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Expression of acetoacetyl-CoA synthetase, a novel cytosolic ketone body-utilizing enzyme, in human brain☆

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Abstract

Acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16) is a ketone body-utilizing enzyme, the physiological role of which remains unclear yet in mammals, particularly has never been studied in human. In order to investigate the tissue distribution of AACS in human, cDNA encoding AACS was isolated from HepG2 cells. Amino acid sequence of human AACS deduced from the open reading frame showed high homology (89.3%) with that of rat AACS and much less homology (43.7%) with that of bacterial AACS. The expression level of the AACS mRNA was high in kidney, heart and brain, but low in liver, and the expression profile of AACS in the human brain was quite similar to that of 3-hydroxy-3-methylglutaryl-CoA reductase.

Keywords: Acetoacetyl-CoA synthetase; Ketone body; Acetoacetate; 3-Hydroxy-3-methylglutaryl-CoA reductase; Tissue distribution; Human

1. Introduction

Acetoacetyl-CoA synthetase (acetoacetate-CoA ligase, EC 6.2.1.16; AACS) is a cytosolic ligase that specifically activates acetoacetate to its coenzyme A ester [1,2]. In bacteria, AACS is known to exhibit important roles in the metabolism of poly-β-hydroxybutyrate, an intracellular reserve of organic carbon and/or chemical energy of some microorganisms [1,3]. While, in mammalian tissues, the physiological role of AACS has not been fully clarified yet compared to the bacterial enzyme, although Endemann *et al*. have demonstrated that acetoacetate is activated by the action of this enzyme and effectively incorporated into cholesterol and fatty acids in the liver of rat [4]. They proposed that AACS has the function of direct supply of acetyl units from acetoacetate in the cytosol for the synthesis of the lipidic substances and that such cytosolic activation could bypass the pathway involving ATP-citrate lyase which supplies acetyl units from mitochondria to cytosol [4].

Previously we have shown that the enzyme activity in the liver of rats remarkably increased and then decreased during the animal's development [5] and increased upon the administration of hypocholesterolemic compounds, cholestyramine and/or pravastatin, to rats [6]. Furthermore, we have demonstrated that hepatic AACS specific activity markedly decreased in streptozotocin-induced diabetic rats as in the case of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) or acetyl-CoA carboxylase [7]. These results suggest that direct activation of acetoacetate by AACS in the cytosolic compartment of the cells for cholesterol and/or fatty acids biosynthesis is an important step for the regulation of ketone body utilization and involved in the significant functions in mammals.

In order to clarify the physiological roles of AACS in mammals, we recently cloned the AACS cDNA from the liver of rat fed with hypocholesterolemic agents, and deduced its cDNA-derived amino acid sequence [6]. Since none of the molecular properties, physiological roles or even the presence of AACS in human has been reported before, in the present study we describe the cDNA cloning of human AACS, its molecular characterization and its distribution in the tissues of human.

☆ The nucleotide sequence reported in this paper has been submitted to the DDBJ data base under accession number AB054121.

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Abbreviations: AACS, acetoacetyl-CoA synthetase; SCOT, succinyl-CoA:3-oxoacid CoA-transferase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

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2. Materials and methods

2.1. Cloning of human AACS

Human total RNA was prepared from cultured HepG2 cells derived from human hepatocellular carcinoma using Quickprep Total RNA Extraction kit (Amersham Biosciences). This RNA (4 μg) was incubated for 60 min at 37° in the reaction mixture (20 μL) containing 300 units of moloney murine leukemia virus reverse transcriptase (Gibco BRL), 15 units of human placenta RNase inhibitor (WAKO) and 0.5 μg of random hexadeoxynucleotide primer (Takara). To amplify the human AACS cDNA fragment, PCR was performed for 30 cycles in the reaction mixture containing an aliquot of the above cDNA solution, 0.05 units/μL EX Taq DNA polymerase (Takara) and 4 pmol/μL each of sense and antisense degenerate primers representing all possible codons corresponding to the sequences of amino acids 177–183 (GAIWSST) and 313–318 (KEHVLH), respectively, of rat AACS [6]. Then nested PCR was performed using the above PCR product and sense (185–190, SPDFGVN) and antisense (299–304, CMVHSA) degenerate primers. The amplified DNA of expected size (362 bp) was cloned into the pGEM-T vector (Promega Corporation). The cloned DNA was sequenced with a 377 automated DNA sequencer (PE Applied Biosystems). To determine the entire coding region, the cDNA was analyzed by the rapid amplification of cDNA ends (RACE) method [8].

2.2. Northern blot analysis in tissues of human and various regions of human brain

The membrane blotted human RNA samples (human multiple tissue Northern blots membrane; MTN blots membrane) was purchased from Clontech Corporation. The membrane was prehybridized at 42° for 4 hr in a hybridization solution (10% dextran sulfate, 50% formamide, 2% SDS, 5 × SSPE, 5 × Denhardt's solution, 100 μg/mL heat-denatured salmon sperm DNA), followed by hybridization at 42° for 18 hr in the hybridization solution containing a ^{32}P-labeled probe encoding human AACS or human HMGCR [9] labeled by Ready-To-Go DNA Labeling Beads (Amersham Biosciences) with cytidine 5′-α-[^{32}P] dCTP (~30 TBq/mmol, Amersham Biosciences). The membrane was then washed at room temperature three times for 5 min each time in 2 × SSC/0.1% SDS, and at 65° twice for 30 min each time in 0.25 × SSC/0.1% SDS. The washed membrane was analyzed with a radioimaging analyzer (BAS2000, Fuji Photo Film).

3. Results and discussion

3.1. cDNA for human AACS

Fig. 1 shows the isolated cDNA for human AACS. The restriction map of cDNA for human AACS is shown in Fig. 1a. It is our understanding that so far nucleotide sequence of a cDNA coding AACS has been published for rat liver [6] and *Sinorhizobium meliloti*, a poly-β-hydroxybutyrate-accumulating bacterium [3]. The coding region for the human AACS was composed of 2016 bp (672 amino acid residues) with a calculated molecular weight of 75,143 (Fig. 1b). This nucleotide length was identical to that of rat AACS cDNA, of which calculated molecular weight was 75,039 [6]. While, nucleotide length of the microbial cDNA (1950 bp, 650 amino acid residues) and calculated molecular weight (71,370) [3] were considerably different from those for animals.

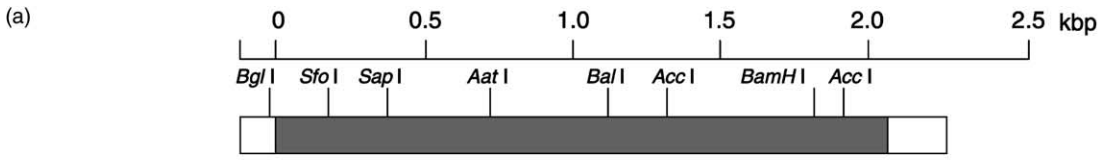
3.2. Homology of AACS between human, rat and bacteria

The amino acid sequence of human AACS deduced from the open reading frame showed high homology (89.3%) with that of rat enzyme [6] and much less homology (43.7%) with that of *S. meliloti* enzyme [3]. Homology alignments of three AACSs are shown in Fig. 2. Amino acid sequences of AACSs have AMP-binding motifs ([LIVMFY]-x(2)-[STG]-[STAG]-G-[ST]-[STEI]-[SG]-x-[PASLIVM]-[KR]) in the same region (human and rat AACSs, I287-K298; *S. meliloti* I268-K279). This motif is known to be present in a number of prokaryotic and eukaryotic enzymes which act *via* an ATP-dependent covalent binding of AMP to their substrate [10,11].

These enzymes also share a region of methionine- and tryptophan-rich sequence in the same region (human and rat AACSs, 335–341, WMMWNWM; *S. meliloti* 316–321, WMMWNW). Since a similar sequence was also found in microbial acetyl-CoA synthetases [6,12], this sequence was searched in BLAST protein sequence database. It turned out that this unique sequence is only observed in those of AACSs and acetyl-CoA synthetases. Since methionine and tryptophan are rarely distributed amino acids in common protein sequence, it is possible that this region plays an important role for the function related to the activation of short chain fatty acids.

3.3. Tissue distribution of AACS

In nonhepatic tissues of mammals, acetoacetate is known to be activated to its CoA ester by mitochondrial succinyl-CoA:3-oxoacid CoA-transferase (EC 2.8.3.5; SCOT) and regarded to be mainly used for energy source in mitochondria [13,14]. On the other hand, in the cytosol of lipogenic tissues of the rat, acetoacetate is demonstrated to be activated by the action of AACS for the synthesis of cholesterol and fatty acids [4]. In order to investigate physiological roles of human AACS, tissue distribution of the human enzyme was examined by Northern blot analysis with a ^{32}P-labeled human AACS cDNA probe (2176 bp portion; nucleotides −107 to 2069 in Fig. 1) using human MTN blots. As shown in Fig. 3, expression of AACS was detected in various tissues of human. Interestingly, expression level



(b)

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-107  AGGCGCTCCTGACCGTCGCTTCCTCCGGTCCCAGGTCCCCGGCCCTCGCCTCAGCCCCGGCCCCTGGTCCCCAGCCCTCGTCGCAGCCCCGGCCGCCCGC  -8
  -7  CGCCGCCATGTCCAAGGAGGAGCGCCCCGGTCGGGAGGAGATCCTGGAGTGCCAGGTGATGTGGGAGCCTGACAGTAAGAAGAACACGCAGATGGACCGC  93
          M  S  K  E  E  R  P  G  R  E  E  I  L  E  C  Q  V  M  W  E  P  D  S  K  K  N  T  Q  M  D  R

   94  TTCCGGGCGGCTGTGGGCGCCGCCTGCGGCCTGGCGCTGGAGAGTTATGATGACTTGTACCATTGGTCCGTTGAGTCATATTCAGACTTCTGGGCAGAGT  193
       F  R  A  A  V  G  A  A  C  G  L  A  L  E  S  Y  D  D  L  Y  H  W  S  V  E  S  Y  S  D  F  W  A  E  F

  194  TCTGGAAATTCAGTGGAATTGTCTTCTCACGTGTGTATGATGAGGTTGTGGACACATCGAAAGGAATCGCAGATGTCCCCGAGTGGTTCAAAGGCAGTCG  293
         W  K  F  S  G  I  V  F  S  R  V  Y  D  E  V  V  D  T  S  K  G  I  A  D  V  P  E  W  F  K  G  S  R

  294  GCTCAACTATGCAGAAAACCTCCTGCGGCACAAAGAGAATGACAGAGTTGCCCTTTACATTGCAAGGGAAGGCAAAGAGGAAATTGTGAAGGTGACTTTT  393
       L  N  Y  A  E  N  L  L  R  H  K  E  N  D  R  V  A  L  Y  I  A  R  E  G  K  E  E  I  V  K  V  T  F

  394  GAAGAGCTGAGGCAAGAAGTGGCTTTGTTTGCAGCAGCAATGAGGAAAATGGGTGTGAAGAAAGGAGATCGGGTTGTTGGTTATTTACCCAACAGTGAGC  493
       E  E  L  R  Q  E  V  A  L  F  A  A  A  M  R  K  M  G  V  K  K  G  D  R  V  V  G  Y  L  P  N  S  E  H

  494  ACGCTGTCGAGGCGATGCTGGCTGCGGCAAGCATTGGTGCCATCTGGAGCTCCACGTCCCCGGACTTCGGTGTGAATGGTGTGCTGGACCGGTTTTCTCA  593
         A  V  E  A  M  L  A  A  A  S  I  G  A  I  W  S  S  T  S  P  D  F  G  V  N  G  V  L  D  R  F  S  Q

  594  AATTCAGCCAAAGCTCATCTTCTCTGTGGAGGCTGTTGTCTATAATGGCAAAGAGCACAACCACATGGAAAAGCTGCAGCAGGTGGTTAAAGGCCTACCA  693
         I  Q  P  K  L  I  F  S  V  E  A  V  V  Y  N  G  K  E  H  N  H  M  E  K  L  Q  Q  V  V  K  G  L  P

  694  GACTTGAAGAAAGTGGTGGTGATTCCTTATGTGTCCTCCAGAGAGAACATAGACCTTTCAAAGATTCCAAACAGTGTGTTTCTGGATGACTTTCTTGCCA  793
       D  L  K  K  V  V  V  I  P  Y  V  S  S  R  E  N  I  D  L  S  K  I  P  N  S  V  F  L  D  D  F  L  A  T

  794  CCGGCACCAGTGAGCAGGCCCCGCAGCTGGAGTTCGAGCAGCTGCCCTTCAGCCACCCACTGTTCATCATGTTCTCATCGGGCACCACGGGCGCACCCAA  893
         G  T  S  E  Q  A  P  Q  L  E  F  E  Q  L  P  F  S  H  P  L  F  I  M  F  S  S  G  T  T  G  A  P  K

  894  GTGCATGGTGCATTCCGCTGGGGGCACCCTCATCCAGCATCTGAAGGAGCACCTGCTGCACGGCAACATGACCAGCAGTGACATCCTCCTGTGCTACACC  993
         C  M  V  H  S  A  G  G  T  L  I  Q  H  L  K  E  H  L  L  H  G  N  M  T  S  S  D  I  L  L  C  Y  T

  994  ACGGTCGGCTGGATGATGTGGAACTGGATGGTGTCCCTTCTGGCCACAGGAGCGGCCATGGTCTTGTACGATGGCTCCCCCCTGGTGCCCACGCCCAATG  1093
       T  V  G  W  M  M  W  N  W  M  V  S  L  L  A  T  G  A  A  M  V  L  Y  D  G  S  P  L  V  P  T  P  N  V

 1094  TGCTCTGGGACCTGGTTGACAGGATAGGCATCACTGTCCTGGTAACTGGGGCCAAGTGGCTGTCAGTGCTGGAAGAGAAGGCCATGAAGCCGGTGGAAAC  1193
         L  W  D  L  V  D  R  I  G  I  T  V  L  V  T  G  A  K  W  L  S  V  L  E  E  K  A  M  K  P  V  E  T

 1194  CCACAGTCTCCAGATGCTCCACACGATCCTGTCCACTGGCTCCCCACTGAAAGCCCAGAGCTACGAGTATGTCTACAGGTGCATCAAGAGCAGCATCCTC  1293
         H  S  L  Q  M  L  H  T  I  L  S  T  G  S  P  L  K  A  Q  S  Y  E  Y  V  Y  R  C  I  K  S  S  I  L

 1294  CTGGGCTCCATCTCAGGAGGCACCGACATCATCTCCTGCTTCATGGGCCACAATTTTTCTCTTCCTGTGTATAAAGGGGAGATTCAGGCCCGGAACCTGG  1393
       L  G  S  I  S  G  G  T  D  I  I  S  C  F  M  G  H  N  F  S  L  P  V  Y  K  G  E  I  Q  A  R  N  L  G

 1394  GCATGGCCGTGGAAGCGTGGAACGAGGAAGGAAAGGCGGTCTGGGGAGAGAGCGGCGAGCTGGTGTGTACTAAGCCGATCCCTTGCCAGCCCACACACTT  1493
         M  A  V  E  A  W  N  E  E  G  K  A  V  W  G  E  S  G  E  L  V  C  T  K  P  I  P  C  Q  P  T  H  F

 1494  CTGGAACGATGAGAACGGCAACAAGTACAGGAAGGCGTATTTCTCCAAATTCCCAGGTATCTGGGCTCATGGCGACTACTGCAGAATCAACCCCAAGACC  1593
         W  N  D  E  N  G  N  K  Y  R  K  A  Y  F  S  K  F  P  G  I  W  A  H  G  D  Y  C  R  I  N  P  K  T

 1594  GGGGGCATCGTCATGCTTGGCCGGAGTGACGGCACCCTCAACCCCAACGGGGTGCGGTTCGGCAGCTCGGAAATCTATAACATTGTGGAATCCTTCGAGG  1693
       G  G  I  V  M  L  G  R  S  D  G  T  L  N  P  N  G  V  R  F  G  S  S  E  I  Y  N  I  V  E  S  F  E  E

 1694  AGGTGGAGGACAGCCTGTGTGTCCCCCAGTATAACAAGTACAGGGAGGAGAGGGTGATCCTCTTCCTGAAGATGGCCTCCGGGCACGCCTTCCAGCCTGA  1793
         V  E  D  S  L  C  V  P  Q  Y  N  K  Y  R  E  E  R  V  I  L  F  L  K  M  A  S  G  H  A  F  Q  P  D

 1794  CTTGGTTAAGAGGATCCGTGACGCCATCCGCATGGGCTTGTCTGCGCGACACGTGCCCAGCCTCATCCTGGAAACCAAGGGCATCCCGTATACGCTCAAC  1893
       L  V  K  R  I  R  D  A  I  R  M  G  L  S  A  R  H  V  P  S  L  I  L  E  T  K  G  I  P  Y  T  L  N

 1894  GGCAAGAAAGTGGAAGTTGCCGTCAAACAGATCATCGCTGGAAAAGCCGTGGAGCAAGGAGGTGCTTTCTCGAACCCCGAGACCCTGGATCTGTACCGGG  1993
       G  K  K  V  E  V  A  V  K  Q  I  I  A  G  K  A  V  E  Q  G  G  A  F  S  N  P  E  T  L  D  L  Y  R  D

 1994  ACATCCCTGAGCTGCAGGGCTTCTGAGTCAGACTGCCTGGCGTGTCACTCAGCCGCACCCGTGTGCACTGTAACTTTTGTGTGCTCAAGAAATTATACAG  2093
         I  P  E  L  Q  G  F  *

 2094  AAACCTACAGCTGTTGTAAAAGGATGCTCGCACCAAGTGTTCTGTAGGCTTGGGGAGGGATCGTTTCTCTGTTTTGTTAAATCTGGTGGGTACCTGGATC  2193
 2194  TTCCACACGAGTGGGATTCTGGCCTTCAGAGACA  2227
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Fig. 1. Structure of human AACS cDNA. (a) Restriction map of the cDNA. The solid box represents the open reading frame. (b) Nucleotide sequence of the human AACS cDNA and the predicted amino acid sequence of the protein.

of the enzyme was highest in kidney, and the level in heart was also as high as in brain, but that in liver was much lower. It was reported that the activity and immunologically determined level of the enzyme in the rat was high in the lipogenic tissues, such as liver, brain and adipose tissue, but considerably low in kidney and heart [15]. To our knowledge, up to the present there is no information on the physiological roles of AACS in kidney and heart of animals, and on the difference in the tissue distribution of the enzyme between human and rat, so that, this is the first report on the demonstration of AACS in human and AACS mRNA levels in animal tissues.

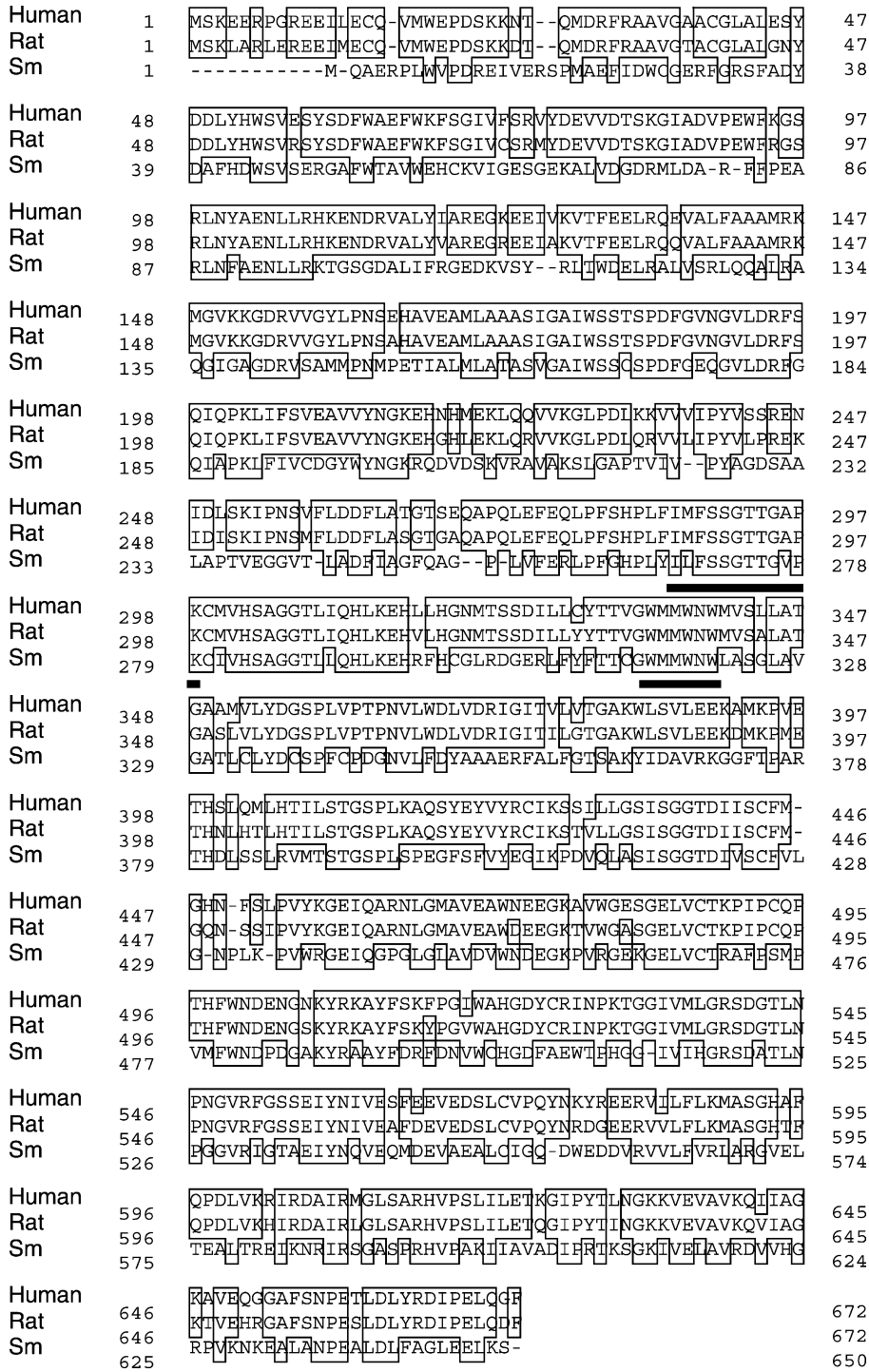

Fig. 2. Homology alignments of AACSs of rat and *Sinorhizobium meliloti* (indicated as Sm). Boxes indicate identical amino acid residues of the sequences. Underlines indicate the sequence specific to AACS and acetyl-CoA synthetase, or the sequence for AMP-binding site.

As to the ketone body metabolism in kidney and heart, Marcondes *et al*. reported that catalytic activity of renal SCOT was impaired by the administration of endotoxin to rats and that this impairment was accompanied by SCOT tyrosine nitration [16]. They also demonstrated such decrease in the catalytic activity and tyrosine nitration of SCOT in the heart of streptozotocin-induced diabetic rats and suggested that the impairment of the ketone body-utilizing enzyme is a contributing factor to the derangements in myocardial substrate utilization in the diabetic status [17]. We previously showed that specific activity of AACS markedly decreased in the liver of streptozotocin-induced diabetic rats correspondingly to the immunologically determined protein level of AACS [7]. Thus, although the regulatory mechanism of AACS activity seems to be different from that of SCOT activity, the high expression of

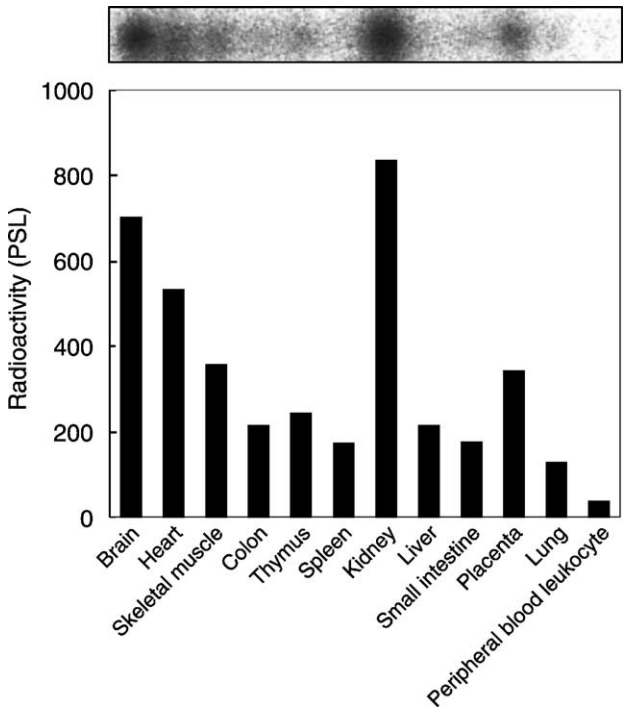

Fig. 3. Expression of AACS mRNA in various tissues of human. Northern blot analysis was carried out with the ^{32}P-labeled human AACS cDNA probe using human multiple tissue Northern blots as described in Section 2. The radioactivity was quantified as photostimulated luminescence (PSL, Fuji BAS system) for each band.

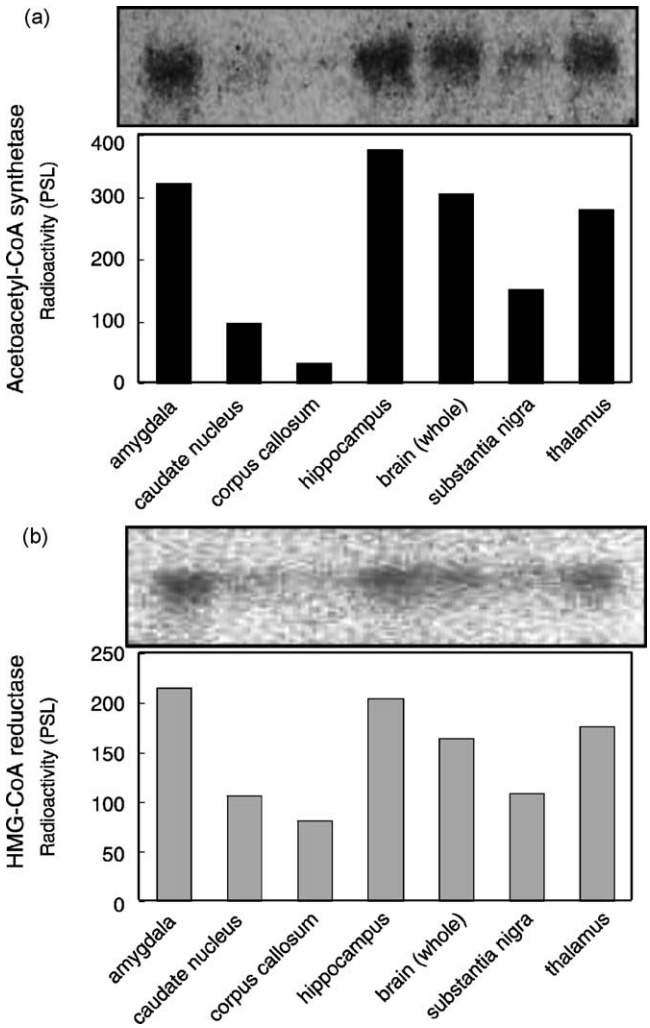

Fig. 4. Expression of AACS and HMGCR mRNAs in various regions of human brain. Northern blot analysis was carried out with the ^{32}P-labeled human AACS (upper) or HMGCR (lower) cDNA probe using human multiple tissue Northern blots as described in Section 2. The radioactivity was quantified as photostimulated luminescence (PSL, Fuji BAS system) for each band.

human AACS observed in the kidney and heart indicates the possibility that the enzyme is also involved in the important parts of the cellular functions of human, even in the tissues different from the lipogenic ones, including the production of energy source from the ketone body, which has been regarded as the specific role of SCOT in mammals.

3.4. Expression profiles of AACS and HMGCR in human brain

The high expression of AACS in the human brain (Fig. 3) led us to the investigation of AACS distribution in the various regions of the brain using human MTN blots. As shown in Fig. 4, there were high levels of expression of AACS in hippocampus, amygdala and thalamus, and the expression profile of AACS in the human brain was quite similar to that of HMGCR, the rate-limiting enzyme of cholesterol biosynthesis. Recently much attention has been focused on the relationship between cholesterol metabolism and Alzheimer disease [18]. Michikawa and Yanagisawa reported that viability of neurons, different from that of nonneuronal cells, depends on the intracellular cholesterol content [19]. Furthermore, it was indicated that there is a lower prevalence of diagnosed probable Alzheimer disease in patients taking the HMGCR inhibitors (statins) [20,21]. As to an action of ketone body on neurons, Kashiwaya *et al*. recently demonstrated that D-β-hydroxybutyrate protects cultured rat hippocampal cells from the toxicity of a fragment of amyloid protein, $A\beta_{1-42}$ [22]. Taken together of the role of AACS in the ketone body utilization to supply acetyl units directly from acetoacetate in the cytosolic compartment for the cholesterol biosynthesis [4] and our results of the quite similar expression profiles between AACS and HMGCR in human brain, it is possible that regulation of AACS activity is one of the important targets for the study on the Alzheimer disease.

Acknowledgments

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