

A PLASMA-BORNE SPECIFIC INHIBITOR OF THE PROLIFERATION OF HUMAN ESTROGEN-SENSITIVE BREAST TUMOR CELLS (ESTROCOLYONE-I)

ANA M. SOTO,* RENÉE M. SILVIA and CARLOS SONNENSCHNEIN

Tufts University Health Science Schools, Department of Anatomy and Cellular Biology, Boston, MA 02111, U.S.A.

(Received 28 October 1991; received for publication 10 August 1992)

Summary—Charcoal-dextran stripped serum/plasma supplemented media specifically inhibit the proliferation of estrogen-sensitive cells in culture conditions; estrogens cancel this effect. Here, we further characterize this phenomenon using human estrogen-sensitive breast cancer MCF₇ cells and human serum/plasma. The serum/plasma-borne inhibitory activity (estrocolyone-I) is a non-dialyzable, heat-stable (60°C × 2 h), protease-sensitive macromolecule and it is not extractable by organic solvents. Estrocolyone-I activity is retained after dialysis against 6 M urea or 10–100 mM dithiothreitol; however, simultaneous treatment with 6 M urea and 10–100 mM dithiothreitol completely abolishes its inhibitory activity. The inhibitory effect of serum is not due to serum albumin, nor to estrogen trapping by albumin or by sex hormone-binding globulin. Substantial purification was achieved by a combination of chromatographic techniques (dye-affinity, ion exchange, hydrophobic interaction chromatography). Estrocolyone-I activity seems to be due to a protein of an apparent native M_w of 70–80 kDa and an isoelectric point of 4.5–4.8.

INTRODUCTION

The regulation of cell proliferation by estrogens is incompletely understood. This subject is currently being explored under three different working hypotheses. The direct-positive hypothesis that proposes that estrogens *per se* trigger the multiplication of their target cells [1–4]. Alternatively, the indirect-positive hypothesis that proposes that estrogens induce the synthesis and/or release of growth factors [5] which, in turn, cause proliferation of their target organs. The autocrine mechanism [6], a variation of this latter hypothesis, proposes that estrogens would induce the synthesis and secretion of a variety of growth factors by their own target cells. In turn, these autocrine growth factors would elicit the proliferation of the same cells which secreted them [7, 8]. In contrast to predictions of the positive hypotheses, several reports showed that cells that are estrogen-dependent when inoculated into animals proliferate maximally in serumless medium regardless of the presence of estrogen [9–13]. Because of these data, we have been engaged in exploring the indirect-negative hypothesis: it proposes that estrogens neutralize the action

of blood-borne specific proliferation inhibitor(s) [10, 11, 14–16].

MCF₇ cells were chosen as a model because (a) they grow as estrogen-dependent tumors in athymic mice [17–19], (b) charcoal-dextran stripped human serum inhibits their proliferation in a dose-dependent pattern [10, 11], and (c) estrogens cancel the inhibitory effect of serum at physiological concentrations. Because these cells also proliferate maximally in serumless medium regardless of the presence of estrogens, we concluded that serum contains a specific inhibitor of the proliferation of estrogen-sensitive cells, estrocolyone-I; estrogens would merely neutralize this inhibitory effect [10, 11, 16]. Here, we describe the characterization and partial purification of estrocolyone-I.

METHODS

Cell culture procedures

A clonal population of the human breast cancer MCF₇ cell line was used in these experiments [10]. To assess the inhibitory effect of serum/plasma or partially purified serum/plasma fractions, 5×10^4 cells were seeded in Costar 3512 Multiplates in 5% fetal bovine

*To whom correspondence should be addressed.

serum (FBS); they were allowed to attach for 24 h. Then, 1 ml of Dulbecco's modification of Eagle medium (DME) plus 2 μ g transferrin (T)/ml and 100 ng insulin (I)/ml (IT-DME) alone or IT-DME plus (i) charcoal-dextran stripped (CD) serum or (ii) partially purified preparations were placed in each well. Each preparation was tested by triplicate in the presence and absence of 30 pM 17 β -estradiol (E₂). Phenol-red free media were used in all experiments [20]. At selected time intervals cells were lysed [10% Zapoglobin (Coulter Electronics, Hialeah, FL)] and nuclei counted on a Coulter Counter Model Zf₁. Previous to testing the inhibitory potency of partially purified estrocolyone-I, fractions were concentrated by ultrafiltration (YM30 Amicon membranes, nominal cutoff = 30 kDa) and dialyzed through cellulose membranes (nominal cutoff = 25 kDa) against a buffer suitable for tissue culture (TC buffer: 100 mM NaCl, 25 mM HEPES, pH 7.4). Estrogens were removed from serum and from partially purified plasma fractions by charcoal-dextran stripping [10, 11].

Purification procedures

Plasma coagulation factors were removed by precipitation with 80 mM BaCl₂ [21]. Matrex-green chromatography (Amicon Corp., Lexington, MA) of BaCl₂-treated plasma was used to remove human serum albumin (HSA). The column was equilibrated with 25 mM bistris-propane (BTP), pH 7.0, and 0.6 ml plasma were loaded/ml packed gel. The breakthrough was used for estrocolyone-I purification and the bound fraction was eluted with 2 M NaCl, 25 mM BTP, pH 7.0. This fraction was used in protease digestion experiments. For anion exchange chromatography, start buffers were 50 mM piperazine pH 6.0 and BTP, pH 7.0 and 9.5. Limit buffers contained 1 M NaCl. 50 to 200 mg Matrex-green breakthrough protein were injected into a FPLC MonoQ Hr10/10 column equilibrated with start buffer; separation was carried out by linear gradient elution. For hydrophobic interaction chromatography, samples were equilibrated in start buffer, injected into a phenyl-Superose HR5/5 column and resolved by linear gradient [50 mM sodium phosphate buffer containing 0.4 to 2 M (NH₄)₂SO₄ as start buffer and 20% acetonitrile-50 mM Na phosphate pH 7.0 as limit buffer]. To determine the pI of estrocolyone-I, chromatofocusing was performed in a MonoP HR25/5 column; pH gradients from 7.0 to 4.0 and from 5.5 to 4.0 were

generated with 10% Polybuffer 74. Separation by gel filtration in FPLC Superose 12 HR 16/50 (bed volume: 98 ml) and in Superdex 200 XK 26/60 (bed volume: 320 ml) columns were used for assessing the native molecular weight of estrocolyone-I. Molecular weight standards were used to monitor the elution pattern. FPLC instruments and reagents were from Pharmacia-LKB (Piscataway, NJ). All buffers contained 1 mM benzamidine, a protease inhibitor.

Protease hydrolysis

Charcoal-dextran stripped rooster serum was incubated at 37°C for 2 h with and without 0.25 mg trypsin/ml (8000 BAEE U/mg). 5000 U soybean trypsin inhibitor/ml were added to stop the proteolytic effect; after dilution to 10% with IT-DME, sera were tested for estrocolyone-I activity. Proteases were removed from human plasma by Matrex-green chromatography; aliquots were dialyzed against 50 mM CaCl₂-50 mM Tris-HCl, pH 8.0 for alpha-chymotrypsin and against 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM piperazine, pH 6.3 for papain digestion. Aliquots were incubated at 37°C for 16 h with 1 ml plain agarose or 100 U of protease coupled to 1 ml agarose (Sigma Chemical Co, St Louis, MO). The digested preparations were dialyzed against TC buffer, diluted with IT-DME and assayed for estrocolyone-I activity.

Hexane, dichloromethane and acetone extraction of serum

5 ml CDHuS aliquots were acidified with 0.5 ml 10 N HCl and extracted with 5 ml hexane or dichloromethane; the solvent phase was evaporated and resuspended in 5 ml of 2 mg HSA/ml in IT-DME. 5 ml acetone cooled at -20°C were added dropwise to 5 ml CDHuS cooled at 4°C; after centrifugation (2000 g \times 15 min) the precipitate was resuspended and dialyzed against TC buffer. The supernatant was evaporated in a Speed Vac concentrator (Savant Instruments, Long Island, NY) and resuspended in 2 mg/ml HSA in IT-DME.

Acid extraction of serum

To determine whether or not estrocolyone-I activity was due to chalone-like oligopeptides [22], CDHuS was dialyzed against 50 mM NH₄HCO₃, lyophilized, resuspended in water and extracted with 2 M acetic acid. Precipitated proteins were removed by centrifugation at 100,000 g and the supernatant was dialyzed

twice against 10 vol of 0.5 M acetic acid. These supernatants were dialyzed against TC buffer, brought to original volume and assayed for cell proliferation at concentrations of 5 and 10%.

Denaturation studies

The effect of urea and DTT on estrocolonyone-I activity was studied as follows: (a) urea crystals (ultrapure grade, Schwarz-Mann Biotech, Cleveland, OH) were added to 10 ml of CDHuS to a final concentration of 6 M. Comparable aliquots were treated with (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT. After 2 h incubation at room temperature, these preparations were dialyzed twice at 4°C for 16 h against 10 vol of 25 mM BTP containing: (a) 6 M urea, (b) 10 mM DTT, (c) 100 mM DTT, (d) 6 M urea plus 10 mM DTT and (e) 6 M urea plus 100 mM DTT, respectively. Precipitated material was removed by centrifugation and each preparation was dialyzed against TC buffer.

Cell cycle analysis

Cells were harvested in 0.2% triton X-100 in Hanks' balanced salt solution containing 2 μ g 4'-6-diamidino-2-phenylindol dihydrochloride/ml [23]. 10^4 cells were analyzed by flow cytometry with a 350/356 krypton laser [24].

HSA

Depending on the concentration range expected, two methods were used. Levels between 5 and 60 mg/ml were measured by the standard bromocresol green method [25]. Levels below 5 mg/ml were measured by a double antibody ELISA based on the assay described for fibronectin by Rennard *et al.* [26]. Briefly, plate coating was performed using 100 ng HSA/0.2 ml for 24 h at 4°C; simultaneously, the first antibody (rabbit anti-HSA 1:4000) was incubated with antigen in liquid phase. After washing the plates and saturating plastic binding sites with 0.5% gelatine, the HSA-first antibody mixture was placed into the coated wells, incubated for 2 h at 22°C; after washing, 0.2 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma) (1:500) were added for 2 h, washed and incubated for 30 min with 0.2 ml of 1 mg/ml *p*-nitrophenyl phosphate; the reaction was stopped with 0.05 ml 3N NaOH and read at 405 nm. This indirect ELISA was very sensitive (10 ng/ml) and reproducible. Anti-HSA serum was obtained as described previously [27].

Sex hormone binding globulin (SHBG)

Purification was done by ligand affinity chromatography [28]; the column breakthrough was used as SHBG-free plasma.

Unless specified otherwise, all chemicals were purchased from Sigma; TGF- β was from Collaborative Research (Waltham, MA). Porcine insulin was a gift from the Eli Lilly Co. (Indianapolis, IN).

RESULTS

Effects of serumless medium, CDHuS and E₂ on proliferation

Cell proliferation curves revealed the following pattern: proliferation rates were maximal for the first 48 h regardless of the presence of E₂ or CDHuS and later, they decreased significantly only in CDHuS supplemented medium [Fig. 1(A)]. At 96 h, 95% of cells in CDHuS had a G₀/G₁ DNA content [Fig. 1(B), bottom panel] while in IT-DME with or without E₂ and CDHuS plus E₂ cells proliferated maximally; the DNA content distribution was 57% in G₀/G₁, 36% in S, and 7% in G₂ [Fig. 1(B), top panel].

Estrocolonyone-I bioassay

The best estimate of proliferative activity is doubling time (t_D) measured during the exponential phase [29]. For practical reasons, a single time point assay was developed based on the evidence presented in Fig. 1. This assay measures the cell number achieved by an inoculum of 5×10^4 cells after 96 h of exposure to plasma fractions added to IT-DME both in the presence and absence of 30 pM E₂. A control for maximal proliferation yield is given by a similar cell inoculum grown in IT-DME. A standard curve of estrocolonyone activity is simultaneously run using serial dilutions of CDHuS. One unit of estrocolonyone-I activity is defined as the amount necessary to inhibit a population doubling of an inoculum of 5×10^4 cells/well in 1 ml of IT-DME after 96 h of exposure. Estrocolonyone-I was measured as described above because cell proliferation is an exponential function; therefore, conditions had to be set to allow maximal proliferation rates in the absence of inhibitor as depicted in Fig. 1(A) (cells cultured in IT-DME proliferated maximally as did cells in CDHuS plus E₂). One estrocolonyone-I unit is contained in 3 mg serum protein (5% serum). Estrocolonyone-I content is measured by serial dilution; the assay measures the lowest dilution to show an activity equal to 1 U.

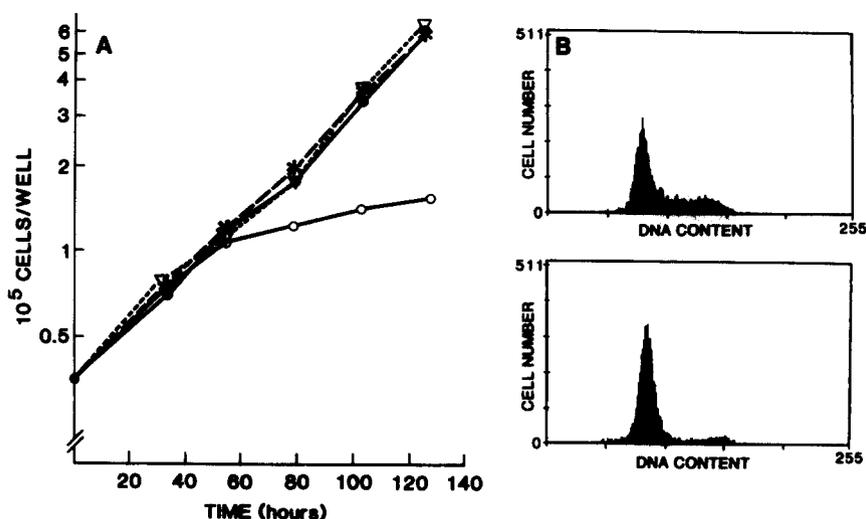


Fig. 1. (A) Cell proliferation curves of cloned MCF₇ cells grown in: IT-DME with (*—*) and without 30 pM E₂ (▽—▽); 10% CDHuS, both in the presence (●—●) and absence (○—○) of 30 pM E₂. Values indicate the mean of 4 experimental measurements; because SD was less than or equal in magnitude to the size of the plotted data point bars were not plotted. (B) Cell cycle profile by flow cytometry. Upper panel, cells in 10% CDHuS plus 30 pM E₂. Lower panel, cells in 10% CDHuS show a G₀/G₁ profile.

On the nature of estrocolonyone-I activity

To assess whether or not estrocolonyone-I is a small molecule, serum was extensively dialyzed against 100 mM NaCl, 25 mM HEPES, pH 7.4; membranes of nominal M_w cut-off ranging from 3.5 to 25 kDa retained estrocolonyone-I activity (not shown). These results indicate that either estrocolonyone-I is not a small molecule, or that it is tightly bound to a macromolecule. In addition, organic solvents such as acetone (Table 1), hexane and dichloromethane failed to

extract estrocolonyone-I activity from serum; this observation suggests that estrocolonyone-I is not a steroid-like molecule.

Acid extraction of CDHuS was performed to assess whether or not estrocolonyone-I activity was due to small chalone-like peptides; these peptides bind to macromolecules and are released from them by acid treatment [22]. Estrocolonyone-I, unlike chalones, was retained in the macromolecular compartment (Table 1).

Dialysis against 6 M urea, 25 mM HEPES, pH 7.4 also retained estrocolonyone-I activity (not

Table 1. Characterization of estrocolonyone-I: effect of serum components on the proliferation of MCF₇ cells

Source	Cell number ^a			Control ^b
	Without E ₂	0.3 nM E ₂	E ₂ + / E ₂ - ratio	
5% CDHuS ¹	130 ± 7	389 ± 31	3.0	375 ± 2
25 kDa membrane cutoff ¹	216 ± 4	482 ± 2	2.2	381 ± 18
6 M urea ²	83 ± 2	217 ± 27	2.6	375 ± 2
6 M urea, 10 mM DTT ²	370 ± 12	381 ± 18	1.0	375 ± 2
100 mM DTT ²	215 ± 1	521 ± 5	2.4	520 ± 9
2 M acetic acid extract ²	67 ± 2	160 ± 2	2.4	424 ± 13
Acetone extract ³	333 ± 12	351 ± 8	1.1	310 ± 3
Acetone pellet ⁴	125 ± 9	320 ± 8	2.6	310 ± 3
30 nM SHBG ⁵	133 ± 8	165 ± 10	1.2	318 ± 3
5% SHBG-free plasma ⁶	242 ± 8	472 ± 5	2.0	524 ± 5
2 mg HSA/ml ⁷	361 ± 12	389 ± 3	1.1	341 ± 9

^aCell number is expressed as cells/well × 10³ ± SD. ^bControl is the cell yield of a similar inoculum grown simultaneously in IT-DME. ¹: 5% CDHuS. ²: CDHuS aliquots were treated as indicated; retained proteins were dialyzed against TC buffer and assayed at 3 mg/ml IT-DME. ³: extracts were dried down and resuspended to the original serum volume with IT-DME containing 2 mg/ml HSA (Sigma, Cat No. A8763). ⁴: pellet was resuspended to the original serum volume with TC buffer, dialyzed against TC buffer and diluted to 5% with IT-DME. ⁵: SHBG was purified, molarity was calculated from its androgen binding capacity; after dialysis against DB, SHBG was diluted with IT-DME. ⁶: The affinity column breakthrough was dialyzed against DB and diluted with IT-DME. ⁷: Sigma, Cat No. A8763.

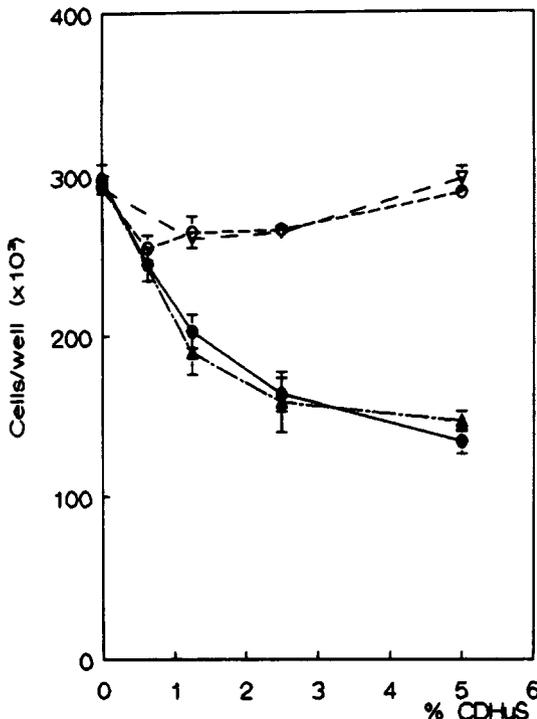


Fig. 2. MCF₇ cell yields were measured after 96 h of exposure to variable concentrations of: (i) CDHuS (normal donor) alone (●—●), or (ii) plus 30 pM E₂ (○—○), (iii) CDHuS (analbuminemic donor) alone (▲—▲), or (iv) plus 30 pM E₂ (▽—▽).

shown). Activity was not affected by dialysis against 10–100 mM DTT, 100 mM NaCl, 25 mM HEPES, pH 7.4; however, dialysis against 6 M urea, 10–100 mM DTT, 25 mM HEPES, pH 7.4 resulted in complete loss of estrocolyone-I activity (Table 1). These results are consistent with the notion that estrocolyone-I is a macromolecule; the inactivation effect of DTT only in the presence of 6 M urea suggests that a disulfide bond necessary for estrocolyone-I activity is either not accessible or it regenerates readily in non-denaturing conditions.

To assess whether or not proliferation inhibition by serum was due to trapping of estro-

gens by the serum proteins SHBG and HSA, these purified proteins were added to IT-DME. Neither inhibited MCF₇ cell proliferation; moreover, SHBG-free plasma obtained by adsorption of SHBG to cortisol-Sepharose retained the estrocolyone-I activity. TGF- β was marginally inhibitory at 0.1 nM but this effect was not cancelled by E₂. Cell yields were: 118,000 \pm 11,000 in IT-DME; 86,000 \pm 7000 in IT-DME + TGF- β and 72,000 \pm 4000 in IT-DME + TGF- β + 0.3 mM E₂.

Estrocolyone-I activity in analbuminemic human serum

To ascertain whether or not estrocolyone-I activity is due to HSA as proposed by Laursen *et al.* [30], three strategies were implemented: (a) chromatographic separation as reported below, (b) testing the inhibitory activity of commercially obtained HSA (Table 1), and (c) testing the inhibitory activity of serum from an analbuminemic donor [31]. The HSA concentration in analbuminemic serum was <0.1 mg/ml, while in the normal serum used as control the HSA concentration was 40 mg/ml. Figure 2 compares the dose-response curve to normal and analbuminemic CD serum; both curves are superimposable indicating that estrocolyone-I activity is present in analbuminemic serum.

Protease treatment

Human serum contains macromolecular protease inhibitors such as α_1 -anti-trypsin; this precludes the use of trypsin treatment of whole serum to assess whether or not estrocolyone-I is a trypsin-sensitive macromolecule. CD-avian serum also inhibits the proliferation of MCF₇ cells but, unlike human serum, it does not contain α_1 -anti-trypsin. Trypsin digestion of rooster serum resulted in loss of inhibitory

Table 2. Protease sensitivity of serum estrocolyone-I activity

Treatment	Control			Enzyme-treated		
	No E ₂	0.3 nM E ₂	E ₂ + / E ₂ - ratio	No E ₂	0.3 nM E ₂	E ₂ + / E ₂ - ratio
Trypsin ^a	180 \pm 8	355 \pm 14	2.0	285 \pm 16	324 \pm 24	1.1
α -Chymo-trypsin ^b	91 \pm 3	287 \pm 76	3.2	174 \pm 32	265 \pm 7	1.5
Trypsyn ^b	114 \pm 3	259 \pm 13	2.3	168 \pm 33	219 \pm 8	1.3
Papain ^b	144 \pm 2	395 \pm 10	2.7	161 \pm 3	168 \pm 37	1.0

^aCD-rooster serum was treated with 0.25 mg trypsin/ml, 2 h at 37°C; 5000 U soybean trypsin inhibitor/ml were added, and serum was assayed at 10%.

^b1 ml agarose (control) or immobilized enzyme (100 U/ml agarose) were incubated with 5 ml Matrex-green fraction; after incubation for 16 h, at 37°C and dialysis samples were treated in culture at 10% dilution. Results are expressed as the mean \pm SD of a set of three experiments.

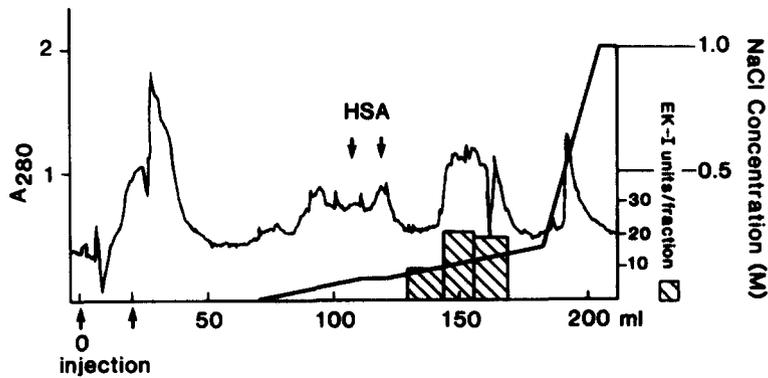


Fig. 3. 50 mg Matrex-green eluate were chromatographed through a MonoQ HR10/10 column. Start buffer: 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6. Limit buffer: 1 M NaCl in start buffer.

activity (Table 2). Human plasma was treated with 80 mM BaCl_2 to remove endogenous proteases and chromatographed through Matrex-green agarose to remove endogenous protease inhibitors. The retained fraction containing 50% of the estrocolyone-I activity and most HSA was eluted with 2 M NaCl, 25 mM HEPES, pH 7.0, dialyzed, and digested with proteases covalently bound to the agarose gel as described in Methods. Protease-treated human estrocolyone-I fractions no longer inhibited MCF₇ cell proliferation (Table 2).

Separation of estrocolyone-I and HSA

Estrocolyone-I and HSA coprecipitated with $(\text{NH}_4)_2\text{SO}_4$ between 60 and 70% saturation. HSA and estrocolyone-I were retained by and coeluted from blue-Sepharose. Matrex-green also bound HSA; the breakthrough fraction contained only 15% of the HSA load with an estrocolyone-I yield of 50%. This fraction was used for further estrocolyone-I purification. A 1.5- to 3-fold purification was achieved by this procedure.

Ion exchange chromatography of the Matrex-green breakthrough

Estrocolyone-I was adsorbed to cation exchangers at pH 4.0 and to anion exchangers at pH 6.0. Chromatographic titration curves were performed on a MonoQ column; estrocolyone-I and HSA coeluted at pH 7.0 and 8.0 and were resolved at pH 6.0 and 9.5. Binding at pH 6.0 and adding 20% acetonitrile and 2 mM DTT to the start and limit buffers improved separation (Fig. 3). Estrocolyone-I activity was eluted between 120 and 170 mM NaCl. In a series of 3 experiments the purification achieved through this step was 30- to 40-fold; the overall purification was 40- to 60-fold. A representative experiment is shown in Table 3.

Hydrophobic interaction chromatography (HIC)

Complete estrocolyone-I adsorption was achieved at 2 M $(\text{NH}_4)_2\text{SO}_4$; estrocolyone-I was eluted with a linear gradient. This chromatographic step resulted in a 7- to 14-fold purification when performed after Matrex-

Table 3. Estrocolyone-I purification by anion exchange chromatography

Fraction	Protein content ^a	Specific activity ^b	Total yield ^c	Yield (%) ^d	Purification ^e
BaCl_2 -plasma	155.00	1.33	199.0	100	1.0
Matrex-green breakthrough	50.00	2.00	100.0	50	1.5
MonoQ fraction No. 5	1.38	11.40	15.7	8	7.5
MonoQ fraction No. 6	0.25	80.00	20.0	10	60.0

Purification procedures are described in Methods. Anion exchange chromatography was performed in a MonoQ HR10/10 column; the start buffer was 20% acetonitrile, 2 mM DTT, 1 mM benzamidine, 50 mM piperazine, pH 6, and the limit buffer was 1 M NaCl in start buffer.

^aProtein content is expressed in mg.

^bSpecific activity is expressed in estrocolyone-I U/mg protein.

^cTotal estrocolyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolyone-I preparation.

^dPercent yield is calculated as $100 \times$ total estrocolyone yield of each purification step/estrocolyone-I yield of the starting material (BaCl_2 -plasma).

^ePurification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

Table 4. Estrocolyone-I purification by hydrophobic interaction chromatography

Fraction	Protein content (mg)	Specific activity ^b	Total yield ^c	Yield (%) ^d	Purification ^e
BaCl ₂ -plasma	110.00	1.00	110.0	100	1.0
Matrex-green breakthrough	31.96	2.20	70.3	63	2.2
2 M (NH ₄) ₂ SO ₄ supernatant	13.60	5.00	68.0	61	5.0
Phenyl Superose fraction No. 6	0.18	41.70	7.5	10	41.7
Phenyl Superose fraction No. 7	0.21	35.70	7.5	10	35.7

Purification procedures are described in Methods. Hydrophobic interaction chromatography was performed in a Phenyl Superose HR5/5 column; the start buffer was 2 M (NH₄)₂SO₄, 50 mM sodium phosphate pH 7 and the limit buffer was 20% acetonitrile, 50 mM sodium phosphate, pH 7.

^aProtein content is expressed in mg.

^bSpecific activity is expressed in estrocolyone-I U/mg protein.

^cTotal estrocolyone-I yield is calculated as the product of the protein content by the specific activity of the estrocolyone-I preparation.

^dPercent yield is calculated as 100 × total estrocolyone-I yield of each purification step/estrocolyone-I yield of the starting material (BaCl₂-plasma).

^ePurification is calculated as the ratio between the specific activity achieved after a purification step and the initial specific activity of the starting material.

green chromatography; the overall purification achieved was 37- to 47-fold. A representative experiment is shown in Table 4. Phenyl Superose chromatography of the MonoQ estrocolyone-I fraction resulted in a further 10-fold purification (Fig. 4).

Estrocolyone-I isoelectric point by chromatofocusing

HSA-free estrocolyone-I obtained by MonoQ chromatography was chromatofocused using a 5.5 to 4.0 pH gradient. The inhibitory activity eluted at pH 4.5 to 4.8 (Fig. 5).

Estrocolyone-I molecular weight

Chromatography of CDHuS through a Superdex-200 column resulted in coelution of HSA and estrocolyone-I (not shown). Estrocolyone-I purified by anion exchange chromatography eluted from a Superose 12 column at volumes corresponding to a M_w of 70–90 kDa (Fig. 6).

Comparable molecular weight was obtained using 6 M urea in the running buffer.

DISCUSSION

We described the inhibitory effect of serum on the proliferation of both human and rodent estrogen sensitive cells [10, 11, 14–16]. Estrogens cancel this inhibitory effect by mechanisms not fully understood [16]. Herein, we describe physicochemical properties of the molecule putatively responsible for the inhibitory effect of serum, namely, estrocolyone-I. The proliferation-inhibiting activity remained in the macromolecular compartment after extensive dialysis against 0.5 M acetic acid through membranes of a nominal cutoff = 25 kDa and was not extracted by organic solvents (Table 1). Protease treatment of unfractionated mammalian serum was hindered by endogenous protease inhibitors; removal of protease inhibitors from human

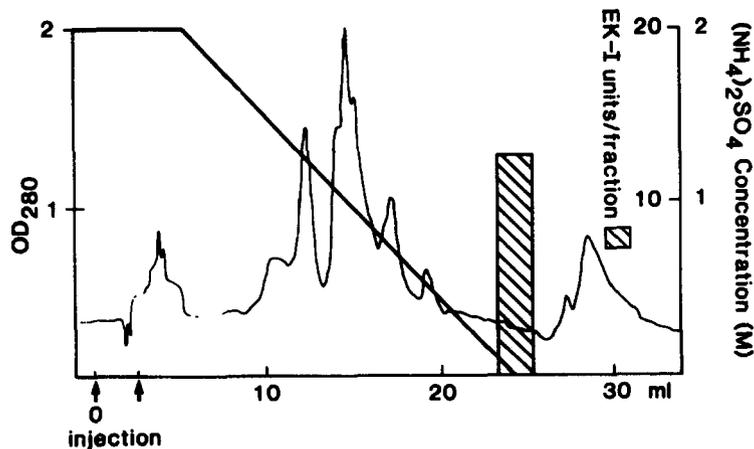


Fig. 4. 10 mg MonoQ fraction were chromatographed through a phenyl Superose HR5/5 column. Start buffer: 2 M (NH₄)₂SO₄, 50 mM sodium phosphate pH 7. Limit buffer: 20% acetonitrile, 50 mM sodium phosphate, pH 7.

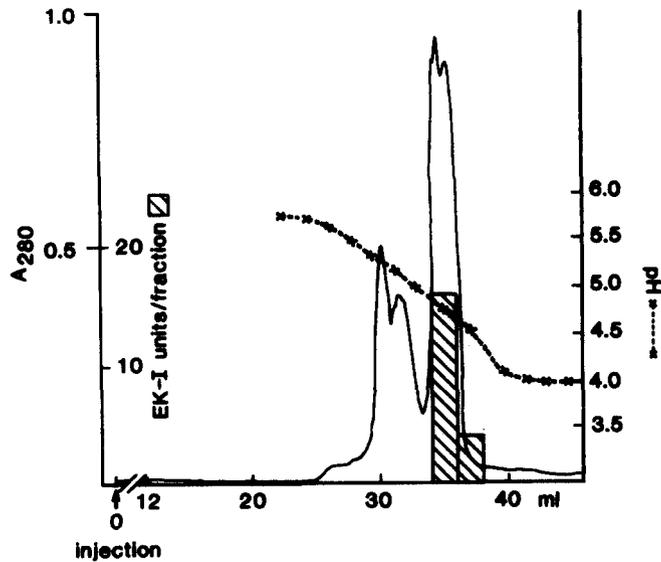


Fig. 5. Chromatofocusing profile of the MonoQ fraction. 25 mg protein were injected into a MonoP HR25/5 column equilibrated with 50 mM piperazine pH 5.5. After isocratic elution with this buffer, separation was achieved with a self generated pH 5.5–4 gradient by 10% Polybuffer pH 4.

plasma by Matrex-green chromatography followed by protease treatment completely abolished the inhibitory activity (Table 2). Trypsin treatment of chicken serum, which lacks anti-trypsin activity, significantly reduced estrocolyone-I activity. Reduction of disulfide bonds by DTT destroyed estrocolyone-I activity only when performed in the presence of denaturing concentrations of urea, suggesting that either critical disulfide bonds are not accessible in the native conformation or that they regenerate correctly only in this state. These results strongly suggest that estrocolyone-I is not a TGF- β -like molecule, since TGF- β activity is irreversibly destroyed by 10–100 mM DTT. Moreover, TGF- β did not significantly inhibit the proliferation of MCF₇ cells. This is an agreement with the results reported by Laursen

et al. [30]. Acid extraction was performed to determine whether or not estrocolyone-I was a chalone-like oligopeptide; these experiments confirmed the macromolecular nature of estrocolyone-I activity. These data suggest that estrocolyone-I is a polypeptide; henceforth, protein purification methods were used for estrocolyone-I characterization.

(NH₄)₂SO₄ precipitation is often used to reduce the contaminant burden to the following chromatographic step. Estrocolyone-I and HSA coprecipitated with (NH₄)₂SO₄; because HSA is the most abundant plasma protein, this fractionation procedure was ruled out. These two moieties also coeluted by Cibacron blue chromatography. To determine whether or not the inhibitory activity of serum was due to albumin [30], the inhibitory activity of HSA

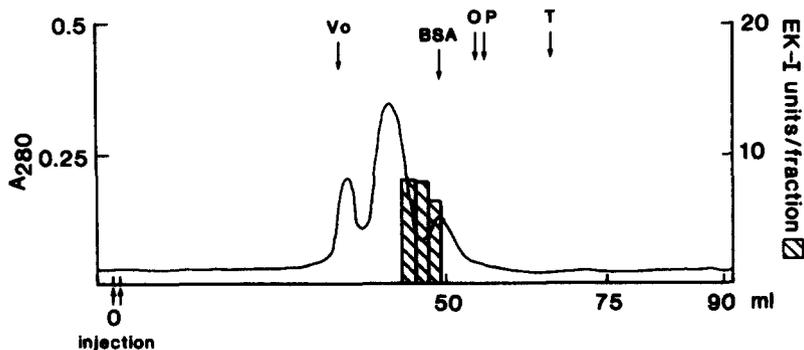


Fig. 6. Gel filtration profile of the MonoQ fraction. 30 mg protein (2 ml) were injected into a Superose 12 HR16/50 equilibrated with 100 mM NaCl, 50 mM BTP pH 7. Arrows indicate the elution of molecular weight markers. V₀: void volume (blue dextran), BSA: bovine serum albumin (66 kDa), O: ovalbumin (45 kDa), P: pepsin (34.7 kDa) and T: trypsinogen (24 kDa).

(Table 1) and of serum from an analbuminemic donor were assessed (Fig. 2). These results indicated that estrocolyone-I activity was not due to HSA.

HSA was removed by adsorption to Matrex-green. Estrocolyone-I isoelectric point was 4.5–4.8; the native molecular weight was 70–80 kDa. Both results were obtained using chromatographic techniques; since estrocolyone-I detection relies on a bioassay, the use of analytical gel electrophoresis and isoelectric focusing is precluded. Partial purification was obtained by HIC and anion exchange chromatography of the Matrex-green fraction. Substantial purification was achieved by using Matrex-green to remove HSA, followed by anion exchange chromatography and HIC. These results suggest that (1) estrocolyone-I is a protein, and (2) its inhibitory effect is not due to mere estrogen trapping since SHBG and albumin fail to inhibit MCF₇ cell proliferation (Table 1).

Finally, these data strengthen the notion that a plasma-borne factor, estrocolyone-I, is responsible for the serum-induced inhibition of the proliferation of estrogen-sensitive MCF₇ cells. Further purification of estrocolyone-I is being pursued, a step necessary for a mechanistic understanding of its action and that of estrogens on the control of cell proliferation.

Acknowledgements—We recognize the capable technical support of Jonathan W. Wray. We are grateful to Dr H. Shapiro for performing the cytometry studies, to Dr D. A. Damassa for providing purified SHBG and to the analbuminemic serum donors. We appreciate the discussions held with Dr Barbara C. Furie on plasma protein purification strategies. This work was supported by NSF DCB-8711746, NIH CA 13410 and Aid for Cancer Research.

REFERENCES

- Katzenellenbogen B. S.: Dynamics of steroid hormone receptor action. *A. Rev. Physiol.* **42** (1980) 17–35.
- Darbre P., Yates J., Curtis S. and King R. J. B.: Effect of estradiol on human breast cancer cells in culture. *Cancer Res.* **43** (1983) 349–355.
- Stack G. and Gorski J.: Direct mitogenic effect of estrogen on the prepubertal rat uterus: Studies on isolated nuclei. *Endocrinology* **115** (1984) 1141–1150.
- Jordan V. C.: Biochemical pharmacology of antiestrogen action. *Pharmac. Rev.* **36** (1984) 245–276.
- Sirbasku D. A.: Estrogen-induction of growth factors specific for hormone-responsive mammary, pituitary and kidney tumor cells. *Proc. Natn. Acad. Sci. U.S.A.* **75** (1978) 3786–3790.
- Sporn M. B. and Todaro G. J.: Autocrine secretion and malignant transformation of cells. *New Engl. J. Med.* **303** (1980) 878–882.
- Rocheffort H., Chalbos D., Capony F., Garcia M., Veith F., Vignon F. and Westley B.: Effect of estrogen in breast cancer cells in culture: released proteins and control of cell proliferation. In *Hormones and Cancer* (Edited by E. Gurpide, R. Calandra, C. Levy and R. J. Soto). Alan R. Liss Inc, New York (1984) pp. 37–51.
- Lippman M. E., Dickson R. B., Bates S., Knabbe C., Huff K., Swain S., McManaway M., Bronzert D., Kasid A. and Gelmann E. P.: Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res. Treat.* **7** (1986) 59–70.
- Butler W. B., Kirkland W. L., Gargala T. L., Goran N., Kelsey W. H. and Berlinski P. J.: Steroid stimulation of plasminogen activator production in a human breast cancer cell line (MCF7). *Cancer Res.* **43** (1983) 1637–1641.
- Soto A. M. and Sonnenschein C.: Mechanism of estrogen action on cellular proliferation: Evidence for indirect and negative control on cloned breast tumor cells. *Biochem. Biophys. Res. Commun.* **122** (1984) 1097–1103.
- Soto A. M. and Sonnenschein C.: The role of estrogens on the proliferation of human breast tumor cells (MCF-7). *J. Steroid Biochem.* **23** (1985) 87–94.
- Barnes D. and Sato G.: Growth of a human mammary tumor cell line in serum-free medium. *Nature* **281** (1980) 388–389.
- Briand P. and Lykkesfeldt A. E.: Effect of estrogen and antiestrogen on the human breast cancer cell line MCF-7 adapted to growth at low serum concentration. *Cancer Res.* **44** (1984) 1114–1119.
- Soto A. M., Murai J. T., Siiteri P. K. and Sonnenschein C.: Control of cell proliferation: evidence for negative control on estrogen-sensitive T47D human breast cancer cells. *Cancer Res.* **46** (1986) 2271–2275.
- Soto A. M., Bass J. C. and Sonnenschein C.: Proliferative behavior of the cloned Syrian hamster tumor cells H301. *Cancer Res.* **48** (1988) 3676–3680.
- Soto A. M. and Sonnenschein C.: Cell proliferation of estrogen-sensitive cells: the case for negative controls. *Endocrine Rev.* **8** (1987) 44–52.
- Soule H. D. and McGrath C. M.: Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett.* **10** (1980) 177–189.
- Soule H. D., Vazquez J., Long A., Albert S. and Brennan M. J.: A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natn. Cancer Inst.* **51** (1973) 1409–1413.
- Brooks S. C., Locke E. R. and Soule H. D.: Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J. Biol. Chem.* **248** (1983) 6251–6253.
- Berthois Y., Katzenellenbogen J. A. and Katzenellenbogen B. S.: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 2496–2500.
- Blanchard R. A., Furie B. C., Kruger S. F., Waneck G., Jorgensen M. J. and Furie B.: Immunoassays of human prothrombin species which correlate with functional coagulant activities. *J. Lab. Clin. Med.* **101** (1983) 242–255.
- Eljio K. and Reichelt K. L.: Purification and characterization of a mitosis inhibiting epidermal peptide. *Cell. Biol. Int. Rep.* **8** (1984) 379–382.
- Taylor I. W.: A rapid single step staining technique for DNA analysis by flow microfluorometry. *J. Histochem. Cytochem.* **28** (1980) 1021–1024.
- Schapiro H. M.: *Practical Flow Cytometry*. Alan R. Liss, New York (1985).
- Rodkey F. L.: Direct spectrophotometric determination of albumin in human serum. *Clin. Chem.* **11** (1965) 478–487.
- Rennard S. I., Berg R., Martin G. R., Foidart J. M. and Gehron Robey P.: Enzyme-linked immunoassay for connective tissue components. *Analyt. Biochem.* **104** (1980) 205–214.

27. Reny J. L. and Soto A. M.: Human serum does not contain a high affinity estrogen-binding glycoprotein different from sex hormone-binding globulin. *J. Clin. Endocr. Metab.* **68** (1989) 938–945.
28. Ferlund P. and Laurell C. B.: A simple 2-step procedure for the simultaneous isolation of corticosteroid-binding globulin and sex hormone-binding globulin from human serum by chromatography on cortisol-Sepharose and phenyl-Sepharose. *J. Steroid Biochem.* **14** (1981) 545–552.
29. Sonnenschein C. and Soto A. M.: But . . . are estrogens *per se* growth promoting hormones? *J. Natn. Cancer Inst.* **64** (1980) 211–215.
30. Laursen I., Briand P. and Likkesfeldt A. E.: Serum albumin as a modulator on growth of the human breast cancer cell line, MCF-7. *Anticancer Res.* **10** (1990) 343–352.
31. Boman H., Hermodson C. A. and Motulsky A. G.: Analbuminemia in an american indian girl. *Clin. Genet.* **9** (1976) 513–526.