RADIOIMMUNOASSAY FOR AQUAPORIN-9

CHANG LI[1], YOSHIFUMI HIROOKA[1], TOMOKO YASAKA[2], JUNKO TAKAGI[1], MINEHIRO GOTOH[1], TSUYOSHI NOGIMORI[3]

[1]Department of Laboratory Medicine, [2]Division of Endocrinology, Metabolism and Diabetology, Department of Internal Medicine, Aichi Medical University School of Medicine, Nagakute, Aichi, Aichi and [3]Department of Internal Medicine, Konanshowa Hospital, Konan, Aichi (Japan)
E-mail: labmed@aichi-med-u.ac.jp

Objective. To develop radioimmunoassay for aquaporin-9(AQP9) and search for its presence in certain rat tissues.

Methods. Anti-AQP9 antiserum has been raised in New Zealand white rabbits immunized with a conjugate of synthetic AQP9 with bovine serum albumin. Radioiodination of AQP9 was performed by chloramin T method followed by purification of radioiodinated material on Sephadex G-25 column.

Results. The obtained antibody did not crossreact with other aquaporins, hypothalamic hormones, pituitary hormones, neuropeptides or gut hormones. The assay was performed with a double antibody system. AQP9 was extracted from the tissues with acid acetone. The dilution curve of acid acetone extracts of rat liver in the radioimmunoassay system was parallel to the standard curve. The recovery of tissue AQP9 was about 90 %, and the intra-assay and inter-assay variations were 4.8 % and 7.9 %, respectively. AQP9 was found in the liver, testis and brain.

Conclusion. These data suggest that this assay system is suitable for the estimation of AQP9 in the tissues.

Key words: Aquaporin-9 - Liver - Testis - Brain - Radioimmunoassay

At least 10 mammalian aquaporins have been identified (AGRE et al. 2002), and these are selectively permeated by water or water plus glycerol. The rat aquaporin-9 (AQP9) gene and the human AQP9 gene were cloned (ISHIBASHI et al. 1998; TSUKAGUCHI et al. 1998) and later on by others (KO et al. 1999). AQP9 cDNA was found to encode a 295 amino acid protein in both rat and human (ISHIBASHI et al. 1998; TSUKAGUCHI et al. 1998; KO et al. 1999). Rat AQP9 has the greatest homology with human AQP9 (KO et al. 1999). AQP9 mRNA has been found in rat liver (TSUKAGUCHI et al. 1998; KO et al. 1999; GARCIA et al. 2001; NIHEI et al. 2001; CARBREY et al. 2003), testis (TSUKAGUCHI et al. 1998; KO et al. 1999; GARCIA et al. 2001; NIHEI et al. 2001), brain (KO et al. 1999; GARCIA et al. 2001), and lung (TSUKAGUCHI et al. 1998; KO et al. 1999). In human, AQP9 mRNA has been detected in leukocytes, liver, lung, spleen (ISHIBASHI et al. 1998; TSUKAGUCHI et al. 1998) and bone marrow (TSUKAGUCHI et al. 1998). Thus, AQP9 mRNA has been shown to be present in different tissues, but there have not been any reports concerning the tissue localization of the quantitative level of protein. Therefore, this study was undertaken to examine the tissue distribution of AQP9 protein by newly developed radioimmunoassay system.

Materials and Methods

Animals. Male Wistar rats weighing 250 g were housed in a temperature (22 °C) and humidity (60 %) controlled room with 12 h illumination cycle. They were fed with laboratory chow and water ad libitum. After twelve hours fasting, the rats were sacrificed under sodium pentobarbital anesthesia (60 mg/kg) and the tissues (liver, testis and brain) were obtained. The brain was removed and the hypothalamus, cerebrum and cerebellum were separated according to the method previously described (MITSUMA et al. 1991). The study was approved by the Animal Ethical Committee of Aichi Medical University School of Medicine.

Hormones and chemicals. Synthetic hypothalamic hormones, pituitary hormones, neuropeptides and gut hormones were purchased from the Protein Research Foundation (Osaka, Japan). Bovine serum albumin (BSA) and glutaraldehyde were obtained from the Wako Chem. Co. Ltd. (Tokyo, Japan). Sephadex G-25 was purchased from Phamacia (Sweden). Other substances were obtained from Sigma (Mo. USA).

Generation and characterization of anti-AQP9 antibody. Peptides corresponding to the following sequences of AQP9 were synthesized using a solid phase method by an automated peptide synthesizer, followed by purification with HPLC:VFKAEQSEDKPEKYE, according to the method previously described (Hirooka et al. 1993). The peptides were conjugated on an equal weight basis to bovine serum albumin by the method previously described for anti-GHRH antibody (Mitsuma et al. 1983), using glutaraldehyde. New Zealand white rabbits were immunized with the emulsion of 1 mg of this conjugate in 1 ml water in complete Freund's adjuvant (1:2, v/v) which was injected into the foot pad at intervals of three weeks. Blood was drawn one week after each injection. The presence of anti-AQP9 antiserum was checked by immunoprecipitation method as reported elsewhere (Hirooka et al. 1992).

Radioiodination of AQP9 and purification. Radioiodination of AQP9 was performed with the chloramin T method according to the report by Greenwood et al. (1963). The radioiodinated materials were chromatographed on Sephadex G-25 (1x20 cm), eluted with 0.01 M phosphate buffer (pH 7.5) and collected in one ml fractions. The first peak was I-125 labeled AQP9 and the second peak was free I-125. Specific activity was calculated to be approximately 200 micro Ci /micro g.

Assay buffer. The assay buffer consisted of 0.01 M phosphate buffer (pH 7.4) with 0.1 % BSA, 0.1 % mercaptoethanol and 0.1 % triton X-100.

Assay procedure. Double antibody radioimmunoassay system was used and the schematic diagram of the procedure was shown in Table 1.

Extraction of AQP9 from the tissues. The extraction of AQP9 was performed by the method previously described (Mitsuma et al. 2000). The freshly obtained tissues were weighed and placed in 0.5 ml acid acetone, homogenized and centrifuged. The supernatants were dried under the air stream in a water bath (56°C). The recovery of this extraction method was evaluated by adding a known amount of synthetic AQP9 to the tissues. The recovery was found to be approximately 90 %.

Results

Preparation of anti-AQP9 antiserum. All three rabbits responded to the immunization and developed antibodies at a final dilution 1:1000 or higher. The antiserum used in this study was obtained one week after the third injection and showed a specific binding at a final dilution of 1:8000.

Specificity of antiserum. The specificity of anti-AQP9 is shown in Table 2. No crossreactivity was observed with AQP1, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, AQP8, hypothalamic hormones, pituitary hormones, neuropeptides and gut hormones.

Standard curve and dilution curve of acid acetone-extracted liver. The detection limit in this system was caluculated as 10 pg/ml. Parallel curve was obtained for the dilution of acid acetone-extracted liver.

Recovery experiment. Recovery of AQP9 was evaluated with known amount of AQP9 to the liver and measured with the radioimmunoassay. The recovery was approximately 100 %.

Intra-assay and inter-assay variation. The intra-assay variation was 4.8 %, and inter-assay variation was 7.9 %.

AQP9 in the tissues. AQP9 was found in the liver, testis and brain shown in Table 3.

Discussion

The present study demonstrated that AQP9 was found in rat liver, testis and brain by newly developed radioimmunoassay system. These data support the previous report which showed that AQP9 mRNA is present in rat liver (Tsukaguchi et al. 1998; Ko et al. 1999; Garcia et al. 2001; Nihei et al. 2001; Carbrey et al. 2003), testis (Tsukaguchi et al. 1998; Ko et al. 1999; Garcia et al. 2001; Nihei et al. 2001), and brain (Tsukaguchi et al. 1998; Ko et al. 1999; Garcia et al. 2001). We confirmed this especially in quantitative protein levels. AQP9 was found to be abundant in liver where it may be involved in transmembrane water or solute transport including a potential role for bile formation. AQP9 has been reported to have high permeability to solute as well as water (Tsukaguchi et al. 1998; Ko et al. 1999). Since the liver is a major site of urea production (Ishibashi et al. 1998, Tsukaguchi et al. 1998), a function of AQP9 in the liver as urea channel has been proposed. In addition to urea, it also has been suggested that AQP9 may provide exit routes for solutes such as purins and pyrimidines derived from nucleotide syn-

Table 1
A schematic diagram of the assay procedure for AQP9

Standard or samples	0.1 ml
Antibody (1 :1000)	0.1 ml
AQP9-I-125	0.1 ml
Buffer	0.5 ml

Incubated for 24 hours at 4° C
Added second antibody solution at 4° C
Centrifuged at 3000 rev / min at 4° C
Decanted supernatants
Counted (precipitates)
Calculated bound / total count (B / T)

Table 2
Relative reactivity of aquaporins, hypothalamic hormones, pituitary hormones, neuropeptides or gut hormones in AQP9 radioimmunoassay system

AQP9	100	Adrenomedullin	0.001
AQP1	0.001	Endothelin-1	0.001
AQP2	0.001	Endothelin-2	0.001
AQP3	0.001	Endothelin-3	0.001
AQP4	0.001	ACTH	0.001
AQP5	0.001	TSH	0.001
AQP6	0.001	LH	0.001
AQP7	0.001	FSH	0.001
AQP8	0.001	Prolactin	0.001
TRH	0.001	GH	0.001
Somatostatin	0.001	Alpha-MSH	0.001
GHRH	0.001	Vasopressin	0.001
LHRH	0.001	Oxytocin	0.001
Neurotensin	0.001	Secretin	0.001
Beta-endorphin	0.001	Glucagon	0.001
Leucine-enkephalin	0.001	Gastrin	0.001
Dynorphin	0.001	GLP-1	0.001
Alpha-neoendorphin	0.001	GLP-2	0.001
Beta-neoendorphin	0.001	rat Ghrelin	0.001
Substance P	0.001	human Ghrelin	0.001
VIP	0.001	Resistin	0.001
CGRP	0.001		
ANP	0.001		
BNP	0.001		
CNP	0.001		

Arbitary values of AQP9 = 100

Table 3
Immunoreactive AQP9 in rat organs (ng / g wet weight)

Liver	3.12±0.15 ng / g
Testis	1.47±0.13 ng / g
Hypothalamus	0.62±0.14 ng / g
Cereberum	0.01±0.08 ng / g
Cerebellum	0.09±0.01 ng / g

The values are shown by mean±SE in each group of seven rats

thesis de novo, lactate and ketone bodies (Tsukaguchi et al. 1998). However, further studies need to be done in order to elucidate this. The protein level of AQP9 in testis was found less than that in liver. The role of testis AQP9 still remains obscure. Regarding the endocrine component of testis, it synthesizes and secretes the male sexual hormone androgen. There are conflicting results as to the effect of androgen on AQP9 (Pastor-Soler et al. 2002; Badran and Hermo 2002). Further studies need to be done to elucidate the significance of the localization of AQP9 in testis. Several groups have shown the presence of AQP9 mRNA in the rat brain (Tsukaguchi et al. 1998; Ko et al. 1999; Garcia et al. 2001). In the present study, AQP9 at the protein level was found in the hypothalamus, cerebrum and cerebellum. The presence of AQP9 in the hypothalamus raises the possibility, that the water channel may be involved in the extrachoroidal production and the extraarachnoidal reabsorption of the cerebrospinal fluid. Concerning the existence of AQP9 in the cerebrum and cerebellum, no report has been shown. In order to elucidate the significance of the localization of AQP9 in the above tissues, further studies need to be done. The present study suggest that this assay system is suitable to measure AQP9 in the tissues.

Acknowledgments

The authors acknowledge Ryo Kojima and Kazuko Fujisaki for the excellent secretarial assistance.

References

AGRE P, KING LS, YASUI M, GUGGINO WB, OTTERSEN OP, FUJIYOSHI Y, ENGEL A, NIELSEN S: Aquaporin water channels- from atomic structure to clinical medicine. J Physiol **542,**13-16, 2002

BADRAN HH, HERMO LS: Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. J Androl **23**, 358- 373, 2002

CARBREY JM, GORELICK-FELDMAN DA, KOZONO D, PRAETORIUS J, NIELSEN S, AGRE P: Aquaglyceroporin aqp9: solute permeation and metabolic control of expression in liver. Proc Natl Acad Sci USA **100**, 2945-2950, 2003

GARCIA F, KIERBEL A, LARCOCCA MC, GRADILONE SA, SPLINTER P, LARUSSO NF, MARINELLI RA: The water channel aquaporin 9 is mainly intracellular in rat hepatocytes, and its plasma membrane insertion is stimulated by cyclic AMP. J Biol Chem **276,** 12147-12152, 2001

GREENWOOD FC, HUNTER WM, GLOVER JS: The preparation of 131-I labeled human growth hormone of high specific radioactivity. Biochem J **89**, 114-123, 1963

HIROOKA Y, MITSUMA T, NOGIMORI T, ISHIZUKI Y, SAKAI J, SUGIE I , NARUSE M, FUKAYAMA M: Generation of bioactive rabbit antibodies against the extracellular domains of human thyrotropin receptor peptides and detection of hypertriiodothyroninemia in the rabbit. Med Sci Res **20**, 639-640, 1992

HIROOKA Y, MITSUMA T, NOGIMORI T, ISHIZUKI Y, SAKAI J, NARUSE M, FUKAYAMA M: In vivo stimulation of thyroid hormone release by rabbit antibodies with thyroid stimulating activity raised against the mutated receptor peptide TSH-R-HIS 17. Life Sci **53**, 659- 664, 1993

ISHIBASHI K, KUWAHARA M, GU Y, TANAKA Y, MARUMO F, SAKAI S: Cloning and functional expression of a new aquaporin(AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. Biochem Biophys Res Commun **244**, 268-274, 1998

KO SB, UCHIDA S, NARUSE S, KUWAHARA M, ISHIBASHI K, MARUMO F, HAYAKAWA T, SASAKI S: Cloning and functional expression of rAQP9L a new member of aquaporin family from rat liver. Biochem Mol Biol Int **47**, 309-318, 1999

MITSUMA T, SUN DH, NOGIMORI T, CHAYA M, MURAKAMI K, MARUTA K, TERAO S, MORISHIMA T: J Aichi Med Assoc **14,** 243-251, 1983

MITSUMA T, ADACHI K, YAMASHITA K, TOMITA T: Concentration of thyrotropin-releasing hormone, substance P and somatostatinn in the brain of the gracil axonal dystrophy mouse. Med Sci Res **19,** 207-208, 1991

MITSUMA T, HIROOKA Y, KAYAMA M, MORI Y, YOKOI Y, RHUE N, PING N, IZUMI M, IKAI R, ADACHI K, NOGIMORI T: Radioimmunoassay for orexin A. Life Sci **66,** 897-904, 2000

NICCHIA GP, FRIGERI A, NICO B, RIBATTI D, SVELTO M: Tissue distribution and membrane localization of aquaporin-9 water channel: evidence for sex-linked differences in liver. J Histochem Cytochem **49**, 1547-1556, 2001

NIHEI K, KOYAMA Y, TANI T, YAOITA E, OHSHIRO K, ADHIKARY LP, KUROSAKI I, SHIRAI Y, HATAKEYAMA K, YAMAMOTO T: Immunolocalization of aquaporoin-9 in rat hepatocytes and leydig cells. Arch Histol Cytol **64**, 81-88, 2001

PASTOR-SOLER N, ISNARD-BAGNIS C, HERAK-KRAMBERGER C, SABOLIC I, VAN HOEK A, BROWN D, BRETON S:Expression of aquaporin 9 in the adult rat epididymal is modulated by androgens. Biol Reprod **66**, 1716-1722, 2002

TSUKAGUCHI H, SHAYAKUL C, BERGER UV, MACKENZIE B, DEVIDAS S, GUGGINO WB, VAN HOEK AN, HEDIGER MA: Molecular characterization of a broad selectivity neutral solute channel. J Biol Chem **273**, 24737-24743, 1998

Corresponding author: Yoshifumi Hirooka, MD, PhD
Department of Laboratory Medicine
Aichi Medical University School of Medicine
Nagakute, Aichi, Aichi, 480-1195, Japan
E-mail: labmed@aichi-med-u.ae.jp
Fax: +81-561-62-3075