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Cloning, functional expression and tissue distribution of human cDNA for the vascular-type vasopressin receptor

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We have cloned a human vasopressin receptor from human mesenteric artery using RACE (Rapid Amplification of cDNA Ends) methods. The deduced amino acid sequence of the clone (HV-RACE) encodes a protein of 418 amino acids that showed a strong sequence homology to the previously cloned rat V_{1A} vasopressin receptor. The [^{3}H] arginine vasopressin (AVP) binding to HV-RACE expressed in COS-7 cells was potently inhibited by AVP (K_i = 2.9 nM). Interestingly, a new non-peptide "V_1-selective" antagonist OPC-21268 exhibited markedly higher affinity for rat V_{1A} receptor (K_i = 57 nM) rather than for HV-RACE (K_i = 56 μM). With the reverse-transcription polymerase chain reaction assay, we observed a large amount of HV-RACE transcripts in the mesenteric artery, while a small amount in a variety of other tissues. The data show that the clone HV-RACE encodes a human vascular-type vasopressin receptor cDNA.

The neurohypophyseal hormone arginine vasopressin (AVP) has diverse actions, including the inhibition of diuresis, contraction of smooth muscle, stimulation of liver glycogenolysis and modulation of adrenocorticotropic hormone release from the pituitary. AVP exerts its actions through binding to specific membrane receptors coupled to distinct second messengers. Vasopressin receptors can be classified into at least three subtypes - the V_{1A}, V_{1B} and V_2 receptors (1, 2). The V_{1A} receptors mediate vasoconstriction and hepatic glycogenolysis, while the V_{1B} receptors modulate adrenocorticotropin hormone secretion from the anterior pituitary and the V_2 receptors mediate antidiuretic effects (3, 4). In general, activation of the V_1 receptors results in phosphatidylinositol hydrolysis and mobilization of intracellular calcium, while V_2 receptors are associated with an increase in the intracellular cyclic AMP. Recently, cDNAs encoding the rat vasopressin V_{1A} and V_2 receptors have been cloned (5 - 7), and both of the receptors are found to

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Abbreviations: AVP, arginine vasopressin; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

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be members of the G protein-coupled receptors with putative seven transmembrane domains. However, very few informations are still available regarding the pharmacological properties and the tissue distribution of vasopressin receptor subtype in human. Further examination of the human vasopressin receptor cDNA, V_1 receptor in particular, seems to be of importance, since recent pharmacological studies indicated a marked species difference in the effects of several vasopressin receptor antagonists (8).

We have recently demonstrated that the reverse transcription-polymerase chain reaction (RT-PCR) method is advantageous in detecting rare transcripts such as hormone receptor from a small amount of source and also in identifying specific mRNA (9 - 11). Here we report the cloning and functional expression of human vasopressin receptor from cDNA pool of human mesenteric artery using 5'- and 3'-RACE method. Using the cloned receptor, we investigated the effects of the non-peptide antagonists, OPC-21268 and OPC-31260, and also the tissue distribution of the receptor in human.

Materials and Methods

5'-RACE & 3'-RACE cDNA cloning of human vascular-type vasopressin receptor : Human tissues were obtained at surgical operation after informed consent was obtained. Total cellular RNA from mesenteric artery was isolated by the cesium chloride gradient method. We performed the RT-PCR on human mesenteric artery using Vz-1, Vz-2, rV1-1, rV1-2 and rV1-4 primers (Fig. 1A). Sequences of all the primers used in the present study were summarized in Table 1. The primers were synthesized on a model 391A (Applied Biosystems, Inc., Foster City, CA, USA) DNA synthesizer (using β-cyano-methylphosphoramidate derivatives). The PCR amplification profiles consisted of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 2 min. The resulting PCR product was purified after the agarose gel electrophoresis by the SUPREC-01 (TaKaRa, Kyoto, Japan), subcloned into the EcoRV site of pBluescript II KS(+) plasmid by TA-cloning method (12). Five clones highly homologous to rat V_{1A} receptor were selected and designated HV-01 to -05 (Fig. 1). Synthesizing specific primers for HV clones, hV1-4 and hV1-3 for 5'- and 3'-RACE, respectively, the 5'-RACE and 3'-RACE were performed using 1 μg of total RNA according to the protocol of manufacturer (GIBCO BRL Gaithersburg, MD), with RACE, RACE-2 and RACE-T primers (Table. 1).

Table 1

Oligonucleotide primers for PCR

	direction	position	Sequence (5' to 3')
rV1-1	Forward	667-696 (rV1A)	atgctggtggtgatgacagccgaccgctac
rV1-2	Reverse	1200-1171 (rV1A)	catctggacaatgaagaaaggcgcccagca
rV1-3	Forward	232-257 (rV1A)	aggctctgtacggacagcatgagttt
rV1-4	Reverse	1449-1420 (rV1A)	gttgggctccggttgttagaataagaagtc
hV1-1	Forward	680-703 (HV-RACE)	cttctccatgatcgaggtgaacaa
hV1-2	Reverse	945-922 (HV-RACE)	tgacacagggtgcgagcaggaacc
hV1-3	Forward	1178-1202 (HV-RACE)	tctccttcaagactgtgttcaaagc
hV1-4	Reverse	461-438 (HV-RACE)	gtaggtgatgtcccagcacatttg
Vz-1	Forward	544-571 (rV1A)	gt(cg)gciititt(tc)ca(ag)gt(ag)(tc)ticcica
Vz-2	Forward	885-856 (rV1A)	cca(gt)gg(ct)t(cg)iii(ag)aa(gt)ii(ag)gcccagc
β-1	Forward	2158-2187 (human)	atcatgtttgagaccttcaacaccccagcc
β-2	Reverse	2579-2550 (human)	aagagagcctcggggcatcggaaccgctca
RACE			ggccacgcgtcgactagtacgggiigggiigggiig
RACE-2			ggccacgcgtcgactagtac

PCR cloning of rat V_{1A} receptor: Using primers of rV1-3 and rV1-4 which can amplify the full length of coding region (Table 1), cDNA synthesized from rat liver RNA was amplified, and the resulting product (rV_{1A}-10) was subcloned and sequenced. We confirmed that the rV_{1A}-10 clone was identical to the previously reported rat V_{1A} receptor cDNA by sequencing (7).

DNA sequencing: Cloned cDNA, enzyme digested fragments and PCR products were subcloned into pBluescript KS II(+) (Stratagene, La Jolla, CA). Nucleotide sequence analysis was performed by the ABI 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) for both complete strands.

Transient expression in COS-7 cells: To facilitate construction of the expression vector for HV-RACE clone and rV_{1A}-10 clone, EcoRI-XhoI fragments were ligated into the EcoRI-XhoI-digested SRα promoter-based mammalian expression vector pME18S (13). The constructs pME-HV and pME-RV were transfected into COS-7 cells by the DEAE-dextran method, and cells were harvested 48-72 h after transfection.

Rat liver plasma membrane preparation: To compare the binding properties of HV-RACE and the cloned rat V_{1A} receptor with the native rat V_{1A} receptor, rat liver plasma membrane was prepared according to the methods of Nakamura et al. (14).

Radioligand binding assay: COS-7 cell membranes were prepared as described previously(11). Briefly, the cells were collected and disrupted by the Branson sonicator (model SONIFIER 250, setting 5 for 8 s). The mixture was then centrifuged at 3,000 x g for 10 min. The supernatant fraction was centrifuged at 35,000 x g for 20 min. The resulting pellet was resuspended in binding buffer (100 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 1 % (w/v) bovine serum albumin). Membrane aliquots (50 μg of protein) were incubated for 30 min at 30 °C with [^{3}H]AVP, with or without a competing ligand, in a final volume of 250 μl of binding buffer. After dilution with ice-cold buffer, samples were immediately filtered through Whatmann GF/C glass fiber filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, USA). Competition experiments were carried out at about 8.2 nM [^{3}H]AVP which is Kd of the ligand. At this concentration nonspecific binding, defined as that occurring in the presence of 1 μM AVP, represented about less than 10% of total binding. The protein concentration was measured using the BCA protein assay kit (PIERCE, Rockford, IL). Analysis of binding data was performed using a nonlinear reiterative technique with the LIGAND programs (15).

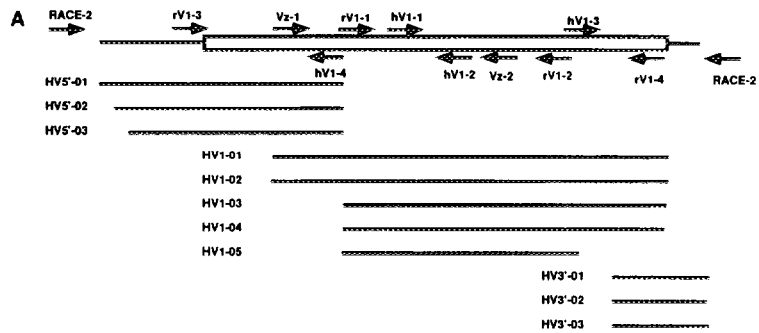
Tissue distribution of mRNA expression: For RT-PCR, total cellular RNA from various human tissues obtained at autopsy was isolated by the cesium chloride gradient method. In preliminary experiments, the integrity of the purified RNA collected with this method was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1 % agarose-formaldehyde ethidium bromide gel.

RT-PCR analysis was performed as described previously (9 - 11). Oligonucleotide primers were constructed from the cDNA sequences of HV-RACE and β-actin cDNA. β-actin served as an internal standard for the efficacy of RNA isolation and cDNA synthesis. Primers specific for HV-RACE (hV1-1, hV1-2) and β-actin (β–1, β–2) were also shown in Table 1. The predicted sizes of the amplified HV-RACE and human β-actin PCR products were 266 and 421 bp, respectively. The PCR amplification profiles consisted of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min for 35 cycles. Negative control reactions without template were routinely included in PCR amplifications with both primer sets.

Drugs: OPC-21268 and OPC-31260 were kindly provided by Otsuka Pharmaceuticals (Tokyo, Japan). AVP was obtained form Sigma (St. Louis, MO, U.S.A.). [^{3}H]AVP (specific activity = 75.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Results and Discussion

Utilizing the PCR homology cloning strategy in combination with the 5'- and 3'- RACE methods, we obtained ten clones (HV1-01 to -05, HV5'-01 to -03 and HV3'-01 to -02; Fig. 1A) from the human mesenteric artery cDNA as a source. To avoid the misincorporation by PCR amplification, all clones obtained were sequenced and confirmed to be identical in overlapping region. Furthermore, we assembled the clones HV5'-01, HV1-01 and HV3'-02 using PCR assembly methods (12), and the resulting construct was designated HV-RACE (Fig. 1A).



B

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      GGGGGGGGGAGACCTGCGTTTCGGACGAGGGAGCCCAAGTCCTCCGAGACGGGGGA  -61
GGGAGCGGCCCGCGAGGGCTGGAGCTCCGAAGAGGGCCGAGTAGGAGCTGCATGGACAGA   -1

ATGCGTCTCTCCGCCGGTCCCGACGCGGGGCCCTCGGGCAACTCCAGCCCATGGTGGCCT   60
M  R  L  S  A  G  P  D  A  G  P  S  G  N  S  S  P  W  W  P

CTGGCCACCGGCGCTGGCAACACAAGCCGGGAGGCCGAAGCCCTCGGGGAGGGCAACGGC  120
L  A  T  G  A  G  N  T  S  R  E  A  E  A  L  G  E  G  N  G

CCACCGAGGGACGTGCGCAACGAGGAGCTGGCCAAACTGGAGATCGCCGTGCTGGCGGTG  180
P  P  R  D  V  R  N  E  E  L  A  K  L  E  I  A  V  L  A  V

ACTTTCGCGGTGGCCGTGCTGGGCAACAGCAGCGTACTGCTGGCTCTGCACCGGACGCCG  240
T  F  A  V  A  V  L  G  N  S  S  V  L  L  A  L  H  R  T  P

CGCAAGACGTCCCGCATGCACCTCTTCATCCGACACCTCAGCCTGGCCGACCTGGCCGTG  300
R  K  T  S  R  M  H  L  F  I  R  H  L  S  L  A  D  L  A  V

GCATTCTTCCAGGTGCTGCCGCAAATGTGCTGGGACATCACCTACCGCTTCCGCGGCCCC  360
A  F  F  Q  V  L  P  Q  M  C  W  D  I  T  Y  R  F  R  G  P

GACTGGCTGTGCCGCGTGGTGAAGCACCTGCAGGTGTTCGGCATGTTTGCGTCGGCCTAC  420
D  W  L  C  R  V  V  K  H  L  Q  V  F  G  M  F  A  S  A  Y

ATGCTGGTAGTCATGACAGCCGACCGCTACATCGCGGTGTGCCACCCGCTCAAGACTCTG  480
M  L  V  V  M  T  A  D  R  Y  I  A  V  C  H  P  L  K  T  L

CAACAGCCCGCGCGCCGCTCGCGCCTCATGATCGCGGCCGCCTGGGTGCTGAGCTTCGTG  540
Q  Q  P  A  R  R  S  R  L  M  I  A  A  A  W  V  L  S  F  V

CTGAGCACGCCGCAGTACTTCGTCTTCTCCATGATCGAGGTGAACAATGTCACCAAGGCC  600
L  S  T  P  Q  Y  F  V  F  S  M  I  E  V  N  N  V  T  K  A

CGCGACTGCTGGGCCACCTTCATCCAGCCCTGGGGTTCTCGTGCCTACGTGACCTGGATG  660
R  D  C  W  A  T  F  I  Q  P  W  G  S  R  A  Y  V  T  W  M

ACGGGCGGCATCTTTGTGGCGCCCGTGGTCATCTTGGGTACCTGCTACGGCTTCATCTGC  720
T  G  G  I  F  V  A  P  V  V  I  L  G  T  C  Y  G  F  I  C

TACAACATCTGGTGCAACGTCCGCGGGAAGACGGCGTCGCGCCAGAGCAAGGGTGCAGAG  780
Y  N  I  W  C  N  V  R  G  K  T  A  S  R  Q  S  K  G  A  E

CAAGCGGGTGTGGCCTTCCAAAAGGGGTTCCTGCTCGCACCCTGTGTCAGCAGCGTGAAG  840
Q  A  G  V  A  F  Q  K  G  F  L  L  A  P  C  V  S  S  V  K

TCCATTTCCCGGGCCAAGATCCGCACGGTGAAGATGACTTTTGTGATCGTGACGGCTTAC  900
S  I  S  R  A  K  I  R  T  V  K  M  T  F  V  I  V  T  A  Y

ATCGTCTGCTGGGCGCCTTTCTTCATCATCCAGATGTGGTCTGTCTGGGATCCCATGTCC  960
I  V  C  W  A  P  F  F  I  I  Q  M  W  S  V  W  D  P  M  S

GTCTGGACCGAATCGGAAAACCCTACCATCACCATCACTGCATTACTGGGTTCCTTGAAT 1020
V  W  T  E  S  E  N  P  T  I  T  I  T  A  L  L  G  S  L  N

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CAAAGCTTCCCATGCTGCCAAAACATGAAGGAAAAATTCAACAAAGAAGATACTGACAGT 1140
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TGGAAGGACTCGCCTAAATCTTCCAAGTCCATCAAATTCATTCCTGTTTCAACTTGAGCC 1260
W  K  D  S  P  K  S  S  K  S  I  K  F  I  P  V  S  T  *

TTGCATTC
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Fig. 1.
A: RACE strategy of human V_1 vasopressin receptor cloning.
B: Nucleotide sequence and deduced amino acid sequence of the human V1 receptor clone. 5'-untranslated region = 116 bp, open reading frame = 1254 bp and 3'-untranslated region = 14 bp. The single-letter amino acid code is used.

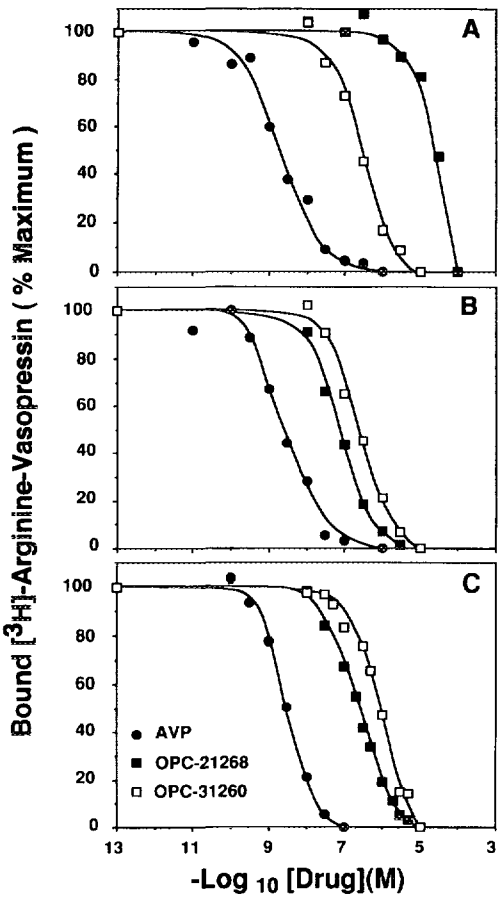

Fig. 2. Specific binding of AVP to COS-7 cells transfected with HV-RACE, RV and rat liver. Transfected COS-7 cells and rat liver membrane were prepared as described in Materials and Methods. The figures show the mean of competition binding experiments performed with one concentration of [^{3}H] AVP and increasing concentrations of AVP or OPC-21268 or OPC-31260 (n=3-4 for each compounds) in HV-RACE (panel A), RV (panel B) and rat liver (panel C).

The clone HV-RACE contains a 1,254 bp open reading frame encoding a protein of 418 amino acids (Fig. 1B). Hydropathicity analysis shows that the translated protein has the typical features of a G protein-coupled transmembrane receptor with seven putative hydrophobic domains, connected by three extracellular and three intracellular loops. The percentage of the amino acid identity of HV-RACE is 72 % compared with the rat V_{1A} receptor (7), while it is 36 % with human V_2 receptor (5) and 45 % with human oxytocin receptor (16). Both the alignment and percentage of amino acid identity relative to other members of the vasopressin receptor family strongly suggest that the clone HV-RACE is belong to the V_1 receptor subtype. Other structural features of this clone are the presence of three potential sites for N-linked glycosylation in the putative extracellular domain (asparagine residues 14, 27 and 196), and the presence of several serine and threonine residues in the carboxyl terminus and intracellular loops, which may serve as potential sites for phosphorylation by protein kinase C and protein kinase A.

pME-HV and pME-RV were transfected COS-7 cells to compare the pharmacological profile of the HV-RACE and rat V_{1A} receptor. Nontransfected COS-7 cells or COS-7 cells transfected with a plasmid pME18S showed no specific [^{3}H]AVP binding. In contrast, COS-7 cells transfected with pME-HV and pME-RV were able to bind the radioligand [^{3}H]AVP in a saturable manner (data not shown). As shown in Fig. 2, the [^{3}H]AVP bindings to both COS-7 cells transfected with pME-HV and pME-RV, and rat liver membrane preparation were potently inhibited by AVP (K_i = 2.9 nM), supporting the idea that cDNA clone HV-RACE encodes the human V_1 receptor. Also, K_i values of AVP and the non-peptide antagonists of OPC-21268 and OPC-31260 for both rat liver membrane were consistent with those of previous reports (Table 2) (17, 18).

Very recently, orally effective, non-peptide antagonists selective for vasopressin receptor subtypes have been developed (17 - 19). Interestingly, the non-peptide "V_1 receptor-selective" antagonist OPC-21268 exhibited a markedly higher affinity to rat V_{1A} receptor (K_i= 57 nM) rather than to the human vascular-type V_1 receptor (K_i= 56 μM), while the "V_2 receptor-selective" antagonist OPC-31260 exerted a potent displacing effect on both rat and human V_1 receptor binding sites (K_i = 339 and 215 nM for human and rat, respectively)(Table 2). Our observation that OPC-21268 has a lower affinity for human V_1 receptor binding sites than for the rat one was in good agreement with the result previously reported by Pettibone et al. (8), who examined the binding properties of primate and human liver membrane. Also, Chiba and Tsukada have shown that AVP-induced vasoconstriction in isolated simian femoral arteries is potently inhibited by OPC-31260 but not by OPC-21268 (20), which functional data is well consistent with our observation that OPC-31260 is more potent on the human V_1 receptor than OPC-21260 (Fig. 2). Taken together, our data suggest that these non-peptide antagonists have a marked species differences in their drug effects on V_1 receptors.

To map the V_1 receptor distribution in human, RT-PCR analysis was performed. The tissue distribution of human V_1 receptor was found to be markedly different from that in rats (10). Using the specific primer sets of hV1-1 and hV1-2, the PCR products were detected abundantly in the mesenteric artery, kidney, adrenal and aorta, and less amounts in the lung, prostate and skeletal muscle (Fig. 3). Similar results were obtained from at least three individuals. In the liver, however, one individual (male) showed a large amount of PCR product; however, the other two individuals (female) had very little or no PCR products detected. This finding may indicate a gender difference

Table 2
Pharmacological characterization of the expressed human vasopressin receptor

	HV-RACE K_i (nM)	rV1A-10 K_i (nM)	rat liver membrane K_i (nM)
AVP	2.9	2.0	1.6
OPC-21268	56,000	57	170
OPC-31260	339	215	595

COS-7 cell membranes transfected with the pME18S expression vector containing human and rat vasopressin receptors and rat liver membranes were incubated with ligand [^{3}H]AVP, in the absence or presence of increasing concentrations of AVP, OPC-21268 and OPC-31260. Each point represents the mean of at least two individual experiments, in duplicate. Ten concentrations of each ligand were tested, and the points were chosen to be on the linear portion of the displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND.

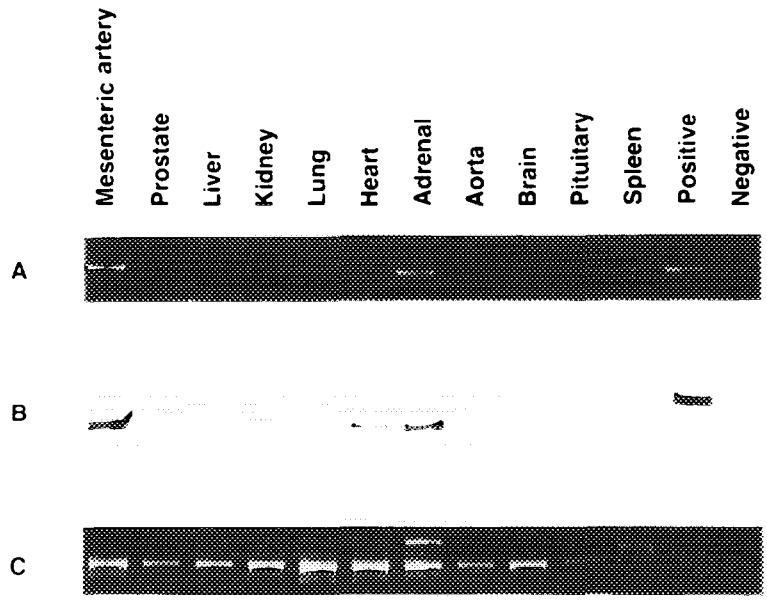

Fig. 3. Tissue distribution of HV-RACE clone.
RT-PCR assay of V_1 receptor mRNA expression in human tissues. RT-PCR was performed with the primer set as described under Material and Methods. cDNAs from various human tissues, genomic DNA and buffer (blank) were used as templates for PCR.

in human V_1 receptor mRNA expression level in the liver. Furthermore, RT-PCR assay using degenerate primers (Vz-1, Vz-2 in Table 1) showed that only V_1 receptor PCR product was detected in the mesenteric artery. Since these primer sets could amplify all V_1, V_2 and oxytocin receptor cDNAs when they exist (data not shown), the RT-PCR data may suggest that only V_1 receptors are exclusively expressing in the human mesenteric artery. Taken together, the present data showed that the human V_1 receptor cDNA we cloned are expressing mainly in the human vasculature, and may play an important physiological role.

In summary, we have isolated a cDNA for human vascular-type vasopressin receptor. With the information regarding pharmacological properties and tissue distribution of the receptor subtype, cloning of the human receptor cDNA would be instrumental in developing selective agents for this receptor subtype.

Note Added in Proof

In preparing this report, human V_{1A} receptor cDNA was cloned from the liver cDNA library (Thibonnier, M. et al. (21)). The sequence is almost identical to our clone.

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