Isolation, characterization and synthesis of α -foetoprotein from neonatal-rat skin

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Monospecific anti-[rat α -foetoprotein(α -FP)] immunoglobulin G was coupled to CNBr-activated Sepharose-4B (4.5 mg/ml packed volume of gel) to yield an adsorbent. The immunoaffinity column was used to isolate α -FP from neonatal-rat skin. Purified skin α -FP was found to be immunologically and electrophoretically similar to serum α -FP. It yielded a single band with mol.wt. 68 000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. However, on polyacrylamide-gel electrophoresis under non-denaturing conditions, the α -FP displayed slow- and fast-moving variants similar to those observed in serum α -FP. A Scatchard plot of oestradiol binding to the α -FP yielded an association constant of $2.5 \times 10^9 \text{ M}^{-1}$ by dextran-coated-charcoal and $0.75 \times 10^8 \text{ M}^{-1}$ by Sephadex-gel-filtration procedures respectively. Skin explants from newborn rats were found to incorporate [^{14}C]leucine into immunoprecipitable intracellular α -FP. Cycloheximide inhibited the synthesis of α -FP in skin explant culture. Our results indicate that newborn-rat skin contains α -FP that is similar to serum α -FP and which may arise in neonatal-rat skin as a result of synthesis *in situ*.

α -Foetoprotein, a major plasma protein of the foetus, is synthesized by yolk sac and liver (Gitlin & Pericelli, 1970). Its concentration is very low in adults, except in patients with teratomas and hepatomas. Monitoring of α -FP concentration in serum provides a useful diagnostic indicator of tumours (Abelev, 1971; Alexander, 1972; Ruoslahti *et al.*, 1974).

α -FP binds oestrogens with high affinity (Liang-Tang & Soloff, 1972; Aussel & Masseyeff, 1978). Its presence as well as synthesis has already been demonstrated in some extrahepatic developing tissues such as uterus and brain. It has been proposed that oestrophilic α -FP may be involved in the differentiation and growth of oestrogen target tissues (Attardi & Ruoslahti, 1976; Smalley & Sarcione, 1980; Toran-Allerand, 1980; Ali *et al.*, 1981*b,c*, 1983*a*). Epidermal cells of the adult rat skin are also sensitive to steroid hormones (Rampini *et al.*, 1971).

The presence of an oestradiol-binding protein has already been demonstrated in adult-rat skin cytosol (Eppenburger & Hisa, 1972). However, no information is available regarding the identity and origin of

oestradiol-binding protein in skin during early development. We observed previously that α -FP is also present in immature-rat skin (Ali *et al.*, 1983*b*).

The aim of the present study was to isolate α -FP from neonatal-rat skin and compare its properties with α -FP present in serum. Our results indicate that skin α -FP is similar to serum α -FP in many properties and that the pool of oestrophilic α -FP in skin might be the result of its independent synthesis in skin.

Materials and methods

Sepharose-4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose DE-52, cytochrome *c* and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Agarose, oestradiol, oestrone and Coomassie Brilliant Blue were purchased from Sisco Research Laboratories, Bombay, India. Acrylamide and *NN'*-methylenebisacrylamide were the products of Koch-Light, Poole, Dorset, U.K. Ammonium persulphate, *NNN'*-tetramethylethylenediamine and SDS from BDH, Poole, Dorset, U.K., were also used in the present study. Lysozyme and Amido Black were obtained from E. Merck, Darmstadt, Germany. [$2,4,6,7\text{-}^3\text{H}$]oestradiol (sp. radioactivity 100 Ci/mmol) was obtained from New England Nuclear Corp., Boston,

Abbreviations used: α -FP, α -foetoprotein; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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MA, U.S.A. [^{14}C]Leucine was purchased from Bhabha Atomic Research Centre, Bombay, India. All other chemicals and reagents used were of AR grade.

Animals

Neonatal albino rats (1-day-old Charles Foster strain of either sex) maintained in the animal colony of this Institute were used throughout the study.

Foetal serum

Foetuses were decapitated and their blood collected. Sera thus obtained were stored frozen at -20°C .

Purification of serum α -FP and preparation of anti-(rat α -FP) serum

Serum α -FP was purified and thereafter antiserum against α -FP was raised as described by Ali *et al.* (1981a), who also investigated the specificity of the antiserum. The IgG fraction was prepared from anti- α -FP serum by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and ion-exchange chromatography [see Ali *et al.* (1981a) for details].

Preparation of anti-(rat α -FP)-Sephacrose

Sephacrose-4B was activated with CNBr as described by March *et al.* (1974). The extent of activation of Sepharose was monitored colorimetrically as described by Kohn & Wilchek (1978). Usually 6–8 μmol of CN^- ions were bound per ml packed gel volume (Kohn & Wilchek, 1978).

Anti-(rat α -FP) IgG was dissolved in 0.1 M-sodium bicarbonate buffer, pH 8.3, and mixed with the activated Sepharose (about 10 mg of protein/ml of gel) and left for 24 h at 4°C with constant and gentle stirring. After incubation, the gel was thoroughly washed with coupling buffer (0.1 M-sodium bicarbonate buffer) and the remaining active groups were blocked with 0.2 M-glycine. The gel was finally washed with 0.1 M-bicarbonate buffer, pH 8.3, and 0.1 M-sodium acetate buffer (pH 4.0) containing 0.5 M-NaCl; 4.5 mg of IgG was coupled/ml packed volume of gel.

Preparation of immature-rat skin cytosol

Newborn rats were killed by decapitation. The skin was removed from the dorsal side and washed thoroughly with cold 10 mM-phosphate-buffered saline, pH 7.4. The chilled tissue was homogenized (50%, w/v) in phosphate-buffered saline/EDTA (0.01 M) with a motor-driven Potter–Elvehjem homogenizer with a Teflon-coated pestle. The homogenate was centrifuged at 22 000 g for 10 min. The supernatant obtained was re-centrifuged at 100 000 g for 1 h. The clear supernatant (skin cytosol) was dialysed extensively against phosphate-buffered saline and was used for the isolation of skin α -FP by the immunoaffinity procedure.

Preparation of skin explants

Skin explants were prepared by using the procedure of Halprin *et al.* (1979). The dorsal side of the skin was removed by dissection, washed first with 70% (v/v) ethanol and then thoroughly with phosphate-buffered saline. The skin was placed on a Petri dish, stratum-corneum-side down, and freed of subcutaneous tissue by scraping to produce thin pieces of skin. These were then chopped into small pieces (2 mm^2) and used for investigating the synthesis of α -FP by skin.

α -FP synthesis and secretion by immature-rat skin

Newborn-rat skin explants were maintained in leucine-free Eagle's minimum essential medium containing 1.0 μCi of [^{14}C]leucine/ml of medium, 1 mM-glutamine, 20 mM-Hepes and antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml) in culture flasks. Cycloheximide 100 μg /ml of culture medium (Sigma) was added to some flasks 15 min before the addition of labelled leucine. Culture flasks were incubated at 37°C . Incubation was terminated after 2, 4 or 6 h and the culture medium recovered by decantation. Skin explants were washed several times with phosphate-buffered saline containing 0.1% leucine and homogenized in phosphate-buffered saline/0.01 M-EDTA. The homogenate was centrifuged at 22 000 g for 30 min at 4°C . The clear supernatants were treated as 'skin lysate'.

Portions of cell lysate and culture medium was mixed with 100 μl of a 1:10 dilution of foetal serum to provide sufficient carrier α -FP for immunoprecipitation of the labelled α -FP. The α -FP was precipitated by the addition of 0.5 ml of anti-(rat α -FP) and the mixture was kept at 37°C for 1 h and at 4°C overnight. The amount of anti-(rat α -FP) serum needed was determined by preliminary titrations to give maximum precipitation of the labelled α -FP. The immunoprecipitate was washed several times with phosphate-buffered saline/leucine to remove [^{14}C]leucine adsorbed non-specifically. Radioactivity was determined in a liquid-scintillation counter (Packard model 3330). Controls employed to check the immunoprecipitation procedure were: (i) fresh culture medium containing [^{14}C]leucine was subjected to immunoprecipitation with carrier α -FP and anti-(rat α -FP) serum, and (ii) fresh medium or culture medium containing labelled leucine was subjected to immunoprecipitation with rabbit IgG and guinea-pig anti-(rabbit IgG). No significant radioactivity was observed in these immunoprecipitates, thereby indicating that non-specific precipitation is negligible.

Analytical techniques

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a

standard. Ouchterlony (1958) double diffusion and immunoelectrophoresis (Grabner & Williams, 1955) were used to evaluate the homogeneity of purified α -FP; Laurell (1966) rocket immunoelectrophoresis was adopted to quantify α -FP. Polyacrylamide (10%, w/v)-gel electrophoresis was performed as described by Bennassayang *et al.* (1975). The oestradiol binding assay was carried out with dextran-coated-charcoal (Baxter & Tomkins, 1971) and Sephadex-gel-equilibrium-dialysis (Pearlman *et al.*, 1969) methods. Molecular weight was determined by the method of Weber & Osborn (1969), with cytochrome *c* (mol.wt. 11 700), lysozyme (14 200), deoxyribonuclease (38 000) and bovine serum albumin (66 000) as standards.

Results

Purification of α -FP from neonatal-rat skin cytosol

Skin cytosol was applied to an anti-(α -FP)-Sephacrose-4B column (14.5 cm $\times$ 1.5 cm) pre-equilibrated with 10 mM-phosphate-buffered saline, pH 7.4. The unadsorbed proteins were washed off the column with 3 bed vol. of phosphate-buffered saline. The adsorbed protein was eluted from the column with 0.2 M-glycine/HCl, pH 2.4, containing 0.5 M-NaCl, at a flow rate of 10 ml/h. Fractions (2.5 ml) were collected in 0.5 ml of 0.5 M- K_2HPO_4 for immediate neutralization. The elution profile was monitored spectrophotometrically at 280 nm. Protein-rich fractions were analysed by double diffusion. The α -FP-positive fractions were pooled, dialysed extensively against phosphate-buffered saline, concentrated and stored frozen at -20°C .

Table 1 shows that about 43% of the α -FP was recovered by the immunoaffinity procedure.

Characterization of purified skin α -FP

The purity of the α -FP was checked by various immunochemical and analytical techniques.

Purified skin α -FP gave a single precipitin line against anti-(rat α -FP) serum on Ouchterlony double diffusion, with a perfect line of immunological identity with the α -FP species in other compartments (Fig. 1).

Skin α -FP also formed a single arc on immunoelectrophoresis and had an electrophoretic mobility similar to that of serum α -FP (Fig. 2).

Polyacrylamide-gel (10%) electrophoresis of skin

Fig. 1. Ouchterlony (1949) double-diffusion analysis using 1.25% agarose in phosphate-buffered saline 1, Serum α -FP; 2, skin cytosol; 3, purified serum α -FP; 4, serum α -FP; 5, purified skin α -FP; central well, anti-(rat α -FP).

Fig. 2. Immunoelectrophoresis of purified skin α -FP on 1% agarose buffered at pH 8.6 with 0.05 M-Veronal buffer, pH 8.6

The trough contains anti-(rat α -FP) serum, foetal serum.

α -FP yielded slow- and fast-moving variants, thereby indicating that it exhibits molecular heterogeneity similar to that observed in serum α -FP (Fig. 3). However, when analysed by SDS/polyacrylamide-gel electrophoresis under denaturing conditions, the purified α -FP yielded a single band with mol.wt. 68 000, a value which is slightly lower than that of serum α -FP. Samples were reduced with

Table 1. Purification of α -FP from neonatal-rat skin

Procedure	Volume (ml)	[α -FP] ($\mu\text{g/ml}$)	Total (μg)	Total protein (mg/ml)	α -FP (mg/mg of protein)	Yield (%)
100 000 g supernatant (skin cytosol)	21	20	420	5.00	0.004	—
Eluate from anti-(rat α -FP) IgG-Sephacrose-4B column	9	20	120	0.02	1.000	42.7

β -mercaptoethanol when analysed by SDS/polyacrylamide-gel electrophoresis.

Scatchard (1949) analysis of oestradiol binding to α -FP yielded association constants of $2.5 \times 10^9 \text{ M}^{-1}$ and $0.75 \times 10^8 \text{ M}^{-1}$ by dextran-coated-charcoal by Sephadex-gel-filtration procedures respectively (Fig. 4a and 4b).

Synthesis and secretion of α -FP by neonatal-rat skin explants in culture

The time course of [^{14}C]leucine incorporation into immunoprecipitable α -FP in skin lysate (synthesis)

and in medium (secretion) during a 6 h culture period is presented in Table 2. The incorporation of [^{14}C]leucine into α -FP by neonatal-rat skin explant culture was linear for 6 h. Addition of cycloheximide inhibited the incorporation of [^{14}C]leucine into α -FP (synthesis in cell lysate) and secretion (in medium). It thus appears that [^{14}C]radioactivity observed in the immunoprecipitable α -FP represents synthesis and secretion *in situ* of α -FP in newborn-rat skin explants in culture.

Fig. 3. Polyacrylamide-slab-gel electrophoresis on 10% (w/v) gel

Constant current of 2.5 mA/lane; A, purified skin α -FP (50 μg); B, foetal serum (70 μg); C, serum α -FP (50 μg). Constant current of 7.0 mA/lane: D and E, α -FP (purified) was applied as duplicate samples.

Fig. 4. Scatchard plot of oestradiol (E_2) binding to skin α -FP measured by dextran-coated charcoal (a) and Sephadex-gel-equilibrium-filtration procedures (b) Abbreviations used: Sb, bound steroid; Su, free steroid; Sb^* , inert steroid (in b).

Table 2. Time course of [^{14}C]leucine incorporation into α -FP by newborn-rat skin explants: synthesis and secretion Values are means $\pm$ S.D. of at least six observations. A, in the absence of cycloheximide; B, in the presence of cycloheximide.

Incubation time (h)	$[^{14}\text{C}]$ Leucine incorporation into immunoprecipitable α -FP			
	Synthesis (d.p.m./mg of tissue protein)		Secretion (d.p.m./mg of secretion protein)	
	A	B	A	B
2	640 $\pm$ 480	240	960 $\pm$ 220	320
4	1440 $\pm$ 400	280	2480 $\pm$ 240	280
6	2480 $\pm$ 400	320	4480 $\pm$ 320	320

Discussion

α -FP was found to be present in very high concentrations in immature rat skin. It thus served as an important source material for purification of α -FP. α -FP isolated from immature-rat skin by immunoaffinity chromatography was found to be similar to serum α -FP electrophoretically as well as immunologically. It was found to be homogeneous on double diffusion, immunoelectrophoresis and SDS/polyacrylamide-gel electrophoresis. However, on polyacrylamide-gel electrophoresis it displays slow- and fast-moving variants, thereby indicating that it exhibits molecular heterogeneity similar to that reported for serum α -FP by other investigators (Karckaert *et al.*, 1979; Aussel & Masseyeff, 1978). α -FP purified from immature-rat skin was found to bind oestradiol with high specificity, association constants of $2.5 \times 10^9 \text{ M}^{-1}$ and $0.75 \times 10^8 \text{ M}^{-1}$ being determined by dextran-coated-charcoal and Sephadex-gel-filtration procedures respectively. These values are similar to those observed for serum α -FP (Liang-Tang & Soloff, 1972; Aussel & Masseyeff, 1978).

An oestradiol-binding protein observed in the cytosol of adult-rat skin and having mol.wt. 58000 is different from the oestradiol-binding protein from immature-rat skin, which has mol.wt. 68000. Hence there would now seem to be at least two types of oestradiol-binding protein in skin during development: one is a α -FP (foetal type), which is predominantly present during embryonic and early postnatal development, and the other is oestradiol-binding-protein (adult type), which appears somewhat later in development.

Our observations, derived from [^{14}C]leucine incorporation into immunoprecipitable α -FP in cell lysate (synthesis) and in culture medium (secretion), clearly indicate that α -FP is indeed synthesized *in situ* by skin explants. The immunoprecipitate obtained, when analysed by SDS/polyacrylamide-gel electrophoresis after dissolution, yielded radioactivity in the band corresponding to the electrophoretic mobility and molecular weight of α -FP (Ali *et al.*, 1983b). [^{14}C]Leucine incorporation into α -FP by skin explants was inhibited by cycloheximide, a protein-synthesis inhibitor. This observation clearly rules out the possibility that the radioactivity observed in α -FP is the result of artefact of culture.

The physiological importance of the existence of an independent pool of oestrophilic α -FP in neonatal-rat skin is not known. It appears that it may be involved in the binding and extracellular sequestration of excess of oestrogens infiltrating from the maternal circulation.

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