Research Paper

Distribution of the AQP4 Water Channel in Normal Human Tissues

Protein and Tissue Microarrays Reveal Expression in Several New Anatomical Locations, including the Prostate Gland and Seminal Vesicles

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ABSTRACT
Aquaporins facilitate osmotically driven water movement across cell membranes. Aquaporin 4 (AQP4) is a major water channel in the central nervous system where it participates in cerebral water balance. AQP4 is also present in basolateral membranes of lower respiratory tract airway and renal collecting duct epithelial cells, gastric parietal cells and skeletal muscle cells. However, the distribution of AQP4 in many other tissues is still unknown. The aim of this study was to determine the expression and relative abundance of AQP4 in human Tissue MicroArrays (TMAs) and human protein microarrays by immunohistochemistry and chemiluminescence. In the central nervous system AQP4 was abundantly expressed in the cerebral cortex, cerebellar cortex (purkinje/granular layer), ependymal cell layer, hippocampus and spinal cord. Lower levels were detected in choroid plexus, white matter and meninges. In the musculoskeletal system AQP4 was highly expressed in the sarcolemma of skeletal muscle from the chest and neck. In the male genital system AQP4 was moderately expressed in seminiferous tubules, seminal vesicles, prostate and epididymis. In the respiratory system AQP4 was moderately expressed in lungs and bronchus. AQP expression was abundant in the kidney. In the gastrointestinal system AQP4 was moderately present in basolateral membranes of parietal cells at the base of gastric glands. AQP4 was also detected in salivary glands, adrenals, anterior pituitary, prostate and seminal vesicles. Human protein microarrays verified the TMA data. Our findings suggest that AQP4 is expressed more widely than previously thought in human organs and may be involved in prostatic and seminal fluid formation.

INTRODUCTION
Aquaporins (AQPs) are members of a recently expanded superfamily of membrane proteins that facilitate water and small solute movement across biological membranes in microorganisms1 animals2-4 and plants.5-7 In mammals, members of the aquaporin water channel family are widely distributed in various cells and tissue types and contribute to the water permeability of epithelial and endothelial barriers.5-9 The members of this family may encode two distinct types of channel: 1) the aquaporins, which only transport water and 2) the aquaglyceroporins which also transport small neutral solutes such as glycerol.2 Currently 13 members of the AQP gene family (AQP0-12) have been identified in the human genome. In addition, there are a number of AQP pseudogenes that do not encode aquaporin proteins. These pseudogenes are associated with CDNA clones that differ greatly from the genomic sequences of the protein encoding aquaporin genes.1

The AQP4 gene (also known as HMIWC2 or MIWC) maps on chromosome 18, at 18q11.2-q12.1 (RefSeq) and encodes a protein that is the predominant aquaporin found in the brain. It was cloned in 1994 by two independent groups as a candidate osmoreceptor and regulator of water balance in the central nervous system.10,11 It is now generally accepted that AQP4 plays a crucial role in the formation of the blood brain barrier12-14 and the maintenance of cerebral water balance in response to brain edema formation.15-18 Two alternatively spliced transcript variants encoding distinct isoforms, AQP4a and AQP4b, have been found for this gene in the brain.19,20 In humans variant a of AQP4 has 323 amino acids (34.8 kDa) and is encoded by a 5236 base pair mRNA. Variant b of AQP4 is 301 amino acids long (32.2 kDa) and is encoded by a 5357 base pair mRNA. Functional studies of AQP4 reveal that it is mercurial-insensitive20 and water-selective.21 Early in situ hybridization studies of rat brain localized AQP4 mRNA

1http://www.ncbi.nih.gov/IEB/Research/Assembly/
2http://www.ncbi.nih.gov/IEB/Research/Assembly/av.cgi?exdb=AceView&db=35g&term=AQP4
to ependymal cells lining the aqueduct, glial cells forming the edge of the cerebral cortex and brainstem, vasopressin-secretory neurons in supraoptic and paraventricular nuclei of hypothalamus, and Purkinje cells of cerebellum. Immunohistochemical studies have established that AQP4 is present in astrocytes and immunogold studies have shown that the protein is highly concentrated in astrocyte end-feet that surround capillaries and form the glia limitans and in ependymal cell membranes. RNA interference experiments in primary cultures of astrocytes suggest that knockdown of AQP4 expression results in major alterations in cell morphology, cell growth, and water transport and induces changes in ischemia-related genes.

Detailed immunofluorescence and immunohistochemical studies in other rat tissues have revealed AQP4 expression on the basolateral plasma membrane of collecting duct principal cells and tracheal and bronchial epithelial cells, iris, ciliary body, and neural cell layers in retina. AQP4 is also present in fast-twitch fibers of mammalian skeletal muscle and immunocytochemical studies of dystrophic mdx mice have suggested a progressive reduction in AQP4 expression in fast skeletal muscle fibers.

Expression of sarcolemmal AQP4 together with that of microvascular AQP1 may account for rapid water movement from blood into muscle during intense activity or the rapid exit of metabolic water from skeletal muscle cells. More recent studies also suggest that AQP4 is expressed in acid secreting gastric parietal cells and is important for the formation of gastric secretions. With the exception of the vasopressin-regulated AQP2 water channel, AQPs (including AQP4) are primarily subject to transcriptional-level regulation rather than membrane-vesicle recycling by short-term regulation. However, there is evidence for regulation of AQP4 function by protein kinase C dependent phosphorylation which has been suggested to affect the permeability of the protein.

We have recently taken advantage of Tissue MicroArray (TMA) technology to determine the distribution and relative abundance of AQP1, AQP2 and AQP3 in normal human tissues and studied AQP1 expression in tumors of the lung, colon, prostate, breast and ovary. The picture that emerges from these studies suggests that these aquaporins are differentially distributed in human tissues. In tumors AQP1 expression in microvascular endothelia may be a consequence of angiogenesis and may be important for the formation or clearance of tumor edema. Recent studies also indicate that targeted AQP1 gene disruption impairs cell migration and tumour growth in tumour cell implanted AQP1-null mice. Stable transfection of cells from these animals with AQP1 or the structurally related AQP4 accelerates cell migration in vitro supporting a role for water channels in angiogenesis and tumor metastasis.

However, despite its biological significance and involvement in osmoregulation in the brain, the distribution of AQP4 in the human brain and many other human tissues is still unknown. This lack of knowledge confounds our understanding of the physiological roles of AQP4 in other tissues. Accordingly, in this study we studied the distribution and relative abundance of AQP4 in “body on a slide” and “central nervous system” human TMAs. Initially we tested four antibodies raised against AQP4 on rat TMAs and selected one of them as the antibody of choice for probing human TMAs. We also used human protein microarrays to probe for AQP4 expression and confirm the TMA data in 30 different human tissues. The information presented provides evidence for the expression of AQP4 in several locations not previously described by other investigators as well as confirming its expression as seen in earlier rodent studies.

MATERIALS AND METHODS

Chemicals and antibodies. All chemicals used in this study were molecular biology grade and were purchased from Sigma/Aldrich (Poole, Dorset, UK). Four different antibodies to AQP4 were used in the initial developmental stages of this study: a monoclonal antibody to rat AQP4 from Abcam (ab9512; mouse monoclonal antibody to rat AQP4, amino acids 301-318, intracellular C-terminal epitope), an affinity purified polyclonal antibody to rat AQP4 (Chemicon International; full details provided below) and two polyclonal antibodies developed in our own laboratories against the C-terminus of rat AQP4: SGAQPA4A, peptide sequence: NH2-(C)DNRSQV ETDDILK-COOH and SGAQPB, peptide sequence: NH2-(C)RSQVETEDLILKPG-COOH. Although all four antibodies produced similar results, the rabbit polyclonal antibodies against rat AQP4 were obtained from Chemicon International (Temecula, CA; Catalog No. AB3594, Lot No. 24071165) produced the most specific immunostaining with no background staining. Therefore, this affinity purified antibody was adopted as the probe of choice. This polyclonal antiserum was developed against a highly purified fusion protein corresponding to residues 249-323 of rat AQP4 (SwissProt Accession number P47863). It was expected that this polyclonal antibody would cross-react with human AQP4 due to the high sequence similarity between the rat and human AQP4 peptides corresponding to this region (69 identical amino acid residues out of 75). Nevertheless, we carried out a comprehensive characterization of this AQP4 antibody by Western blot analysis of total protein lysates from selected rat tissues (including kidney and skeletal muscle), immunohistochemical staining of custom-designed rat tissue microarrays and chemiluminescent immunostaining of commercially produced human protein microarrays. A monoclonal antibody to the α1 subunit of Na, K-ATPase (α6F) was used as an internal control for staining Na, K-ATPase in epithelial barriers. The α6F hybridoma originally developed by D. Fambrough (Johns Hopkins University) was obtained from the Developmental Studies Hybridoma Bank (http://www.uiowa.edu/~dshbwww/) developed under the auspices of the National Institute of Child Health and Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Immunohistochemistry was carried out using a DakoCytomation EnVision+ Dual Link System-HRP (DAB+) kit (Code K4065; Ely, Cambridgeshire, UK).

Animals. Adult Sprague-Dawley rats (n = 3, weight ranging from 350 g up to 550 g) were used in this study. The rats were fed standard rat chow and water ad libitum and were maintained under pathogen-free conditions in the animal house facilities of the University of Liverpool. The animals were used in strict accordance with the local ethical guidelines of the University of Liverpool, current UK Home Office and European regulations. The animals were overdosed with ketamine (Parke-Davis, UK; 120 mg/kg).

Rat tissue microarrays. A custom-designed rat tissue microarray containing a careful selection of rat organs was produced in our histology facility for characterization of the Chemicon AQP4 antibody and optimization of the immunohistochemical procedure, which is described in detail below. The rat tissue microarray contained formalin fixed paraffin embedded tissues and organs obtained from three adult Sprague-Dawley rats. The tissues chosen for this array were cardiac muscle, skeletal muscle (diaphragm and gastrocnemius), renal cortex and medulla, duodenum, distal colon, liver, pancreas, testis, cerebellar cortex and forebrain. Full details of the microarray design and the tissues included are included in the supplementary data file 2 accompanying this paper.
Tissue Distribution of AQP4

| Table 1 Summary of the semi-quantitative histomorphometric analysis of the immunohistochemical data obtained with polyclonal antibodies to AQP4 and the complete list of organ systems and tissue types represented on the CHTN2002N1 TMA |
|---|---|
| Tissue | Expression |
| 1 | aorta, smooth muscle |
| 2 | heart, myocardium |
| 3 | lymphatic endothelium |
| 4 | small muscular artery (lung) |
| 5 | small vein (intestine) |
| 6 | esophagus, squamous mucosa |
| 7 | gastric mucosa, antral |
| 8 | gastric mucosa, oxyntic |
| 9 | small intestine, mucosa |
| 10 | small intestine, epithelium |
| 11 | colon, mucosa |
| 12 | anus, mucosa |
| 13 | seminiferous tubules |
| 14 | epididymis |
| 15 | seminal vesicle |
| 16 | prostate |
| 17 | gallbladder |
| 18 | liver |
| 19 | liver |
| 20 | pancreas |
| 21 | pancreas |
| 22 | pancreas |
| 23 | salivary gland (parotid) |
| 24 | tonsil, squamous epithelium |
| 25 | skin, squamous epithelium |
| 26 | subepidermal tissue |
| 27 | subepidermal tissue |
| 28 | breast, epithelium |
| 29 | ectocervix |
| 30 | endocervix |
| 31 | endometrium, secretory |
| 32 | fallopian tube |
| 33 | adrenal gland, cortex |
| 34 | adrenal gland, medulla |
| 35 | parathyroid adenoma |
| 36 | pituitary, anterior |
| 37 | thyroid |
| 38 | adipose tissue, breast |
| 39 | cartilage, articular |
| 40 | cartilage, bronchial |
| 41 | skeletal muscle |
| 42 | smooth muscle, intestine |
| 43 | smooth muscle, uterus |
| 44 | synovium |
| 45 | ovary, primary oocytes |
| 46 | ovary, corpus luteum |
| 47 | ovary, epithelium |
| 48 | ovary, stroma |
| 49 | amniotic membrane |
| 50 | placenta, villi |
| 51 | autonomic ganglia & nerves, intestinal |
| 52 | peripheral nerve |
| 53 | cerebral cortex |
| 54 | cerebellar cortex, purkinje/granular layer |
| 55 | choroid plexus |
| 56 | ependymal cells |
| 57 | meninges |
| 58 | motor neurons (spinal cord) |
| 59 | white matter (subcortical) |
| 60 | hippocampus |
| 61 | lymph node |
| 62 | mucosa associated lymphoid tissue, appendix |
| 63 | spleen |
| 64 | thymus |
| 65 | tonsil |
| 66 | alveoli |
| 67 | bronchus, epithelium |
| 68 | bronchus, epithelium |
| 69 | kidney, cortex |
| 70 | kidney, medulla |
| 71 | bladder, transitional epithelium |

Human tissue microarrays. Normal human Tissue MicroArrays (TMAs) were obtained from the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute (NCI), the National Institutes of Health, Bethesda, MD, USA (http://faculty.virginia.edu/chtn-tma/home.html). The “body on a slide” CHTN2002N1 TMA contained formalin fixed paraffin embedded samples of 66 nonneoplastic adult tissues obtained from surgical resection specimens, obtained within one hour of surgical removal from anonymous donors. All the tissues represented on the CHTN2002N1 TMA were normal with the exception of parathyroid gland, which was from a benign parathyroid adenoma and lymphatic tissue which was from a benign lymphangioma. The central nervous system tissues on the CHTN2002N1 TMA were obtained from autopsy specimens within 36 hours of death. The tissues were grouped into cardiovascular, respiratory, gastrointestinal, hepatic and pancreatobiliary, oral, salivary and nasal, mammary, endocrine, genital tract, central and peripheral nervous systems, urinary tract, skin, cartilage and synovium.

Immunohistochemistry. The immunohistochemical protocol used was initially optimized using “test” human tissue microarrays, which contained selected tissues from the central nervous system and skeletal muscle. These control arrays were used to titrate immunohistochemical assay parameters and antibody dilutions prior to use of the more comprehensive tissue microarrays. TMAs were deparaffinized in xylene for 20 minutes to remove embedding media and washed in absolute ethanol for three minutes. The TMAs were gradually rehydrated in a series of alcohol baths (96%, 85% and 50%) and placed in distilled water for 5 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and the TMAs were then incubated for one hour at room temperature (RT) with 1% bovine serum albumin in TBS containing 0.5% Tween 20 (TBS-T) to block non-specific antibody binding. Slides were incubated overnight at 4°C with polyclonal antibodies to AQP4 (affinity purified antibody, diluted 1:200 in TBS-T) and Na, K-ATPase (hybridoma supernatant, diluted 1:10 in TBS-T) to block non-specific antibody binding. Slides were incubated overnight at 4°C with polyclonal antibodies to AQP4 (affinity purified antibody, diluted 1:200 in TBS-T) and Na, K-ATPase (hybridoma supernatant, diluted 1:10 in TBS-T). The diluted AQP4 antibody was centrifuged (10,000 x g for 5 min) at 4°C before use. After 24 hrs at 4°C the slides were washed three times for 5 min each in TBS-T before incubation with horseradish peroxidase (HRP) labeled polymer conjugated to affinity purified goat anti-rabbit and goat anti-mouse immunoglobulins for 30 minutes at RT. The sections were washed three times for 5 min in TBS-T before applying liquid DAB+ Chromogen (DAKO; 3,3'-diaminobenzidine) for up to 10 minutes. The development of the brown colored reaction was stopped by rinsing with distilled water. The stained slides were immersed for 5 min in a bath of aqueous hematoxyl (DakoCytomation, Code No. S3309) to counterstain cell nuclei. Finally the slides were washed for 5 min in running water and dehydrated in a series of graded ethanol baths before rinsing in three xylene baths and mounting in DPX (BDH laboratories, UK). Control experiments were performed by omitting primary antibody and exposing TMA slides to secondary antibody only.
Data acquisition and analysis. The stained TMA slides were visually scored in a double-blind fashion by two independent investigators. Staining results were then compared and recorded directly into a Microsoft Excel worksheet using a color coded key as follows: zero (blue) no expression; one (green) low expression; two (yellow) moderate expression; three (orange) high expression; four (red) abundant expression. After the two observers independently completed the analysis and semi-quantitative scoring of the same slides the results were compared. Variation in histomorphometric scoring between TMAs and between observers was infrequent. In cases where variation was found, the difference was never more than one unit. In rare instances where this occurred the process was overseen by a histopathologist who eliminated any remaining ambiguity, by providing a definitive score. The data obtained was linked to a database of digital images captured using a Nikon Microphot-FX microscope fitted with a Nikon DXM1200 digital camera.

Rat tissue homogenization and extraction of total protein. Rat tissue samples were processed by homogenization in liquid nitrogen with a pestle and mortar and the ground powder was resuspended in RIPA buffer consisting of phosphate buffered saline (PBS), 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) sodium dodecysulfate (SDS), 1 mM sodium orthovanadate and freshly added protease inhibitors: 4 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ pepstatin A, 10 μg ml⁻¹ leupeptin and 1 mM phenylmethylsulfonylfluoride (PMSF) on ice for 30 min. Insoluble material was removed by centrifugation at 10,000 g and the supernatant containing total proteins was stored at -80°C until assayed for protein content.

Protein determination. The protein concentration of the rat tissue homogenates was determined spectrophotometrically using a Bio-Rad detergent compatible (DC) protein assay kit. Bovine serum albumin was used as the protein standard.

SDS-PAGE and western blotting. Rat protein extracts (50 μg/lane) were solubilized in Laemmli buffer by heating at 95°C for 3 minutes, resolved on 4–20% SDS-PAGE minigels (AMS Biotechnology, Abingdon, UK) and transferred to PVDF membrane (AMS Biotechnology, Abingdon, UK). Membranes were blocked in TBS (pH 7.4) containing 0.5% Tween-20 and 10% nonfat dried milk powder (Marvel) and incubated in primary AQP4 antibody diluted.
Human protein microarrays. Human protein microarrays (also known as Mega Western protein Arrays; n = 3) were obtained from the BioChain Institute (Hayward, CA; Cat. No. W8234480, Lot# A801015; and distributed by AMS Biotechnology, Abingdon, UK). The 1 x 2 inch human protein arrays were manufactured by employing a proprietary technology that uses high quality denatured protein lysates from well-documented, ethically acceptable tissue sources. The protein arrays contained 32 different human tissue samples arranged on a PVDF membrane array. All protein samples were prefractionated into 24 fractions according to molecular weight by the manufacturer.

Chemiluminescence detection of AQP4 in human protein Microarrays. Protein microarrays were blocked with 5% BSA in TBS-T for 1 hour at room temperature before incubation with primary AQP4 antibody (diluted 1:1,000 in TBS-T) overnight at 4°C with gentle agitation. After 3 x 5 minute washes in TBS-Tween the arrays were incubated with HRP-conjugated anti-rabbit IgG for one hr at room temperature. Protein arrays were incubated with SuperSignal West Pico enhanced chemiluminescent substrate (Pierce) and after three x 5 minute washes in TBS-Tween they were exposed to Hyperfilm ECL film (Amersham Biosciences) and developed as described above.

RESULTS

Immunohistochemistry was used semiquantitatively to examine the expression patterns of the AQP4 water channel in 66 different types of normal human tissues represented on CHTN2002N1 CHTN TMAs. Images from all of the tissues present on the CHTN2002N1 CHTN TMA are included in supplementary data file 1 which accompanies this paper. The results of the semi-quantitative immunohistochemical and histomorphometric analysis of AQP4 expression in human TMAs have been summarized in supplementary data file 1 and in Table 1. Selected micrographs from the TMAs immunostained with polyclonal antibodies against AQP4 and a monoclonal antibody to the α1 subunit of Na, K-ATPase are shown in Figures 1–6. The results obtained with the rat TMA are summarized in Figure 7. The protein microarray data is shown in Figure 8.

The major findings of this study are summarized below:

1. AQP4 was abundant in the central nervous system. AQP4 was highly expressed in the cerebral cortex, cerebellar cortex, ependymal...
cell layer, hippocampus and spinal cord. Lower levels were detected in choroid plexus, white matter and meninges.

(2) In the musculoskeletal system AQP4 was highly expressed in the sarcolemma of skeletal muscle from the chest, chest wall and neck.

(3) In other systems AQP4 was moderately expressed in seminiferous tubules, prostate and lung. In the renal system AQP4 was selectively expressed in collecting tubules in the inner renal medulla. No expression was seen in the urinary bladder. In the gut AQP4 expression was only seen in parietal and chief cells at the base of gastric glands. Very weak AQP4 expression was noted in the adrenal cortex and pituitary gland. No expression was detected in cardiac muscle.

(4) Expression of Na, K-ATPase, used as a parallel control, was observed in all tissues especially in heart and skeletal muscle and in basolateral membranes of all epithelial cells except the choroid plexus where it localized to the apical membrane of choroid plexus epithelial cells.

(5) Test TMA slides were used as controls to demonstrate that non-specific immunostaining did not occur with the secondary antibody when primary antibody was omitted from the immunohistochemical protocol.

(6) Similar immunohistochemical results were obtained with rat TMAs. AQP4 immunostaining was abundant in the cerebellar cortex, lung and kidney. AQP4 immunostaining was not detected in rat liver and cardiac muscle.

The expression of AQP4 is now discussed in different organ systems and particular emphasis is placed on new sites of expression.

(7) Human protein microarrays probed with affinity purified polyclonal antibodies to AQP4 (Chemicon International) confirmed the immunohistochemical data obtained using human TMAs and revealed high AQP4 expression in protein fractions 13–18 (molecular weight range 30–46 kDa) in several tissues including placenta, brain (whole), cerebellum, diaphragm, duodenum, kidney, uterus, stomach and lung.

Renal, urinary and respiratory systems. In the kidney AQP4 immunostaining was detected in basolateral membranes of cortical and medullary collecting ducts (Fig. 2 and Supplementary data file 1). There was no expression detected in the urinary bladder and upper respiratory airways. No AQP4 expression was detected in hyaline condrocytes of bronchial cartilage (see Supplementary data file 1). However, high expression was seen in lower respiratory airways and alveoli in the lung. Parallel immunostaining of identical TMAs with a monoclonal antibody to the α1 subunit of Na, K-ATPase revealed abundant expression throughout the nephron, predominantly along basolateral membranes of proximal and distal tubules and medullary

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**Figure 4.** Immunohistochemical staining of AQP4 and the α1 subunit of Na, K-ATPase in selected cardiovascular and musculoskeletal tissues. The controls shown are from experiments where the primary antibody was omitted from the immunostaining procedure. All bars represent 100 μm.

**Figure 5.** Immunohistochemical localization of AQP4 and the α1 subunit of Na, K-ATPase in selected regions of the central nervous system, including cerebral cortex, cerebellar cortex, choroid plexus, ependymal cell layer, and hippocampus. The controls shown are from experiments in which the primary antibody was omitted from the immunohistochemical procedure. All bars represent 100 μm.
Tissue Distribution of AQP4

Figure 6. Localization of AQP4 in selected genital and reproductive systems including prostate (A), seminal vesicles (B) and in endocervix (C), parotid salivary gland (D), adrenal gland (E), anterior pituitary (F). All bars represent 100 μm.

Collecting ducts which is in agreement with early immunohistochemical studies of human and rat kidneys.\textsuperscript{37} However, some Na, K-ATPase immunostaining was also present in the glomerular capillary endothelium. Na, K-ATPase was also abundantly expressed in basolateral membranes of the transitional epithelium of the urinary bladder, upper respiratory airways and alveoli a finding that is consistent with published information in the literature\textsuperscript{38} (Fig. 2).

**Gastrointestinal and pancreatobiliary systems.** In the gastrointestinal system AQP4 was localized only to parietal cells in gastric pits (Fig. 3). No expression was detected in the squamous epithelium of the oesophagus. Very weak diffuse staining was observed in epithelial cells of the ileum. Extremely weak expression was noted in the anal mucosa (Fig. 3) and very weak expression was detected in epithelial cells in the gallbladder and pancreas (see Supplementary data file 1). In contrast Na, K-ATPase was abundantly expressed in basolateral membranes of epithelial cells from the beginning of the gastrointestinal tract (i.e., the oesophagus) to the anus (Fig. 3) and throughout the pancreatobiliary system (see Supplementary data file 1).

**Cardiovascular and musculoskeletal systems.** High AQP4 expression was detected in the sarcolemma of skeletal muscle but no expression was seen in cardiac muscle and intestinal smooth muscle (Fig. 4). No expression was detected in the lymphatic endothelium of the heart (Fig. 4), in aortic smooth muscle (Fig. 4) or in intestinal smooth muscle (see Supplementary data file 1). We found no AQP4 expression in skin and adipose tissues or in articular chondrocytes and synoviocytes (see Supplementary data file 1). Positive AQP4 expression was also noted in the endocervix.

**Central and peripheral nervous systems.** Heavy AQP4 and Na, K-ATPase immunostaining was observed in the central nervous system (Fig. 5). Indeed, this was the most abundant AQP4 expression seen in any part of the human body. AQP4 expression was abundant in cerebral cortex, cerebellum, ependymal cell layer and hippocampus. Expression densities in other areas of the central nervous system were varied (see Table 1). Positive expression was also detected in some cells in the choroid plexus epithelium (Fig. 5). Our data is consistent with many earlier observations in the rat central nervous system. The presence of AQP4 in some choroid plexus epithelial cells is novel and suggests that this water channel may have an important role (along with apical AQP1) in water transport across the human choroid plexus epithelium during CSF secretion. This is the first time AQP4 expression has been observed in the human choroid plexus. Low AQP4 expression was also noted in peripheral nerves (Supplementary data file 1). Expression of Na, K-ATPase was equally plentiful in the same anatomical locations of the human central nervous system (Fig. 5). As expected, Na, K-ATPase was localized to apical membranes of choroid plexus epithelial cells.\textsuperscript{39} Typically, this ion transporting pump is concentrated in the basolateral membrane of epithelial cells, but it is concentrated in the apical membranes of the retinal pigment epithelium and the epithelium of the choroid plexus.

**Genital and reproductive systems.** In the female genital and reproductive systems AQP4 expression was exclusively limited to the endocervix where immunostaining was observed in some muscular structures (uterine smooth muscle) (Fig. 6). There was low expression in the uterus (endometrium or myometrium) and no expression in ovaries. In the male genital and reproductive systems moderate expression was seen in seminiferous tubules, epididymis, seminal vesicles and the prostatic epithelium (Fig. 6).

**Placenta and fetal membranes.** Our immunohistochemical observations suggest that AQP4 is not present in fetal membranes or in the placenta (see Supplementary data file 1).

**Salivary glands, breast, endocrine organs and lymphatic system.** In the parotid salivary gland AQP4 expression was moderate to high and limited to myoepithelial cells (Fig. 6). There was no AQP4 immunostaining in nonlactating breast tissue (see supplementary data file 1). AQP4 expression was also detected in the adrenal cortex.
and the anterior pituitary gland (Fig. 6). No expression was detected in any of the lymphoid organs.

**Summary of the Rat TMA Data.** The commercial AQP4 antibodies from Chemicon International were characterized by western blotting and immunohistochemistry and the results are summarized in (Fig. 7) and in supplementary data file 2. For this purpose we designed a rat TMA containing twelve different tissue types. The layout of the rat TMA is shown in (Fig. 7A). Western blotting of rat kidney and rat diaphragm confirmed the presence of a 34 kDa protein in these samples (Fig. 7B). Immunohistochemistry confirmed AQP4 expression in rat kidney, diaphragm, cerebellar cortex and lung (Fig. 7C).

**Summary of the human protein microarray data.** Immunostaining of the human protein microarray confirmed most of the immunohistochemical data obtained using TMAs (Fig. 8).

**DISCUSSION**

Our understanding of aquaporin physiology has been challenged by the recent expansion of the AQP gene family. Thus far thirteen aquaporins have been cloned and identified in humans and rodents (AQP0-13). However, we still do not have a clear picture of the expression and tissue distribution of these aquaporins in mammalian tissues and even less is known about AQP distribution in human tissues. In this study, we have employed two complementary microarray technologies to study the expression and distribution of AQP4 in human tissues.

The recent development of microarray technologies has allowed the simultaneous analysis of hundreds or thousands of parameters within a single experiment. Investigators can probe arrays consisting of large numbers of genes, proteins or rare tissue specimens for novel diagnostic and prognostic correlations. Using this technology microspots of capture molecules (DNA, protein or tissue specimens) are immobilized onto a solid support (i.e., membranes or charged glass slides) in orderly rows and columns and exposed to samples containing the corresponding binding molecules (i.e., radioactive or fluorescently labelled DNA or antibodies). After stringency washing fluorescence, chemiluminescence or autoradiography is used to detect signals indicating probe-target complex formation within each microspot. Protocol standardization and miniaturization increases the sensitivity and through-put providing extraordinarily powerful tools for biomolecule detection and quantification. The combined use of DNA, tissue and protein microarrays has revolutionized in vitro diagnostics, genomics and proteomics.

The system-specific arrangement of multiple human tissue samples on a single charged glass tissue microarray (TMA) has previously allowed us to successfully study the distribution of AQP128.

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**Figure 7. Summary of AQP4 expression data in selected rat tissues and western blot characterization of the AQP4 antibodies.** (A) Design of the rat TMA used for characterization of the AQP4 antibodies. (B) Western blot showing expression of AQP4 in rat tissues including kidney and skeletal muscle (diaphragm). (C) Localization of AQP4 in selected rat tissues including cerebellar cortex, kidney cortex, kidney medulla and skeletal muscle (diaphragm). AQP4 expression data in rat tissues is summarized in Supplementary data file 2.

**Figure 8. AQP4 expression in a representative human protein microarray.** AQP4 expression was detected in protein fractions ranging from 30-46 kDa in molecular weight in several tissues including placenta, brain (whole), cerebellum, diaphragm, duodenum, kidney, uterus, stomach and lung as indicated by the arrows.
AQP2 and AQP3 in human tissues. Here we have combined the analytical power of human TMAs and human protein microarrays to explore AQP4 expression and distribution in human tissues. Our experience with human TMAs has demonstrated their significant benefits: having samples of multiple human tissue types represented on a microarray slide allows investigators to analyze the expression profile of aquaporins semi-quantitatively across various tissues by high throughput immunohistochemistry. TMAs allow us to assess not only the relative abundance of aquaporin protein expression but also the anatomical sites in which they are localized. This study is part of an ongoing program of research to study the tissue distribution of AQP4 proteins and to identify organs and tissues in which the expression of aquaporins has not previously been described.

AQP4 (originally known as MIWC) was cloned and identified as a candidate osmoreceptor and regulator of water balance in brain in 1994,10 two years after the cloning of AQP1 (originally known as CHIP). In situ hybridization of rat brain with probes for AQP4 revealed the presence of AQP4 mRNA in ependymal cells, glial cells in the cerebral cortex and brainstem, Purkinje cells of cerebellum and vasopressin-secretory neurons in supraoptic and paraventricular nuclei of hypothalamus.10 Immunohistochemical studies in the rat kidney revealed AQP4 labeling in basolateral membranes of collecting duct principal cells, suggesting a possible role in the exit of apically reabsorbed water (via AQP2) from basolateral membranes of inner medullary collecting ducts.43 In mouse, but not in rat, AQP4 was also present in the basolateral membrane of proximal tubular cells.30,44 Northern blotting and RNase protection assays revealed that AQP4 is also expressed in the rat stomach,45 lung and skeletal muscle.46 AQP4 knock-out studies carried out three years later produced mice exhibiting a minor defect in urinary concentrating ability consistent with AQP4 expression in the medullary collecting duct.47 Subsequent studies by Frigeri and colleague presented data to support expression of AQP4 in fast-twitch fibers of rat skeletal muscle, which implicated important roles for aquaporins in skeletal muscle and in the molecular alterations that occur in muscle from Duchenne muscular dystrophy patients and mdx mice. These studies suggested that expression of sarcolemmal AQP4 together with that of vascular AQP1 may be responsible for the rapid water movement from blood into muscle during intense physical activity and that changes in the osmotic equilibrium of the neuromuscular apparatus may be involved in the pathology of muscular dystrophy.25,29

Many of the published reports of AQP4 expression and regulation have been confined to experimental rodents and few studies have been carried out on human tissues. In this study the most abundant AQP4 expression was seen in the central nervous system and in skeletal muscle (see Fig. 5 and Table 1). AQP4 expression was lower in peripheral nerves (supporting studies in mouse spinal cord) and in subcortical white matter and meninges. Apart from the stomach and anus, no significant AQP4 expression was seen along the human gut. There was no AQP4 immunostaining in the liver and lymphoid organs and AQP4 expression was not detected in many reproductive organs except in prostate and seminal vesicles.

In summary, this is the first time human TMAs have been used for a semi-quantitative histomorphometric analysis of AQP4 expression in multiple normal human tissues. The data we have presented in this paper support the idea that AQP4 is abundantly expressed in the human nervous system. The results of this study reinforce the idea that AQP4 has an important role in human brain water homeostasis. AQP4 is highly expressed in the sarcolemma of skeletal muscle and is also present in the human stomach, lung, and kidney in the same locations as rodents. We also made a number of novel observations: AQP4 was detected in several endothrane organs, in the prostate and in seminal vesicles where it may be involved in prostatic and seminal fluid formation. It is hoped that this new data, and the information in two of our recent TMA studies of AQP tissue distribution, will serve as a useful reference for other investigators interested in the distribution of AQP water channels in human tissues and lead to more discussion regarding the role of these water channel proteins in clinical medicine. We also expect that the combination of protein and tissue microarrays will be used by other investigators to study the distribution of recently identified members of the AQP family (AQP7-12) in human tissues in health and disease.

Note
Supplementary data can be found at: www.landesbioscience.com/supplement/mobasherICHAN1-1-sup1.pdf and www.landesbioscience.com/supplement/mobasherICHAN1-1-sup2.pdf

References
1. Calamita G. The Escherichia coli aquaporin-Z water channel. Mol Microbiol 2000; 37:254-62.
2. Agre P, King LS, Yusi M, Guggino WB, Ottersen OP, Fujiyoshi Y, Engel A, Nielsen S. Aquaporin water channels from atomic structure to clinical medicine. J Physiol 2002; 542:3-16.
3. King LS, Kozono D, Agre P. From structure to disease: The evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 2004; 5:687-98.
4. Agre P, Kozono D. Aquaporin water channels: Molecular mechanisms for human diseases. FEBS Lett 2003; 555:72-8.
5. Schroff AR. Aquaporin function, structure, and expression: Are there more surprises to surface in water relations? Planta 1998; 204:131-9.
6. Chrisspeels MJ, Maurel C. Aquaporins: The molecular basis of facilitated water movement through living plant cells? Plant Physiol 1994; 105:9-13.
7. Johansson L, Karlsson M, Johansson U, Larsson C, Kjellbom P. The role of aquaporins in cellular and whole plant water balance. Biochim Biophys Acta 2000; 1465:324-42.
8. Verkman AS, Mitra AK. Structure and function of aquaporin water channels. Am J Physiol Renal Physiol 2000; 278:F13-28.
9. Verkman AS. Aquaporin water channels and endothelial cell function. J Anat 2002; 206:617-27.
10. Jung JS, Bhar RV, Preston GM, Guggino WB, Baraban JM, Agre P. Molecular characterization of an aquaporin cDNA from brain: Candidate osmoreceptor and regulator of water balance. Proc Natl Acad Sci USA 1994; 91:13052-6.
11. Haegewa H, Ma T, Skach W, Mathaaya MA, Verkman AS. Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. J Biol Chem 1994; 269:5497-500.
12. Nicchia GP, Nico B, Camassa LM, Mola MG, Loh N, Dermietzel R, Spray DC, Svelto M, Frigeri A. The role of aquaporin-4 in the blood-brain barrier development and integrity: Studies in animal and cell culture models. Neuroscience 2004; 129:935-45.
13. Tomas-Camardiel M, Venero JL, Herrera AF, De Pablos RM, Pintor-Toro JA, Machado A, Cano J. Blood-brain barrier disruption highly induces aquaporin-4 mRNA and protein in porcine and pericapillary astrocytes: Protective effect by estradiol treatment in ovariectomized animals. J Neurosci Res 2005; 80:235-46.
14. Amiry-Moghaddam M, Xue R, Haug FM, Neely JD, Bhaward AJ, Agre P, Adams ME, Froehner SC, Mori S, Ottersen OP. Alpha-synuclein deletion removes the perivascular but not endothelial pool of aquaporin-4 at the blood-brain barrier and delays the development of brain edema in an experimental model of acute hypotoniaemia. Faseb J 2004; 18:542-4.
15. Thiagarajan JR, Papadopoulos MC, Verkman AS. Noninvasive early detection of brain edema in mice by near-infrared light scattering. J Neurosci Res 2005; 80:293-9.
16. Papadopoulos MC, Verkman AS. Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. J Biol Chem 2005; 280:13906-12.
17. Papadopoulos MC, Saadoun S, Binder DK, Manley GT, Krishna S, Verkman AS. Molecular mechanisms of brain tumor edema. Neuroscience 2004; 129:1011-20.
18. Manley GT, Binder DK, Papadopoulos MC, Verkman AS. New insights into water transport and edema in the central nervous system from phenotype analysis of aquaporins-4 null mice. Neuroscience 2004; 129:983-91.
19. Lu M, Lee MD, Smith BL, Jung JS, Agre P, Verdijk MA, Merks G, Rijs JP, Deen PM. The human AQP4 gene: Definition of the locus encoding two water channel polypeptides in brain. Proc Natl Acad Sci USA 1996; 93:10908-12.
20. Shi LB, Verkman AS. Selected cysteine point mutations confer mercurial sensitivity to the mercurial-insensitive water channel MWW/AQP-4. Biochemistry 1996; 35:538-44.
21. Yang B, van Hoek AN, Verkman AS. Very high single channel water permeability of aquaporin-4 in baculovirus-infected insect cells and liposomes reconstituted with purified aquaporin-4. Biochemistry 1997; 36:7625-32.

22. Nio B, Ribatti D, Frigeri A, Nicchia GP, Corsi P, Svelto M, Roncali L. Aquaporin-4 expression during development of the cerebellum. Cerebellum 2002; 1:207-12.

23. Rash JE, Yasumura T, Hudson CS, Agre P, Nielsen S. Direct immunogold labeling of aquaporin-4 in square arrays of astrocyte and ependymocyte plasma membranes in rat brain and spinal cord. Proc Natl Acad Sci USA 1998; 95:11981-6.

24. Nicchia GP, Frigeri A, Iuzzo GM, Svelto M. Inhibition of aquaporin-4 expression in astrocytes by RNAi determines alteration in cell morphology, growth, and water transport and induces changes in ischemia-related genes. Faseb J 2003; 17:1508-10.

25. Frigeri A, Gropper MA, Türk CW, Verkman AS. Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. Proc Natl Acad Sci USA 1995; 92:4328-31.

26. Frigeri A, Nicchia GP, Verbavatz JM, Valenti G, Svelto M. Expression of aquaporin-4 in fast-twitch fibers of mammalian skeletal muscle. J Clin Invest 1998; 102:695-703.

27. Frigeri A, Nicchia GP, Nico B, Quondamatteo F, Herken R, Roncali L, Svelto M. Aquaporin-4 deficiency in skeletal muscle and brain of dystrophic mdx mice. Faseb J 2001; 15:90-8.

28. Mobaraki A, Marples D. Expression of the AQP-1 water channel in normal human tissues: A semiquantitative study using tissue microarray technology. Am J Physiol Cell Physiol 2004; 286:C529-37.

29. Frigeri A, Nicchia GP, Balena R, Nio B, Svelto M. Aquaporins in skeletal muscle: Reassessment of the functional role of aquaporin-4. Faseb J 2004; 18:905-7.

30. van Hoek AN, Ma T, Yang B, Verkman AS, Brown D. Aquaporin-4 is expressed in basolateral membranes of proximal tubule S3 segments in mouse kidney. Am J Physiol Renal Physiol 2000; 278:F310-6.

31. Huang Y, Tola VB, Fang P, Soybel DI, Van Hoek AN. Partitioning of aquaporin-4 water channel mRNA and protein in gastric glands. Dig Dis Sci 2003; 48:2027-36.

32. Zelenina M, Zelenin S, Bondar AA, Brismar H, Aperia A. Water permeability of aquaporin-4 is decreased by protein kinase C and dopamine. Am J Physiol Renal Physiol 2002; 283:F309-18.

33. Mobaraki A, Wray S, Marples D. Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J Mol Histol 2005; 36:1-14.

34. Mobaraki A, Airley R, Hewitt SM, Marples D. Heterogeneous expression of the aquaporin-1 (AQP1) water channel in tumors of the prostate, breast, ovary, colon and lung: A study using high density multiple human tumor tissue microarrays. Int J Oncol 2005; 26:1149-58.

35. Endo M, Jain RK, Wiertz B, Brown D. Water channel (aquaporin 1) expression and distribution in mammary carcinomas and glioblastomas. Microvasc Res 1999; 58:89-98.

36. Saadoun S, Papadopoulos MC, Hara-Chikuma M, Verkman AS. Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. Nature 2005; 434:786-92.

37. Hayashi T. Immunohistochemical localization of Na+, K+-ATPase in human and rat kidneys. Nippon Ika Daigaku Zasshi 1988; 55:399-405.

38. Mobaraki A, Avila J, Cozar-Castellano I, Brownieelder MD, Trevan M, Francis MJ, Lamb JF, Martin-Vasallo P. Na+-K+-ATPase isozyme diversity: comparative biochemistry and physiological implications of novel functional interactions. Biosci Rep 2000; 20:51-91.

39. Rizzolo LJ. Polarization of the Na+, K+-ATPase in epithelia derived from the neuroepithelium. Int Rev Cytol 1999; 185:195-235.

40. Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. Trends Biotechnol 2002; 20:160-6.

41. Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. Drug Discov Today 2002; 7:815-22.

42. Stoll D, Tempelin MF, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. Front Biosci 2002; 7:c13-32.

43. Nielsen S, Agre P. The aquaporin family of water channels in kidney. Kidney Int 1995; 48:1057-68.

44. Kim SW, Cho SH, Oh BS, Yeum CH, Choi KC, Ahn KY, Lee J. Diminished renal expression of aquaporin water channels in rats with experimental bilateral ureteral obstruction. J Am Soc Nephrol 2001; 12:2019-28.

45. Misuka T, Abe K, Iwasaki K, Kusaba K, Ichihara M, Miki K, Esomi Y, Azai S. A water channel closely related to rat brain aquaporin 4 is expressed in acid- and pepsinogen-secreting cells of human stomach. FEBS Lett 1996; 381:208-12.

46. Umemori F, Verkman AS, Gropper MA. Quantitative analysis of aquaporin mRNA expression in rat tissues by RNase protection assay. DNA Cell Biol 1996; 15:475-80.

47. Ma T, Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS. Generation and phenotype of a transgenic knockout mouse lacking the mercurial-insensitive water channel aquaporin-4. J Clin Invest 1997; 100:957-62.

48. Oshio K, Binder DK, Yang B, Schecter S, Verkman AS, Manley GT. Expression of aquaporin water channels in mouse spinal cord. Neuroscience 2004; 127:685-93.