The developmental profile of lactoferrin in mouse epididymis

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A sandwich e.l.i.s.a. method was developed to examine the distribution of lactoferrin in mouse reproductive tract. The lactoferrin concentration was found to be much higher in oviduct, uterus, vagina, vas deferens or epididymis than in serum, but the concentration in ovary, testis, seminal vesicle, prostate or coagulating gland was comparable with that in serum. The existence of lactoferrin in male sexual organs was confirmed by Western-blot analyses for tissue proteins. Lactoferrin in male sexual organs was shown to have a molecular mass similar to that of the deglycosylated form of lactoferrin purified from mouse uterine luminal fluid. Northern-blot analyses for total RNA prepared from male sexual organs indicated that only epididymis contained the lactoferrin mRNA. The lactoferrin mRNA was found in the prepubertal period and increased with the growth of epididymis. The mRNA level in prepubertal epididymis could be stimulated by 17β-oestradiol, but was not influenced by testosterone.

INTRODUCTION

Lactoferrin (LF), a non-haem iron-binding protein closely related in structure to transferrin, is a member of the transferrin family [1]. It exists mainly in milk [2], various other external secretions [3] and the secondary granules of neutrophils [4]. Unlike transferrin, which is abundant in serum and has a role in iron transport, only traces of LF are normally present in serum. LF has been associated with iron absorption in the newborn infant [5], bacteriocidal activity [6,7] and antiviral activity [8]. Recent evidence implies that LF inhibits myelopoiesis [9] and affects the immune system through regulating the release of cytokines such as tumour necrosis factor-α and interleukins-1 and -2 [10,11]. The diverse functions of LF (for a review, see [12]) show the importance of its gene regulation in individual tissue and cell type under different types of control. However, its diverse functions make it hard to construct a general model consistent with the action of LF at the molecular level.

LF was first isolated from human milk [13], but its importance in sexual organs has not received attention until recently. Since it was identified to be the major protein component of mouse uterine luminal fluid and its gene expression was shown to be regulated by oestrogen [14,15], many attempts have been made to find the functional role of LF in reproduction. For instance, detailed analyses of LF expression during the natural oestrous cycle [16,17] and the preimplantation period [18] reveal its correlation with ovarian steroids, and suggest LF may play an important role in the female mouse reproductive tract and in early pregnancy. Yet few reports have appeared in the literature concerning the existence of this protein in the male reproductive tract, despite the fact that it was found in human seminal plasma [19] and was said to be one of the major sperm-coating antigens [20]. In the present work the protein's distribution in the reproductive tract of mice was surveyed. It was found that epididymis is the main male sexual organ to contain LF mRNA and to produce a high concentration of LF protein, a process which begins prior to puberty. The production of both LF mRNA and LF protein in prepubertal epididymis can be stimulated by oestrogen, but is not influenced by androgen.

EXPERIMENTAL

Materials

Diethylstilboestrol dipropionate, testosterone propionate, 17β-oestradiol, aprotinin, biotin *N*-hydroxysuccinimide ester were obtained from Sigma (St. Louis, MO, U.S.A.). Protein A–Sepharose was purchased from Pharmacia (Uppsala, Sweden). [α-^{32}P]dATP and ^{125}I-labelled anti-rabbit IgG prepared from donkey were obtained from Amersham International (Bucks., U.K.). The streptavidin–horseradish peroxidase conjugate was obtained from GIBCO BRL (Gaithersburg, MD, U.S.A.). Affi gel-10 was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were reagent grade.

Animals and steroid-hormone treatment

Outbred CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.) and were maintained and bred in the animal centre at the College of Medicine, National Taiwan University. Animals were treated following the institutional guidelines for the care and use of experimental animals.

Diethylstilboestrol dissolved in corn oil was administered subcutaneously (s.c.) to female mice (3 weeks old) with a daily dose of 100 ng/g of body weight for 3 consecutive days. Subsequently the animals were killed and uterine luminal fluid was collected. For the investigation of androgen or oestrogen effect on male animals, baby mice (3 weeks old) and adult mice (14 weeks old) received by s.c. injection a daily dose of testosterone propionate (5 μg/g of body weight) or 17β-oestradiol (100 ng/g of body weight) in corn oil for 3 consecutive days. The control animals received corn oil only. The sexual organs were then removed from the animals for further study.

Preparation of antibody to mouse LF

LF was purified from mouse uterine luminal fluid by the method of Teng et al. [15]. Antiserum against LF was raised in New Zealand White rabbits. The LF antibody was isolated by affinity

Abbreviations used: LF, lactoferrin; s.c., subcutaneous(ly).

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chromatography on Protein A–Sepharose and Affi gel-10 to which mouse LF had been covalently linked.

Electrophoresis and Western-blot analysis

Tissues along with the luminal secretion in their ducts from adult mice (14 weeks old) were homogenized in PBS/50 mM EDTA in the presence of aprotinin (1000 Kallikrein-inhibitory units/ml) and centrifuged at 100000 *g* for 20 min in a Beckman Airfuge (Beckman, Palo Alto, CA, U.S.A.). The concentration of protein in the clarified supernatant was determined by the modified Lowry method [21] and resolved by SDS/PAGE [9% (w/v) acrylamide] on a gel slab (13.5 cm × 14 cm × 0.075 cm). Proteins were transferred from the slab gel to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, U.S.A.) in PBS at 4 °C for 18 h by diffusion. Blots were immunodetected by treatment with the affinity-purified antibody to mouse LF, followed by treatment with ^{125}I-labelled donkey anti-rabbit IgG and fluorography.

Chemical modification

Mouse LF was allowed to react with trifluoromethanesulphonic acid to remove its carbohydrate moiety [22]. Biotinylation of the antibody to mouse LF with biotin *N*-hydroxysuccinimide ester was performed by the method of Bayer et al. [23].

Quantification of tissue LF by e.l.i.s.a.

The sandwich method of e.l.i.s.a. [24] was modified to quantify the amount of LF in mouse tissues. Antibody to mouse LF (200 ng in 50 μl) in 50 mM Na_2CO_3 at pH 9.6 was applied to a 96-well e.l.i.s.a. plate and incubated for 16 h at 4 °C. The wells were blocked with 0.1% gelatin in PBS containing 0.05% Tween 20 (PBST), and incubated consecutively with the supernatant fraction of tissue homogenates, the biotinylated antibody to mouse LF (200 ng in 50 μl of PBST) and the streptavidin–horseradish peroxidase conjugate (50 μl; 1:1000 dilution in PBST) for 2 h at room temperature. Between each incubation, wells were washed with PBST three times. The peroxidase reaction was performed by adding 150 μl of 0.1% *o*-phenylenediamine and 0.067% H_2O_2 in 20 mM citric acid/50 mM sodium phosphate at pH 5.0. After 20 min incubation, the reaction was stopped by adding 50 μl of 1 M H_2SO_4 to the reaction solution and the absorbance was read at 490 nm in an EIA Reader (Model EL-307; Bio-Tek Instruments).

RNA isolation and analysis

RNA was prepared from fresh tissues by the acid guanidinium thiocyanate/phenol/chloroform method [25]. RNA samples were analysed by separation in 1% agarose/formaldehyde-containing gel [26] followed by capillary transfer to nylon membrane and hybridization to ^{32}P-labelled nucleic acid probes [27].

^{32}P-labelled random-primed probe was prepared with a Promega random-priming kit (Promega, Madison, WI, U.S.A.), using a template of a cDNA segment of the mouse LF gene (369 bp) [14] or a cDNA segment of the mouse β-actin gene (26 bp) inserted in pGEM 4Z vector. Both were gifts from Dr. K. Nelson (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Science).

RESULTS

Distribution of LF in the reproductive tract

The sandwich method of e.l.i.s.a. was able to detect mouse LF down to a lower limit of 0.3 ng. The LF standard curve constructed with 0.3–100 ng of LF is given in Figure 1. The absorbance at 490 nm was linearly related to LF concentration over the range of 1–10 ng/50 μl, which was suitable for quantifying LF. The antibody of LF showed no cross-reaction with transferrin, and the non-specific effect of transferrin on the assay method was negligible (Figure 1).

Table 1 summarizes the distribution of LF in the mouse reproductive tract as determined from the e.l.i.s.a. The LF concentration was found to be 0.23 and 0.20 ng in 10 μg of serum protein from male and female mice respectively. In comparison with that in serum, much higher LF concentrations were found

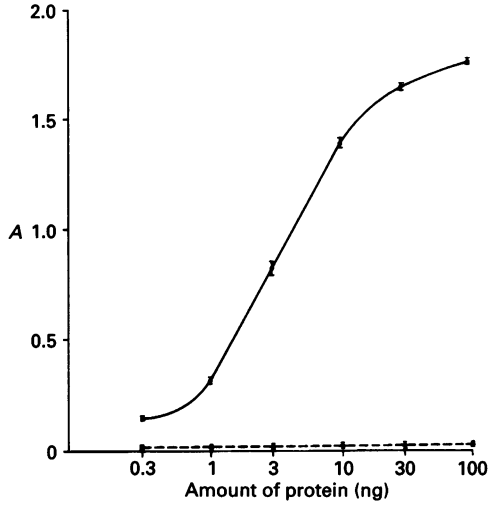

Figure 1 E.l.i.s.a. standard curve

A sandwich method of e.l.i.s.a. was developed for the assay of mouse LF (see the text for details). Calibration curves: ——, mouse LF; – – – –, mouse transferrin. Data were average of three determinations. Error bars represent the S.D.

Table 1 Distribution of lactoferrin in the reproductive tract

The soluble fraction of the tissue homogenate was assayed by the e.l.i.s.a. method. The results presented are averages for four batches of mice. LF was assayed to be 0.23 ± 0.07 ng and 0.20 ± 0.05 ng in 10 μg of serum protein of male and female mice respectively. The amount of lactoferrin in serum was so small that no correction had been made for any contribution to the lactoferrin found in homogenate tissues.

Reproductive organ	Amount of lactoferrin (ng/10 μg of tissue protein)
Testis	0.10 ± 0.02
Epididymis	41.88 ± 12.12
Vas deferns	14.75 ± 3.12
Prostate	0.58 ± 0.13
Seminal vesicle	1.43 ± 0.48
Coagulating gland	0.13 ± 0.04
Ovary	1.11 ± 0.49
Oviduct	14.14 ± 5.81
Uterus	14.12 ± 3.12
Vagina	52.38 ± 16.88

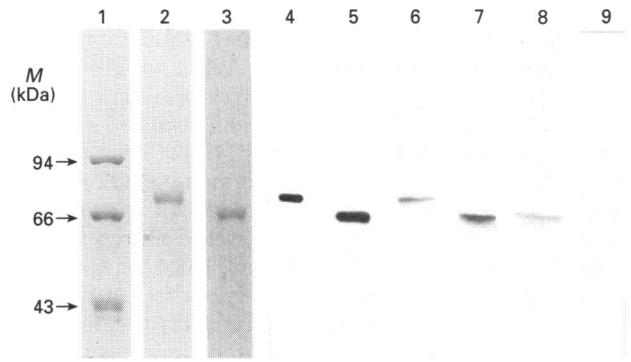

Figure 2 Identification of mouse LF in reproductive organs

LF and the proteins (50 μg) in the soluble fraction of tissue homogenate were resolved by SDS/PAGE: lane 1, the standard protein markers; lanes 2 and 4, LF purified from mouse uterine fluid (lane 2, 2 μg; lane 4, 200 ng); lanes 3 and 5, the deglycosylated derivative of uterine LF; lane 3, 2 μg; lane 5, 200 ng; lane 6, uterus homogenate; lane 7, epididymis homogenate; lane 8, vas deferens homogenate; lane 9, testis homogenate. The proteins in lanes 1–3 were stained with Coomassie Brilliant Blue. The proteins in the gel of lanes 4–9 were transferred to a poly(divinylidene difluoride) membrane and were immunodetected by Western-blot procedures with antibody to mouse LF. Abbreviation: *M*, molecular mass.

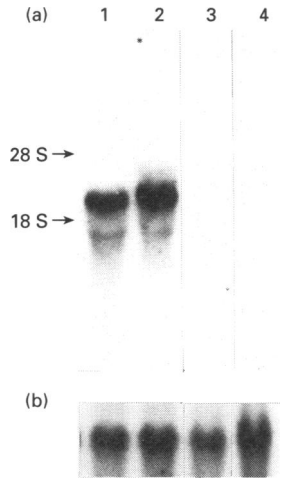

Figure 3 Northern-blot analysis of mouse total RNA from various tissues

Total RNA (100 μg) prepared from each tissue was run on a 1%-agarose/formaldehyde gel, transferred to a nylon membrane and probed with ^{32}P-labelled random-primed DNA to a cDNA segment of either mouse LF (**a**) or β-actin (**b**) (see the text for details). The level of β-actin mRNA was used as an internal control. Tissues examined were uterus (lane 1), epididymis (lane 2), vas deferens (lane 3) and testis (lane 4).

in the oviduct, uterus, vagina, epididymis and vas deferens, while only traces were detected in the ovary, testis, seminal vesicle, prostate and coagulating gland. The vagina was among the female sexual organs to contain a highest LF concentration. It is noteworthy that epididymis had an LF concentration comparable with that found in the vagina.

Demonstration of LF protein and mRNA in male sexual organs

Mouse LF from uterine luminal fluid is a glycoprotein [15] with a molecular mass of 74 kDa by SDS/PAGE (Figure 2, lane 2). The molecular size was decreased to 67 kDa on SDS/PAGE

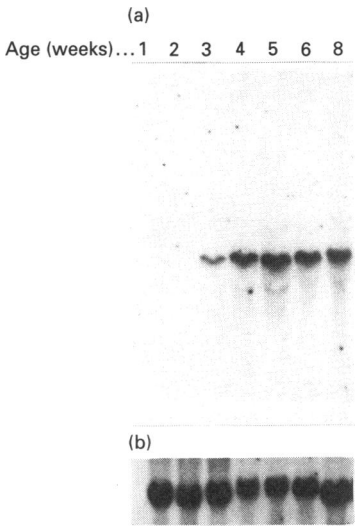

Figure 4 LF synthesis in mouse epididymis at different ages

The Figure shows a Northern-blot analysis for LF mRNA in the total RNA (100 μg) from epididymis of mice at the different ages shown. Detection of LF mRNA (**a**) and β-actin mRNA (**b**) is described in the legend to Figure 3.

when the protein was deglycosylated by reaction with trifluoromethanesulphonic acid (Figure 2, lane 3). Both LF and its deglycosylated derivative were immunoreactive to antibody against LF (Figure 2, lanes 4 and 5). While the 74 kDa LF was found in uterus (Figure 2, lane 6), a smaller LF with the same molecular mass as the deglycosylated uterine LF was immunodetected in both epididymis and vas deferens (Figure 2, lanes 7 and 8). No LF was detected in the testis (Figure 2, lane 9).

Figure 3 displays the results of Northern-blot analysis for LF mRNA in the total RNA of several tissues. The level of β-actin mRNA was used as the internal control when LF mRNA in tissues was examined. The existence of LF mRNA ($\sim$ 2.4 kb) was confirmed in both uterus and epididymis (lanes 1 and 2). No LF mRNA were detected in vas deferens and testis (lanes 3 and 4), indicating that the presence of LF protein in vas deferens probably resulted from epididymal secretion.

Oestrogen-stimulated LF mRNA in epididymis

The level of LF mRNA in mice epididymis at different ages was examined. LF mRNA was not found in mice younger than 3 weeks old (Figure 4, lanes 1 and 2) and appeared when mice were three weeks old (Figure 4, lane 3). The amount of mRNA was greatly increased in the 4-week-old mice (Figure 4, lane 4). Thereafter a highly constant level of LF mRNA maintained. This developmental change of LF mRNA coincided with the growth of the epididymis.

The immature male mice (3 weeks old) and mature male mice (14 weeks old) were injected with testosterone propionate or 17β-oestradiol for 3 consecutive days (see the Experimental section) and the effect of steroid hormones on the production of LF mRNA in epididymis was examined (Figure 5). Irrespective of maturity, the level of LF mRNA in epididymis of the testosterone-treated mice showed no difference from that of the control mice (cf. lanes 1 and 2, lanes 4 and 5). A similar situation occurred in the 17β-oestradiol-treated mature animals (cf. lanes 4 and 6). In contrast, 17β-oestradiol did remarkably increase the

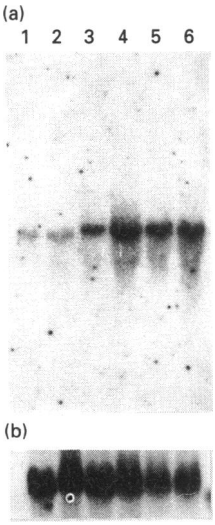

Figure 5 Effect of oestrogen and androgen on the level of LF mRNA in mouse epididymis

The Figure shows a Northern-blot analysis for LF mRNA in the total RNA (100 μg) of immature mice 3 weeks old (lanes 1–3) and mature mice 14 weeks old (lanes 4–6). Mice were injected with testosterone propionate, 17β-oestradiol or corn oil only for 3 consecutive days. Tissues examined were control mice (lanes 1 and 4), androgen-treated mice (lanes 2 and 5) and oestrogen-treated mice (lanes 3 and 6). Detection of LF mRNA (**a**) and β-actin mRNA (**b**) is described in the legend to Figure 3.

LF mRNA level in epididymis of immature animals (cf. lanes 1 and 3).

DISCUSSION

The existence of LF in male sexual organs as determined by e.l.i.s.a. is also supported by the results of Western- and Northern-blot analyses (Figures 2 and 3). Epididymis is apparently the main male sexual organ to produce LF. LF in epididymis might not be glycosylated when it is secreted from epididymis, but the structural differences between epididymal and uterine LF await identification.

Since LF synthesis in mouse uterus was shown to be under the control of oestrogen [14,15], LF has been used as a marker for the oestrogen response of the female reproductive tract. Mouse LF promoter was demonstrated to have an oestrogen-responsive element overlapping with a chicken ovalbumin upstream promoter element in addition to multiple upstream elements that modulated the basal transcriptional promoter activity [28,29]. However, the oestrogen regulation of LF gene expression is tissue-specific; note that LF synthesis in mouse mammary gland is unaffected by oestrogen but is dependent on prolactin [30]. Similarly to the female mouse reproductive tract, LF maintains its oestrogen-responsive character in mouse epididymis, at least in the prepubertal stage, and was not influenced by androgen (Figure 5). Since the oestrogen receptors or the oestrogen-binding sites are widely distributed among male sexual organs such as testis, epididymis, seminal vesicle and prostate [31–33], the differences between the control of LF gene expression in these organs deserve future investigation.

The synthesis of some secretory proteins from epididymis and several aspects associated with the organ growth are androgen-dependent [34–37], whereas some differentiation processes are androgen-independent [38]. It is believed that the normal differentiation and growth of epididymis is controlled by both androgen and oestrogen [39,40]; both hormones may exert their action on different types of cells in the same organ. When rats are at an age of 16–28 days, during which serum androgen is at a low level, cell differentiation and growth from the undifferentiated stem cells proceed in the prepubertal epididymis [41,42]. This coincides with the pattern of LF mRNA level in mouse epididymis (see Figure 4). It is noteworthy that LF production in prepubertal epididymis, which might contain a low level of oestrogen, can be stimulated by injection of 17β-oestradiol. Once the epididymis has completely developed after puberty, oestrogen might be maintained at a certain level and the machinery responsible for LF production no longer promoted by the administration of oestrogen to mature mice. We suspect an important role of oestrogen in the postnatal development of epididymis and suggest LF as a good marker for the oestrogen response of prepubertal epididymis.

Despite the demonstration that oestrogen stimulates LF production in the reproductive tract of both sexes, the functional role of LF in reproduction is currently unclear. With regard to male, more studies are required to determine the function of LF during the developmental change of epididymis as well as its role in the activity of spermatozoa before their ejaculation.

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