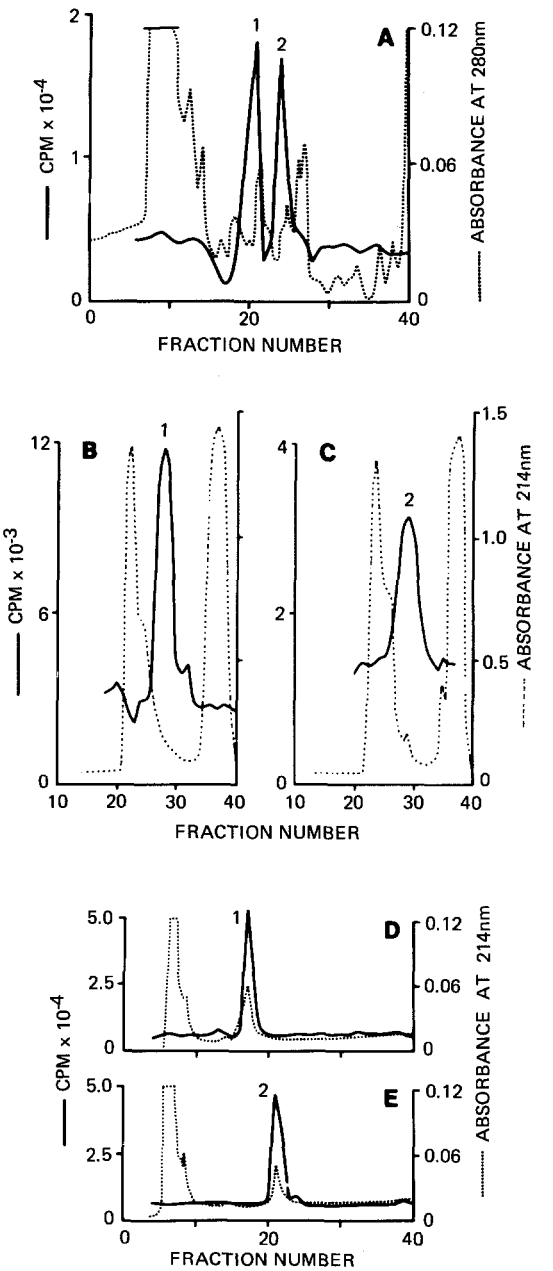

Fig. 1. Purification of erythropoietin-like factors from fetal calf intestine extracts. The continuous line indicates the thymidine incorporated into acid-insoluble cell fractions whereas the dotted line is the absorbance at 280 nm (A) or 214 nm (B-E). A (Step 1), separation of 10 mg ODS-extract from fetal calf intestine on two reversed-phase HPLC columns attached in series. Aliquots of the samples were used for the thymidine incorporation assay and Fractions 1 and 2 from three different chromatographic separations similar to that shown in Fig. 1A were pooled and applied to gel permeation HPLC columns (B and C, step 2). Those fractions with thymidine-incorporation stimulating activity were further purified using two reversed-phase columns as indicated in Methods (D and E, step 3). The activities of the fractions 1 and 2 in U/mg were: Step 1, 60 and 100; Step 2, 1200 and 2000; Step 3, 4500 and 12000, respectively. The recovery of activity in this preparation was 82%.

Table 1

Partial amino acid composition of the HPLC purified erythropoietin-like factors 1 and 2.

Amino Acid	Factor 1	Factor 2
Asx	2.4 ± 0.2	2.1 ± 0.2
Thr	1.1 ± 0.1	1.0 ± 0.04
Ser	3.2 ± 0.2	3.2 ± 0.4
Glx	3.6 ± 0.2	3.7 ± 0.3
Pro	1.3 ± 0.1	1.1 ± 0.1
Gly	4.2 ± 0.4	4.7 ± 0.9
Ala	1.7 ± 0.1	1.5 ± 0.1
Val	1.0 ± 0.1	1.3 ± 0.1
Met	0.3 ± 0.2	0.2 ± 0.3
Ile	0.7 ± 0.02	0.7 ± 0.1
Leu	1.9 ± 0.1	2.1 ± 0.2
Tyr	0.7 ± 0.05	1.0 ± 0.1
Phe	0.9 ± 0.1	0.9 ± 0.1
His	0.5 ± 0.1	0.7 ± 0.04
Lys	1.3 ± 0.2	1.2 ± 0.2
Arg	1.4 ± 0.1	1.2 ± 0.2

The values are the average of three determinations ± S.E.

potent than fraction 1, because losses in activity during the purification of each fraction can not be excluded. The purification obtained compared with the initial ODS extract was 7600- and 20000-fold for fractions 1 and 2, respectively. The partial amino acid analysis of fractions 1 and 2 shown in Table 1 indicated that although very few chromatographic steps have been used it is possible to obtain preparations with very constant amino acid compositions. The values in Table 1 were calculated by taking the average of the absolute amounts of the amino acids Thr, Pro,Val, Ileu and Phe as 1. In this way the addition of the molecular weights of the amino acids adjusted to the nearest integer gives a molecular weight of 3400 Daltons, which is very close to the molecular weight of 3500 daltons determined by gel permeation HPLC.

Figure 2 shows the effects of erythropoietin (EP) and fraction 2 on cell numbers (A) and thymidine incorporation (B) in cultures of fetal rat liver cells. Fraction 2 did not increase the number of cells in cultures of fetal rat liver after 20 h whereas erythropoietin caused a modest increase in the cell numbers ($P < 0.002$). Fraction 2 is less potent on thymidine incorporation in rat cells than in calf cells, because the effect of 0.1 U/ml of

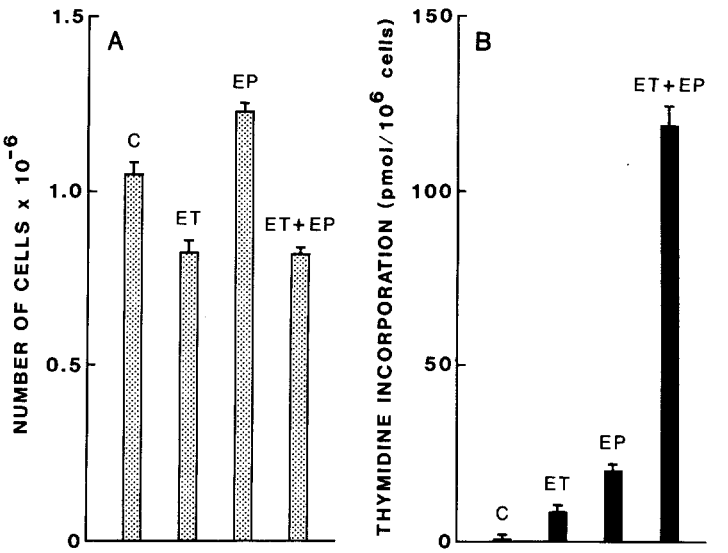

Fig. 2. Effect of fraction 2 (ET) and erythropoietin (EP) on cell numbers (A) and thymidine incorporation into acid insoluble materials (B) in cultures of rat liver cells. Liver cell preparations from rat fetuses (16 day of gestation) were incubated for 20 h in F12 medium with albumin and transferrin (controls, C) or in the same medium containing 0.1 U/ml ET, 0.1 U/ml EP and both ET and EP at the concentration of 0.1 U/ml each (ET + EP). After incubation the cell numbers and the thymidine incorporation were measured as indicated in Methods. The results are the mean ± S.E. of four different experiments each run in duplicates. Fraction 2 significantly increased the action of erythropoietin on thymidine incorporation ($P < 0.001$, Duncan-test).

fraction 2 does not correspond to 0.1 U/ml of erythropoietin. This difference may be explained by a species-specificty of this factor but also by the fact that the rat cells are isolated from rat fetuses at the end of liver erythropoiesis whereas the calf livers come from fetuses at the beginning of this period. The most striking effect of fraction 2 seems to be the potentiation of the erythropoietic response of erythropoietin (Fig. 2B), an effect that can also be observed on the stimulation of hemoglobin synthesis (Congote, in preparation). Fractions 1 and 2 have different physical and biological properties from those of erythropoietin. For this reason the names "erythrotropins I and II" are suggested for these factors because of their activity in erythroid cells and the potentiation by factor 2 of the effect of erythropoietin. The possible effects of these factors on other types of cells are now under investigation in our laboratory. The erythrotropins represent

a new family of compounds acting on erythroid cells which may exert their effects either alone or, together with erythropoietin.

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