A Putative Mouse Oocyte Maturation Inhibitory Protein from Urine of Pregnant Women: N-Terminal Sequence Homology with Human Nonsecretory Ribonuclease

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A putative mouse oocyte maturation inhibitory protein was purified from a urine preparation from pregnant women by Sephadex G-100 gel filtration and reverse-phase chromatography on the basis of inhibitory activity of polar body formation of denuded mouse oocytes in culture. Amino terminal sequence analyses showed that residues 5 to 15 of this protein were identical to residues 1 to 11 of human nonsecretory ribonuclease. Furthermore, residues 1 to 4 of this protein were identical to residues -4 to -1, corresponding to part of a signal peptide region of eosinophil-derived neurotoxin, whose mature sequence is identical to nonsecretory ribonuclease. These results indicate that the protein purified as a putative mouse oocyte maturation inhibitory protein from the urine of pregnant women may be a product of an peculiar processing of a nonsecretory ribonuclease precursor.

Keywords oocyte maturation; inhibitory protein; urinary protein; pregnant women; N-terminal sequence; ribonuclease; nonsecretory ribonuclease; eosinophil-derived neurotoxin

Maturation of the ovarian follicle as well as the oocytes existing in the follicle is regulated by a complex interaction of stimulators and inhibitors in the hypothalamus-pituitary-ovary system. In general, it is accepted that oocyte and follicular maturation is induced by hormones, pituitary gonadotropins and ovarian estrogen. Thus, the germinal vesicle of follicle-enclosed oocytes in animals remains arrested at the dictyate state of meiosis until the occurrence of a gonadotropin surge.^{1,2)} Upon releasing the oocytes from the follicles *in vitro*, the meiotic process resumes, leading to a germinal vesicle breakdown (GVBD) followed by the polar body formation (PBF) of the oocytes,^{3,4)} suggesting that factors in the follicular constituents and/or factors secreted by other tissues sustain the meiotic arrest of oocytes. Such peptide regulators in the control of oocyte and follicular maturation,⁵⁻⁷⁾ as well as an inhibitor of follicle stimulating hormone (FSH) binding to its receptor, have been identified in follicular fluid.^{8,9)} However, the structures of these peptides have not been determined.

In normal pregnancy, although hormonal levels of chorionic gonadotropin (hCG) and estrogen are high, significant maturation of the oocytes and follicles do not occur for a long period during the pregnancy. This phenomenon suggests the possibility of a considerable increase in concentration of the following factors in urine during pregnancy: a factor in the follicular constituents which directly affects oocytes, and/or factors secreted by other tissues, *i.e.* a functional inhibitor of hCG and estrogen, which sustain the meiotic arrest of oocytes. For instance, Soffer *et al.* have reported that a gonadotropin-inhibitory substance has been identified in the urine of normal young children as well as of normal subjects on the basis of its inhibition of mouse uterine-ovary growth stimulated by hCG *in vivo*.^{10,11)} A similar gonadotropin-inhibitory substance was detected in a crude urinary preparation of pregnant women which is a partially purified pharmaceutical preparation of hCG.¹²⁾ In addition to the above studies, Blithe *et al.* reported that hCG itself at low concentrations can inhibit ovarian growth in hypophysectomized rats which have been stimulated with FSH and diethylstilbestrol.¹³⁾

On the other hand, information concerning those substances which directly affect the oocytes in the urine during pregnancy, which have possibly been leaked or excreted into the urine from follicles or other tissues, have not been available.

The purpose of the present study was to determine a factor which directly affects the oocytes and induces their meiotic arrest, if such a factor is present in urine during pregnancy. We describe here how we have identified a source of inhibitory activity on oocyte maturation in urinary extracts obtained from pregnant women using an *in vitro* bioassay based on inhibitions of spontaneously occurring maturation of mouse oocytes (GVBD and PBF) in culture. It was revealed that a purified protein which exhibits inhibitory activity on PBF of a denuded mouse oocyte in culture was a protein related to nonsecretory ribonuclease based on N-terminal amino acid sequence homology.

Experimental

Material A crude urinary preparation from pregnant women, which is a partially purified pharmaceutical preparation of hCG, was a generous gift from Organon.

Culture of Mouse Oocytes Oocytes (40 to 50) from prepubertal ICR mice (15 to 20 g body weight) were prepared by a published method.¹⁴⁾ Oocytes were cultured for 16 h in a 0.2 ml drop of modified Krebs Ringer medium under paraffin oil at 37°C in humidified 5% CO₂ in air. Activities of mouse oocyte maturation inhibition were determined by inhibitory effects on GVBD and PBF of oocytes in culture, as follows. After 3 h of cultivation of the oocytes with the samples, the presence of GVBD was examined under a stereoscopic microscope. PBF was examined after 10 to 12 h of cultivation of the oocytes with the samples. The percentage of inhibition of GVBD and PBF were calculated as follows:

$$\frac{\%GVBD \text{ or PBF (control)} - \%GVBD \text{ or PBF (experiment)}}{\%GVBD \text{ or PBF (control)}} \times 100 = \text{inhibition (\%)}$$

Sephadex G-100 Column Chromatography A crude urinary preparation from pregnant women (100 mg) was dissolved in 5 ml of 0.1% NH₄HCO₃ and the proteins were separated into tubes (7 ml) on a Sephadex G-100 column (2.5 × 100 cm). The tubes were pooled for fractions A to D and lyophilized. Each fraction was rechromatographed on a Sephadex G-100 under the same conditions and lyophilized.

Reverse-Phase High Performance Liquid Chromatography (HPLC) An

aliquot of fraction B (1 mg) from gel filtration was dissolved in purified water and applied to a column (μ Bondasphere C_8 , 3.9×150 mm) of reverse-phase HPLC. The proteins were separated with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid (60 min) at a flow rate of 1 ml/min. Protein content was monitored by measuring the absorbance at 220 nm. Three major peaks of proteins (B1, B2, consisting of three sub-peaks, and B3) were obtained. Each peak was lyophilized and dissolved in water or a 10 mM phosphate buffered saline (pH 7.0).

N-Terminal Sequence Determination Proteins B1, B2-1, B2-2, B2-3 and B3 were analyzed on an automated Applied Biosystems model 477A protein sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer.

Miscellaneous Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Laemmli's method¹⁵⁾ using a 10% gel. Immunoblotting analysis of hCG was performed according to the published method.¹⁶⁾ Protein content was determined by the method of Bradford¹⁷⁾ using bovine serum albumin as the standard. All other chemicals used were standard commercial products.

Results and Discussion

To determine the nature of inhibitory activity in urine from pregnant women on mouse oocyte maturation, a commercial crude preparation of urine from pregnant women (a partially purified pharmaceutical preparation of hCG) was used. The specific activity of hCG in this preparation was 2900 IU/mg, indicating that more than 70% of the protein in this preparation includes urinary constituents other than hCG, since the specific activity of the purified hCG is >10000 IU/mg. A commercial crude preparation of urine from pregnant women, as well

as its fractions B to D obtained from Sephadex G-100 chromatography (Fig. 1), was assayed for inhibitory activities to the GVBD and PBF of mouse oocytes (Fig. 2). Blithe *et al.* reported that a low concentration of hCG is a potent inhibitor of ovarian growth *in vivo* stimulated with FSH and diethylstilbestrol.¹³⁾ Our assay system was conducted *in vitro* using denuded oocytes in culture, in order to delete the effects of contamination with hCG, and fraction B was chromatographed on a Sephadex G-100 column several times until no hCG was detected immunologically using the immunoblotting method. Final yields of protein in fractions B, C and D were approximately 9, 8 and 11 mg, respectively. No further investigation was performed on fraction A since the greater part of it was hCG. When 1 mg/ml of each fraction and the original material were analyzed for mouse oocyte maturation inhibitory activity, neither the individual fractions nor the original materials showed any inhibitory effect on GVBD of mouse oocytes. On the other hand, an inhibitory effect on PBF was detected in fractions B (100%), C (32%), and D (28%), as well as in the original materials (47%). As shown in Fig. 3, the most active fraction, B, inhibited PBF dose dependently. These results suggest that some inhibitory protein to PBF of the mouse oocyte may be present in the urine of pregnant women. At the present time, it remains unknown whether fraction B inhibited only PBF, or both

Fig. 1. Chromatographic Pattern on Sephadex G-100 of the Extracts of Urine Obtained from Pregnant Women

A crude urinary preparation from pregnant women (100 mg) was subjected to a column of Sephadex G-100 (2.5×100 cm), and fractions A to D were obtained under the conditions described in Experimental.

Fig. 2. Inhibition of the Material and Its Fractions (B, C and D) from Fig. 1 on GVBD (■) and PBF (□) of Mouse Oocytes at a Protein Concentration of 1 mg/ml

The numbers in parentheses are the number of oocytes analyzed.

Fig. 3. Dose Dependent Inhibition of Fraction B on GVBD (●) and PBF (○) of Mouse Oocytes

The numbers in parentheses are the numbers of oocytes analyzed.

Fig. 4. Separation Profile of Proteins in Fraction B with HPLC

Fraction B (1 mg) was subjected to a column of μ Bondasphere C_8 (3.9×150 mm) and fractions B1, B2 and B3 were obtained under the conditions described in Experimental.

Fig. 5. SDS-PAGE of Proteins B1 (Lane 2), B2-1 (Lane 3), B2-2 (Lane 4), B2-3 (Lane 5) and B-3 (Lane 6)

Each protein corresponding to 0.1 μ g protein, prepared with reduction by 5% 2-mercaptoethanol and boiling for 5 min, was electrophoresed using a 10% gel. The gel was stained with silver reagents. Lane 1 represents molecular weight marker proteins.

Fig. 6. Inhibition of Proteins in Fractions B, B1, B2 and B3 at a Protein Concentration of 0.3 mg/ml on the PBF of Mouse Oocytes

The numbers in parentheses are the numbers of oocytes analyzed.

GVBD and PBF. However, PBF inhibition by fraction B may not be caused by its cytotoxic effect, since fraction B did not show any cytotoxic effect on other cells tested at the concentration of 1 mg/ml (data not shown).

To purify proteins inhibiting the PBF of mouse oocytes, fraction B was subjected to reverse-phase HPLC (Fig. 4). As shown in Fig. 5, four proteins (B1, B2-1, B2-3, and B3) were purified as single bands with SDS-PAGE. Although peptide B2-2 contained two bands, it was very likely that peptide B2-2 was a mixture of peptides B2-1 and B2-3 (see below). The apparent molecular weights of these peptides were approximately 35000, 37000, 34000 and 32000 daltons (Da) for B1, B2-1, B2-3 and B3, respectively.

When 0.3 mg/ml of each protein peak (B1, B2 and B3) was analyzed for PBF inhibitory activity, only B2 revealed PBF inhibitory activity (approximately 40%) (Fig. 6). PBF inhibitory activity of B2 was not significant compared to fraction B, possibly indicating that factors involving inhibition of PBF may be lost by denaturation during HPLC, or that these effects may result from the mutual interaction of several peptide factors. At the present time, the interaction mechanism of these peptide factors remains unknown.

In this study, we have made efforts to clarify which proteins are present in the active fraction, B, by determining their amino terminal sequences. Approximately 4 μ g of each peptide (B1, B2-1, B2-2, B2-3 and B3) was subjected to N-terminal sequence analysis using an automated sequencer. Each predominant N-terminal sequence except B3 was determined by 15 cycles of Edman degradation as shown in Table I. No N-terminal amino acid was detected in B3, indicating that the N-terminus of B3 may be

TABLE I. N-Terminal Amino Acid Sequence Analyses of Proteins

Peptide	Cycle number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
B1	Lys 116	Glu 53	Ser 36	Arg 28	Ala 169	Lys 90	Lys 114	Phe 101	Gln 60	Arg 31	Gln 78	His 17	Met 101	Asp 48	Ser 23
B2-1	Ser 71	Leu 103	His 10	Val 115	Lys 63	Pro 133	Pro 120	Gln 76	Phe 88	Thr 33	Trp 32	Ala 57	Gln 31	—	—
B2-2	Ser 56	Leu 164	His 27	Val 200	Lys 109	Pro 141	Pro 156	Gln 84	Phe 78	Thr 24	Trp 10	Ala 52	Gln 45	Trp 10	Phe 32
B2-3	Ser 94	Leu 134	His 14	Val 133	Lys 135	Pro 134	Pro 122	Gln 85	Phe 96	Thr 55	Trp 15	Ala 89	Gln 55	Trp 25	Phe 47

Approximately 4 μ g of each protein was subjected to an automated sequencer. Yields of PTH amino acids for cycles (pmol) are shown below each residue. Dashes indicate that the determination of PTH amino acid was not performed.

TABLE II. Comparison of N-Terminal Amino Acid Sequences

B1	1 5 10 15														
	K	E	S	R	A	K	K	F	Q	R	Q	H	M	D	S
HPR	K	E	S	R	A	K	K	F	Q	R	Q	H	M	D	S
B2	1 5 10 15														
	S	L	H	V	K	P	P	Q	F	T	W	A	Q	W	F
HNSR						K	P	P	Q	F	T	?	A	Q	W
EDN	M	-----	G	S	L	H	V	K	P	P	Q	F	T	W	A
	-27			-4				1			5			10	

HPR: Human pancreatic ribonuclease.¹⁸⁾ HNSR: Human non-secretory ribonuclease.¹⁹⁾ EPN: Eosinophil-derived neurotoxin.²⁰⁾

blocked. Sequences of B2-1, B2-2 and B2-3 were identical, indicating that heterogeneity of these peptides on HPLC may be due to C-terminal heterogeneity or other factors such as differences in sugar content which we are now investigating. As shown in Table II, by searching the data base of amino acid sequence (PRF-SEQDB from PRINAS), it was revealed that N-terminal 15 residues of B1 are identical to those of human pancreatic ribonuclease,¹⁸⁾ and residues 5—15 of B2 are identical to the N-terminal sequence of nonsecretory ribonuclease isolated from human urine.¹⁹⁾ Recently, Rosenberg *et al.* reported a precursor structure consisting of signal (27 residues) and mature (134 residues) polypeptide regions deduced from complementary deoxyribonucleic acid (cDNA) of eosinophil-derived neurotoxin (EDN), whose sequence of the mature form is identical to nonsecretory ribonuclease.²⁰⁾ Residues 1 to 4 (S-H-V-L) of B2 were identical to residues -4 to -1 corresponding to part of a signal sequence of EDN. Although the original organ(s) of protein B2 are unknown at present, these results indicate that protein B2 may be produced by an unusual processing of a non-secretory ribonuclease precursor form. The molecular weight of B2 (approximately 34000—37000 Da) was inconsistent with that of nonsecretory ribonuclease (approximately 16000 Da). This may be caused by differences in sugar content, since it is accepted that some ribonucleases are glycoproteins.

In conclusion, it is very likely that the protein B2, purified as a protein having putative oocyte maturation inhibitory activity based on inhibitory effects on PBF of mouse oocytes from urine during pregnancy, is a nonsecretory ribonuclease containing a precursor sequence of residues -4 to -1. Characterization of protein B2 is under investigation and may be of striking interest in understanding the significance

of ribonuclease activity as well as mouse oocyte maturation inhibitory activity.

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