An Enzyme-Linked Immunosorbent Assay System for Quantitative Determination of Calphobindin I, a New Placental Anticoagulant Protein, and Its Application to Various Specimens

Hiroshi NAKAO,*[,a] Takao NAGOYA,[a] Akio IWASAKI,[a] Makoto SUDA,[a] Yukio HATTORI,[a] Yushi SAINO,[a] Yoshihiro SHIDARA[b] and Masahiro MAKI[b]

Department of Cell Biology, Kowa Research Institute, Kowa Co., Ltd.,[a] Tsukuba, Ibaraki 305, Japan and Department of Obstetrics and Gynecology, School of Medicine, Akita University,[b] Akita, Akita 010, Japan. *Received December 12, 1989*

We developed a sandwich enzyme-linked immunosorbent assay (ELISA) system for calphobindin I (CPB-I), a new placental coagulation inhibitor, using two monoclonal antibodies. This ELISA system can detect CPB-I at concentrations of between 0.4 and 25 ng/ml in buffer and allow almost quantitative determination of it in human plasma. Using this ELISA system, CPB-I levels in many kinds of specimens were measured. Levels in the plasma and urine of women were as low as 10 ng/ml, and no significant differences were observed throughout the trimesters of pregnancy and during different stages of the menstrual cycle. Toxemic patients were slightly higher in CPB-I levels than normal pregnant women, and levels in body fluids such as the amniotic fluid, saliva, milk, ascites, and semen were higher than those in the plasma. The high levels of CPB-I were found, being in the order of μg/ml, in the ascites of carcinomatous peritonitis as well as seminal plasma. Measurements of the levels in ovarian follicular fluid samples at different stages of the menstrual cycle showed that those in the immature and atretic stages were higher than those in mature stages. CPB-I levels in many types of cultured human cells ranged from 0.023 to 10.30 μg/mg protein, and levels in cultured human lymphocytes were less than those in other types of cells measured. Little of this inhibitor was secreted into media from cultured human lymphocytes, and it was found in all measured tissues of *Macacus irus* at levels ranging from 0.232 to 1.557 μg/mg protein. From these results, it was suggested that CPB-I might be a ubiquitous protein in the body that has an important physiological role.

Keywords sandwich enzyme-linked immunosorbent assay (ELISA); monoclonal antibody; calphobindin I (CPB-I); CPB-I level; blood coagulation inhibitor protein

Introduction

A novel blood coagulation inhibitor isolated from human placenta was first reported by Maki *et al.* in 1984.[1] Recently, we isolated an anticoagulant protein, calphobindin I (CPB-I),[2] from human placenta; we cloned the complementary deoxyribonucleic acid (cDNA) for this protein and showed that CPB-I had homology with lipocortins[3] and belonged to the annexins family.[4] CPB-I is a potent inhibitor of tissue thromboplastin (TF), inhibiting the blood coagulation pathway triggered by the TF–factor VII complex in the presence of Ca^{2+}.[2] Like calpactin[5] and lipocortins, CPB-I is a calcium- and phospholipid-binding protein and shows phospholipase A_2 inhibitory activity. These proteins reduce the production of prostaglandins and leukotrienes by inhibiting the phospholipase A_2. Although, stated, several properties of CPB-I have been elucidated, its physiological functions are still obscure.

In the present study, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) system for CPB-I and, in order to elucidate the protein's distribution and physiological functions, measured its levels in various types of specimens including plasma and urine samples from women during different stages of their menstrual cycle, plasma and urine samples at various gestational periods during pregnancy, body fluids, cultured human cells, and tissues of *Macacus irus*.

Experimental

Materials RPMI8866, CESS, Daudi, Raji, SKW6-CL4, CEM, MOLT4, U266 and U937 cells were kindly provided by Dr. M. Suemura (Osaka University, Japan). Flow 2000, Flow 4000, Flow 7000, Flow 13000, AV3, WISH, A431 and G361 were purchased from Dainippon Pharmacy Co., Ltd. HUVEC and NHEK were purchased from Sanko Junyaku Co., Ltd. CPB-I was purified as described by Iwasaki *et al.*[2] Chemicals were of the highest grade commercially available.

Preparation of Monoclonal Antibody and Peroxidase Conjugated Fab' Fragment Anti-CPB-I monoclonal antibodies (A46 and A180) were obtained as previously reported.[2,6] The horseradish peroxidase-conjugated Fab' fragment of A180 (HRP-A180) was prepared by the method of Yoshitake *et al.*[7]

ELISA Ninety-six well-microtiter plates (Nunc) were coated with 100 μl of A46 solution (10 μg/ml) diluted with 0.05 M sodium carbonate buffer (pH 9.6) for 2 h at 25 °C and washed with Dulbecco's phosphate buffered saline without calcium and magnesium ions containing 0.05% Tween 20 (PBS-T). Each of these prepared wells was filled with 100 μl of CPB-I or a sample diluted with PBS-T containing 25 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 7.4 (PBS-T-E). After 2 h incubation and washing with PBS-T, 100 μl of HRP-A180 (diluted to 100 ng/ml with PBS-T) was added to each well and incubated at 25 °C for more than 2 h. After washing with PBS-T, each well was filled with 100 μl of 0.1 M citrate–0.2 M phosphate buffer (pH 5.0) containing 0.01% hydrogen peroxide and 0.4 mg/ml *o*-phenylenediamine as a color reagent. After adding 50 μl of 4.5 M sulfuric acid, the absorbance of the developed color was read at 492 nm. CPB-I levels in all of the samples were measured in duplicate and at three dilutions.

Cell Culture Human lymphocytes were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, and 50 μM 2-mercaptoethanol. Other cells were cultured in Eagle's MEM or Dulbecco's MEM supplemented with 10% inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin. Lymphocytes at a density of 1×10^6 cells/ml were grown for 3 d, and after the cells and media had been separated by centrifugation, the cells were washed twice with Hanks' salt solution. Other cells were cultured until they were about 90% confluent, washed three times with Hanks' salt solution and then harvested with a rubber policeman. Cells were sonicated in PBS-T-E, frozen and thawed three times, and centrifuged. The supernatants were used for measurement of CPB-I and protein concentration. Protein concentration was determined by the method of Lowry *et al.*[8]

Preparation of Specimens of *Macacus irus* Organs of *Macacus irus* ($n=1$) were washed with saline, minced with scissors, suspended in 9 volumes (w/v) of PBS-T-E, homogenized with Polytron (Kinematica), sonicated, frozen and thawed three times, and then centrifuged. The supernatants were used for the measurement of CPB-I.

Human Plasma and Other Specimens Plasma ($n=5$) and urine ($n=5$) samples were obtained from apparently normal, non-pregnant women in different stages of their menstrual cycle, normal pregnant women in

different gestational stages and toxemic patients. Amniotic fluids ($n=48$), milk ($n=3$), overian follicular fluid ($n=65$) and ascites ($n=17$) were obtained from normal women and carcinomatous patients. Semen ($n=6$) specimens were obtained from normal men and immediately separated into seminal plasma and sperma by centrifugation. Saliva samples were obtained from normal women ($n=3$) and men ($n=2$).

Results

ELISA for CPB-I Measurement In order to detect CPB-I levels in many types of specimens, we first developed the ELISA system for CPB-I in buffer and determined the concentration and incubation time of antibodies (A46 or HRP-A180) or samples, as described in Experimental (data not shown). The standard curve of CPB-I diluted with PBS-T-E was almost linear in the range of 0.4 to 25 ng/ml (Fig. 1). Therefore, we next wished to measure the CPB-I level in plasma; the curves of the protein in human plasma diluted with PBS-T-E are shown in Fig. 1. Eight-fold or more dilution of plasma allowed a good fit of the concentration–response curves to the standard curve of CPB-I. The intra- and inter-assay coefficient of variations, using plasmas with added CPB-I were 2.38% ($n=12$) and 3.18% ($n=8$) [CPB-I, 10 ng/ml], respectively.

CPB-I Levels in Specimens CPB-I levels were less than 10 ng/ml in the plasma from women at different points of their menstrual cycle and different trimester of pregnancy (Table I). Both the urine and plasma CPB-I levels were less than 10 ng/ml. In toxemic patients, the levels were 23.7 ± 16.3 ng/ml in plamsa and 24.5 ± 10.2 ng/ml in urine. Although these levels were higher than those of normal pregnant women, the differences were not statistically significant in these studies. CPB-I levels in various human body fluids are shown in Table II and these were higher than in plasma: 0.181 ± 0.098 μg/ml in amniotic fluid, 0.104 ± 0.038 μg/ml in saliva and 0.263 ± 0.076 μg/ml in milk. The CPB-I level in the ascites of peritonic patients with malignancies was 1.145 ± 0.465 μg/ml, being more than ten times higher than in normal women ($p<0.001$). The levels in follicular fluids in immature and atretic stages were

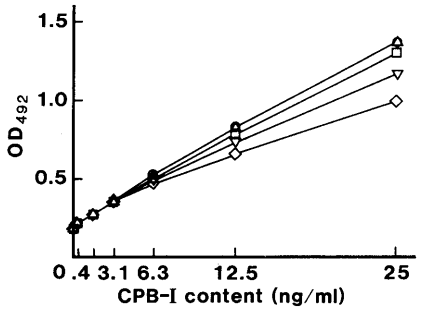

Fig. 1. Measurement of CPB-I Added to Plasma

Ninety-six well-microtiter plates coated with A46 (10 μg/ml) were incubated with human plasma containing CPB-I differently diluted with PBS-T-E (○, not containing plasma (PBS-T-E); ◇, 2-fold; ▽, 4-fold; □, 8-fold; △, 16-fold), following by HRP-A180. See details in the text.

Table I. CPB-I Levels in Plasma and Urine of Different Menstrual Cycles, Pregnant Ages and Toxemic Patients

Sample	CPB-I level (ng/ml)[a]	
	Plasma	Urine
Non-pregnant women		
Menstrual phase	10.4± 3.4[b]	n.t.[c]
Follicular phase	9.5± 1.0	n.t.
Luteal phase	10.0± 2.0	n.t.
Pregnant women		
First trimester	5.2± 0.8	10.9± 3.4
Second trimester	7.2± 0.7	4.6± 2.2
Third trimester	8.0± 1.0	3.9± 1.0
Toxemia	23.7±16.3	24.5±10.2

a) CPB-I levels in the samples were measured by the ELISA system. *b*) Each value is a mean±S.E. of 5 samples. *c*) n.t., not tested.

Table II. CPB-I Levels in Human Body Fluids

Sample	*n*	CPB-I level (μg/ml)[a]	
Amniotic fluid	48	0.181±0.098[b]	
Saliva	5	0.104±0.038	
Milk	3	0.263±0.076	
Semen	6		
Seminal plasma		6.77±0.44	
Sperma		0.13±0.01	
Follicular fluid			
Mature	33	0.029±0.037	
Immature	18	0.072±0.074	[c]$p<0.01$
Atretic	14	0.102±0.080	$p<0.01$
Ascites			
Normal	14	0.129±0.015	
Carcinomatous peritonitis	3	1.145±0.465	$p<0.001$

a) CPB-I levels in the samples were measured by the ELISA system. *b*) Each value is a mean±S.E. *c*) *p* indicates the results of statistical analysis against mature stage or normal ascites by Student's *t* test.

Table III. CPB-I Levels in Cultured Human Lymphocytes

Cell	Type	CPB-I level[a]		
		Cell		Medium (ng/ml)
		(μg/10^7 cells)	(μg/mg protein)	
RPMI8866	B-Lymphoblastoid	0.136	0.115	n.d.[b]
CESS	B-Lymphoblastoid	0.812	0.623	7.0
SKW6-C14	B-Lymphoblastoid	0.412	0.572	n.d.
Daudi	Burkitt's lymphoma	0.051	0.118	n.d.
Raji	Burkitt's lymphoma	0.018	0.183	n.d.
CEM	T-Lymphoma	0.015	0.023	n.d.
MOLT4	T-Lymphoma	0.583	0.680	n.d.
U266	IgE-myeloma	1.146	1.475	11.4
U937	Monocyte/histiocyte	0.632	0.445	n.d.

a) CPB-I levels in the samples were measured by the ELISA system. *b*) n.d., not detected.

Table IV. CPB-I Levels in Various Cultured Human Cells

Cell	Characteristic	CPB-I level[a] (μg/mg protein)
Flow 2000	Embryonic lung	10.30
Flow 4000	Embryonic kidney	4.21
Flow 7000	Foreskin	9.47
Flow 13000	Embryonic liver	2.91
HUVEC	Umbilical vein endothelium	5.04
NHEK	Normal epidermal keratinocyte	3.40
AV3	Amnion	2.33
WISH	Amnion	2.46
A431	Epidermoid carcinoma	2.06
G361	Melanoma	4.50

a) CPB-I levels in the samples were measured by the ELISA system.

TABLE V. CPB-I Levels in Tissues of *Macacus irus*

Tissue	CPB-I level[a]	
	(μg/g wet tissue)	(μg/mg protein)
Brain	19.69	1.557
Heart	22.37	0.708
Thymus	60.78	1.289
Lung	90.99	1.046
Liver	15.39	0.232
Kidney	63.61	0.731
Spleen	210.4	1.092
Intestine	75.53	1.135
Muscle	10.25	0.349

a) CPB-I levels in the samples were measured by the ELISA system.

0.072 ± 0.074 μg/ml and 0.102 ± 0.080 μg/ml, respectively, significantly higher than that in mature stages, which were 0.029 ± 0.037 μg/ml ($p < 0.01$). The CPB-I level in semen was 6.90 μg/ml and was mainly found in seminal plasma. In most kinds of cultured human lymphocytes the levels, based on cell number, were between 0.015 and 1.146 $\mu g/10^7$ cells and, based on protein, between 0.023 and 1.475 μg/mg protein and independent of origin (Table III). CPB-I levels in other kinds of cultured human cells, derived from lung, kidney, foreskin, liver, epidermis, endothelium, amnion and cancer, were between 2.06 and 10.30 μg/mg protein (Table IV). The level fell within the range of 10 to 210 μg/g tissue, or 0.349 to 1.557 μg/mg protein in most tissues from *Macacus irus*, with the muscle showing the lowest level (Table V). CPB-I in *Macacus irus* was confirmed by Western blot analysis (data not shown).

Discussion

In order to measure CPB-I levels in many types of specimens, we developed an ELISA system for CPB-I using two monoclonal antibodies. Good results in terms of both sensitivity and precision were obtained for all the tested specimens. Since we isolated CPB-I from human placenta as an anticoagulant and a phospholipase A_2-inhibitory protein,[2] it was important to determine if CPB-I levels change according to the stage of the menstrual cycle or gestational age. Levels in the plasma and urine of women were as low as 10 ng/ml, and no significant differences were found in the different trimesters of pregnancy and throughout the menstrual cycle. Shirotake *et al.*[9] reported that throughout pregnancy, there were no changes in the plasma level of PP4, one of anticoagulant proteins isolated from human placenta. PP4 is an substance identical to CPB-I, as was demonstrated by recent analysis of its cDNA sequence.[10] These results suggest that CPB-I may not be correlated to the pregnancy and the menstrual cycle. CPB-I levels in the plasma and urine of toxemic patients were higher than those of normal pregnant women, but the differences between the two values were not significant statistically.

Endonexin II has been shown to be a calcium- and phospholipid-binding protein[11] and has recently been demonstrated by DNA sequencing to be an identical substance to CPB-I.[12] Our preliminary studies indicated that CPB-I bound to calcium ($K_d = 2 \times 10^{-4}$ M) and anionic phospholipids in a calcium dependent manner, and, furthermore, bound to CPAE cells (pulmonary artery endothelium, bovine, ATCC No. CCL209) when it was added to culture media (unpublished data). The present observation that CPB-I levels in plasma were as low as 10 ng/ml may indicate that CPB-I easily binds to phospholipids in plasma membranes such as the membranes of endothelial cells on blood vessels.

CPB-I was found in all tissues of *Macacus irus* and all of the cultured human cells measured. PP4 has also been reported to exist in many kinds of human tissues according to Ouchterlony's gel diffusion test.[13] Therefore, CPB-I might be a ubiquitous protein in the body, although it was originally isolated from human placenta and demonstrated to be an inhibitor of tissue thromboplastin (TF).[2] TF has been reported to be requisite for adhesion and spreading in cells cultured in monolayers.[14] Since CPB-I levels in lymphocytes were less than those in other types of cells which proliferate in an anchor dependent manner, it might have an important function in adhesion. It was detected in body fluids such as amniotic fluid, ascites, semen, saliva and milk, and was detected at high level in seminal plasma and ascites of peritonic patients with malignancies. Little CPB-I was secreted from cultured human lymphocytes (Table III), but it was detected in the body fluids measured; thus, it is not clear whether CPB-I is secreted or not. In patients with malignancies, CPB-I might be released into ascites and blood by the destruction of cells. In seminal plasma, on the other hand, it is likely that CPB-I affects the sperma motion, because sperma has the capability of motion for the first time when mixed with seminal plasma. Increased PP4 serum levels were also reported in patients with ovarian, endometrial and cervical cancer.[9] CPB-I in amniotic fluid adjacent to the placenta, which is rich in TF and inhibitors of fibrinolysis, might have an important effect on the coagulant system. The levels in follicular fluids at immature and atretic stages of the menstrual cycle were higher than that in the mature stage, which may indicate that CPB-I is associated with the maturation and the atresia of the ovarian follicle.

In conclusion, CPB-I was distributed in all tissues, cells and fluids in the body, however, we could find no significant relationship between its distribution and its physiological functions. Therefore, our investigations are continuing.

References

1) M. Maki, M. Murata and Y. Shidara, *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **17**, 149 (1984).
2) A. Iwasaki, M. Suda, H. Nakao, T. Nagoya, Y. Saino, K. Arai, T. Mizoguchi, F. Sato, H. Yoshizaki, M. Hirata, T. Miyata, Y. Shidara, M. Murata and M. Maki, *J. Biochem.* (Tokyo), **102**, 1261 (1987).
3) B. P. Wallner, R. J. Mattaliano, C. Hession, R. L. Cate, R. Tizard, L. K. Sinclair, C. Foeller, E. P. Chow, J. L. Browning, K. L. Ramachandran and R. B. Pepinsky, *Nature* (London), **320**, 77 (1986); K. S. Huang, B. P. Wallner, R. J. Mattaliano, R. Tizard, C. Burne, A. Frey, C. Hession, P. McGrey, L. K. Sinclair, E. P. Chow, J. L. Browning, K. L. Ramachandran, J. Tang, J. E. Smart and R. B. Pepinsky, *Cell*, **46**, 191 (1986).
4) K. Weber, N. Johnsson, U. Plesman, P. N. Van, H. D. Soling, C. Ampe and J. Vandekerckhove, *EMBO J.*, **6**, 1599 (1987); H. T. Haigler, D. D. Schlaepfer and W. H. Burgess, *J. Biol. Chem.*, **262**, 6921 (1987).
5) C. J. M. Saris, B. F. Tack, T. Kristensen, J. R. Glenney, Jr. and T. Hunter, *Cell*, **46**, 201 (1986).
6) H. Nakao, T. Nagoya, Y. Saino, Y. Shidara, M. Murata and M. Maki, *Ketsueki To Myakkan*, **19**, 205 (1988).

7) S. Yoshitake, M. Imagawa, E. Ishikawa, V. Niitsu, I. Urushizaki, M. Nishiura, R. Kanazawa, H. Kurosaki, S. Tachibana, N. Nakazawa and H. Ogawa, *J. Biochem.* (Tokyo), **92**, 1413 (1982).
8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) S. Shirotake, N. Inaba, Y. Ohta, I. Fukazawa, H. Takamizawa and H. Bohn, *Chem. Pharm. Bull.*, **34**, 2191 (1986).
10) U. Grundmann, K. J. Abel, H. Bohn, H. Lobermann, F. Lottspreich and H. Kupper, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3708 (1988).
11) D. D. Schlaepfer, T. Mehlman, W. H. Burgess and H. T. Haigler, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6078 (1987).
12) R. Kaplan, M. Jaye, W. H. Burgess, D. D. Schlaepfer and H. T. Haigler, *J. Biol. Chem.*, **263**, 8037 (1988).
13) H. Bohn, W. Kraus and W. Winckler, *Arch. Gynecol.*, **236**, 225 (1985).
14) L. R. Zacharski and O. R. McIntyre, *Blood*, **41**, 679 (1973); *idem*, *Nature* (London), **232**, 338 (1971).