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Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT): development of an antibody to human SCOT and diagnostic use in hereditary SCOT deficiency

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Abstract

Succinyl-CoA:3-ketoacid CoA transferase (SCOT) is a key enzyme for ketone body utilization. Hereditary SCOT deficiency in humans (McKusick catalogue number 245050) is characterized by intermittent ketoacidotic attacks and permanent hyperketonemia. Since previously-available antibody to rat SCOT did not crossreact with human SCOT, we developed an antibody against recombinant human SCOT expressed in a bacterial system. The recombinant SCOT was insoluble except under denaturing conditions. Antibody raised to this polypeptide recognized denatured SCOT and proved useful for immunoblot analysis. On immunoblots, SCOT was easily detectable in control fibroblasts and lymphocytes but was detected neither in fibroblast extracts from four SCOT-deficient patients, nor in lymphocytes from two SCOT-deficient patients. These data indicate that immunoblot analysis is useful for diagnosis of SCOT deficiency in combination with enzyme assay.

1. Introduction

Ketone bodies are important vectors of energy transfer from liver to extrahepatic tissues [1]. Ketosis is a normal physiologic response to fasting and other stresses but under some conditions such as uncontrolled diabetes, severe ketoacidosis may develop.

The utilization of ketone bodies is mediated by two enzymes, succinyl-CoA:3-ketoacid CoA transferase (SCOT, EC 2.8.3.5) which catalyzes CoA transfer from succinyl-CoA to acetoacetate, and mitochondrial acetoacetyl-CoA thiolase (T2, EC 2.3.1.9). Hereditary deficiency of either SCOT (McKusick catalogue number 245050) [2] or T2 (McKusick catalogue number 203750) results in reduced ketolytic capacity, which manifests clinically by intermittent ketoacidotic attacks [3–10].

To aid the diagnosis of the hereditary deficiencies of ketolysis we have characterized the human T2 polypeptide, cDNA and gene [10–13]. We have also cloned a human SCOT cDNA and have identified a nonsense mutation in one case of SCOT deficiency [14]. We showed that SCOT assay is diagnostically useful in lymphocytes, fibroblasts [8] and cultured amniocytes, but not in chorionic villi [15].

In unexplained hyperketotic states the analysis of organic compounds in plasma and urine does not allow a specific diagnosis for SCOT deficiency, although persistent hyperketonemia is believed to be characteristic of SCOT deficiency [3–8]. The assay of enzymatic activity is tedious and mutation analysis is expensive and should be limited to samples where SCOT is known to be deficient. Thus we developed a method for assaying semiquantatively gene products. In this paper, we describe the expression of recombinant human SCOT in bacteria, development of an antibody to this polypeptide and determination of the level of immunoreactive SCOT in normal and SCOT-deficient human cells.

2. Materials and methods

2.1. Antiserum to rat SCOT

This polyclonal antiserum has been described [16].

2.2. Construction of a bacterial expression vector for a human SCOT cDNA

A cDNA fragment spanning residues 115–1586, which includes the entire SCOT peptide less the mitochondrial targeting sequence, was amplified from a plasmid which contains the full-length SCOT cDNA. The following oligomers, which contain an XhoI cloning site (underlined) for ligation into the correct reading frame of the pET16b plasmid (Novagen) were used as sense primer: 5′-TGCCTCGAG[115]CATACCAAGTTTTATACAGAT-3′ and as antisense primer: 5′-TTTCTCG[1586]AGCCTG-GTACAAATATCCATA-3′. PCR was performed as follows, using VENT DNA polymerase (New England BioLabs): 1 min, 94°C; 2 min, 45°C and 3 min, 72°C, for 25 cycles. Three clones were purified through a CsCl density gradient and in vitro transcription and translation were performed according to the manufacturer's instructions (Novagen). Polypeptides produced from each of the three clones showed the size predicted for the recombinant SCOT peptide. These three clones were mixed and used in further experiments.

2.3. Expression and purification of recombinant SCOT protein

Expression was performed according to the manufacturer's instructions (Novagen). For one step purification of the recombinant SCOT protein in 6 M urea a His-Bind column (Novagen) was used.

2.4. Development of rabbit antibody to recombinant human SCOT

About 1 mg of purified recombinant SCOT was suspended in saline with urea, mixed with an equal volume of Complete Freund's Adjuvant and injected subcutaneously at three dorsal sites of a New Zealand white rabbit. Booster doses of 0.5 mg of recombinant SCOT in Incomplete Freund's Adjuvant were injected 2, 6 and 8 wk after the primary immunization. Immune serum was obtained 10 wk after the first immunization and stored at −20°C until use.

2.5. Cell lines and immunoblot analysis

Cells from three previously-described SCOT-deficient patients were available: GS1 [7], GS2 and GS3 [8,15]. GS2 and GS3 are siblings. Patient GS4 has not been reported but we confirmed SCOT deficiency enzymatically (1.6 and 2.3 nmol/min/mg protein; normal, 12.4 ± 4.1 nmol/min per mg protein)

(Wanders et al., unpublished). Fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum and routinely maintained at 37°C in a 5% CO_2 atmosphere. Fibroblasts were harvested with 0.05% trypsin/0.01% EDTA, 2 days after reaching confluency. After three washes in 0.9% sodium chloride, cell pellets were stored at -80°C until use.

Lymphocytes were isolated from heparinized blood samples by Ficoll gradient centrifugation (Pharmacia). Pellets of fibroblasts or lymphocytes were freeze-thawed and suspended in 40 mM sodium phosphate (pH 8.0), 0.1% Triton X-100. After sonication and centrifugation at $10\,000 \times g$ for 10 min, the supernatant was used for enzyme assay and immunoblot analysis.

2.6. Enzyme assay and immunoblot analysis

SCOT assay was performed as described [17], with modifications [8]. Briefly, the assay mixture contained 30 mM acetoacetyl-CoA and 50 mM sodium succinate in 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 4 mM iodoacetamide, and SCOT activity was measured spectrophotometrically as the decrease of acetoacetyl-CoA absorption at 303 nm. The molar extinction coefficient was $21\,400 \ M^{-1} \ cm^{-1}$. Protein concentration was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard.

For immunoblots, human heart muscle was obtained at autopsy and stored at $-80°C$ until use. Heart tissues from human and rat were homogenized in 10 volumes of 50 mM sodium phosphate (pH 8.0), 0.1% Triton-X 100, and used for immunochemical analyses. Immunotitration was performed as described [19]. Briefly, aliquots of 4 μl of heart tissue (3–5 mg protein/ml) were incubated with various amounts of antibody for 1 h at 4°C. After adjusting the volume to 80 μl with 50 mM sodium phosphate (pH 8.0), 0.1% Triton X-100, we added 20 μl of protein A Sepharose, containing about 20 μg of Protein A (Sigma Chemicals, St. Louis, MO, USA) and the preparation was again incubated at 4°C for 1 h. The preparation was centrifugated at $10\,000 \times g$ at 4°C for 10 min and the supernatant was assayed for SCOT activity. Immunoblotting was performed according to Towbin et al. [20], using the ProtoBlot Western Blot AP System (Promega, Madison, WI).

3. Result

3.1. Immunoblot analysis using antibody to rat SCOT

Extracts from rat and human heart muscles were studied by immunoblot analysis using antibody to rat SCOT prepared by Zhang et al. [16]. The rat SCOT polypeptide was detected but the antibody did not crossreact with human SCOT despite loading of up to 200 μg of human heart protein and the use of dilutions of antiserum as small as 1/100 (data not shown).

3.2. Recombinant human SCOT.

SCOT polypeptides expressed from each plasmid had the predicted molecular mass of ~ 55 kDa, showing that no premature termination mutation had been introduced during PCR (data not shown). The results of the purification are presented in Fig. 1. As shown, the recombinant SCOT protein was insoluble in a solution of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), 0.1% NP40 and 1 mM PMSF (lane 2). We therefore solubilized the recombinant SCOT in the same solution with 6 M urea (lane 3), and purified this polypeptide by column chromatography with a His-Bind column (lane 7). Antibody was raised against this denatured recombinant SCOT protein.

3.3. Immunoblot analysis of antibody to human SCOT

The polyclonal antiserum to recombinant human SCOT detected not only the human heart SCOT protein (Fig. 2, lanes 5–7), but also detected rat heart SCOT protein (lanes 1–3) with similar affinity. Both rat and human liver samples had no SCOT protein (lanes 4 and 8). Preimmune serum detected neither rat nor human SCOT protein (data not shown).

3.4. Immunotitration analysis

We tested its interaction with nondenatured SCOT polypeptide, to see whether the antibodies would be useful for immunotitration and pulse-chase experiment. As shown in Fig. 3, human SCOT activity
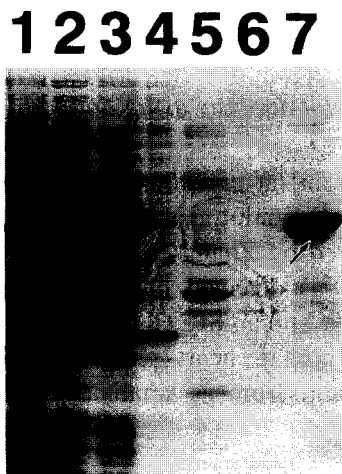
1 2 3 4 5 6 7

Fig. 1. Purification of recombinant SCOT protein. Samples from each of the induction and purification steps were subjected to electrophoresis on a 10% SDS-PAGE. Lanes 1–3 concern the sample preparation and about 1/6000 total amount was applied in each lane. Lanes 4–7 concern the chromatography on a His Bind column and about 1/1600 total amount was applied in each lane. Lane 1 is the starting material for purification, total homogenate obtained 3 h after IPTG induction. Lane 2: supernatant of the homogenate with a solution of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), 0.1% NP40 and 1 mM PMSF; lane 3: supernatant extracted from the pellets of the above homogenate with the same solution plus 6 M urea; lane 4: a pass-through fraction following application of the supernatant described for lane 3 to a His-Bind column; lanes 5 and 6: eluted solutions in washing with a solution of 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), and 6 M urea, and with a solution of 20 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), and 6 M urea, respectively; lane 7: an eluted solution by 1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), and 6 M urea. Arrows indicate the recombinant SCOT protein.

kDa 1 2 3 4 5 6 7 8

202.0-
133.0-
71.0-
41.8-
30.6-

Fig. 2. Immunoblot analysis with antiserum to recombinant human SCOT. Immune sera were used 1/10000 dilution as the first antibodies. 5, 10, and 15 μg of rat heart muscle protein, 15 μg of rat liver protein, 5, 10, and 15 μg of human heart muscle protein, and 15 μg of human liver protein were applied to lanes 1–8, respectively. Arrows indicate SCOT protein. Kaleidoscope Prestained Standards (Bio-Rad) were used as sizemarkers.

3.5. Application of the antibody to human SCOT for diagnosis of SCOT deficiency

We measured SCOT levels in the following samples, using immunoblot analysis: fibroblasts from two

could not be immunotitrated by addition of antibody to denatured human SCOT (H1-3). In contrast, the antibody to rat SCOT immunoprecipitated rat SCOT activity (R1 and 2). In our standard conditions for pulse-chase experiments, labelled protein is immunoprecipitated in a buffer of 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.1% SDS, 0.1% Triton X-100, and 10 mM methionine. Human SCOT was not immunoprecipitated by our anti-[human SCOT] antibody under these conditions determined by immunoblot analysis (data not shown), indicating that the antibody was not useful for pulse-chase experiments in our system. We conclude that the human SCOT antibody did not significantly react with native SCOT.
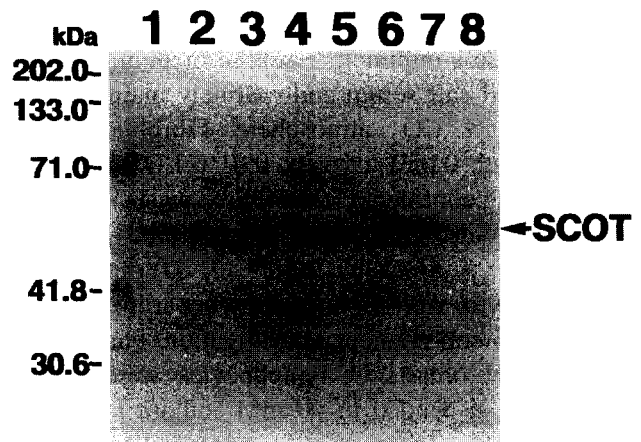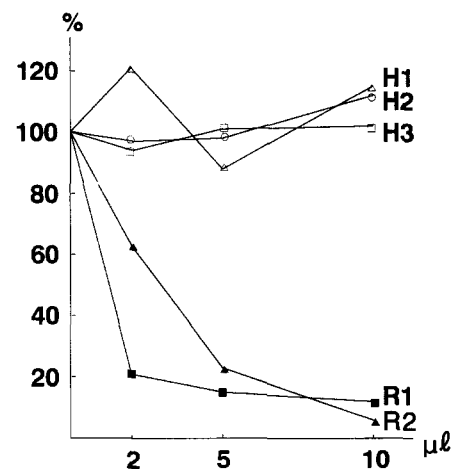

Fig. 3. Immunotitration analysis. 1, 5 and 10 μl of antiserum to SCOT were incubated with a fixed amount (12–20 μg protein) of heart extract. We assayed SCOT activity in the supernatant following incubation with Protein A Sepharose. H1, H2 and H3: human heart extract was immunotitrated with anti-[human SCOT] antibody three times; R 1,2: rat heart extract was done with anti-[rat SCOT] antibody.
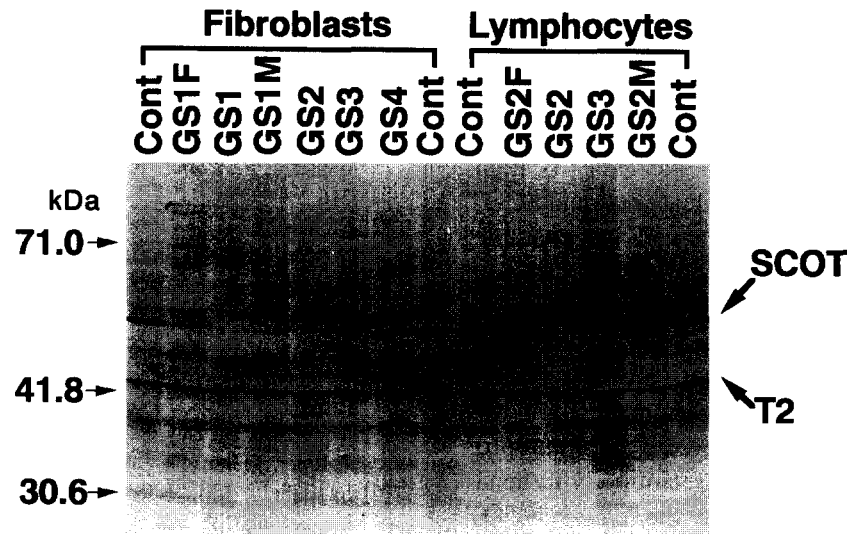
Fig. 4. Immunoblot analysis of fibroblasts and lymphocytes from controls and SCOT-deficient families. Each lane contains 30 μg protein from fibroblasts or lymphocytes extracts. The first antibody was a mixture of two antisera, to human SCOT (10 000-fold dilution) and to rat T2 (20 000-fold dilution). Cont. indicates control fibroblasts or lymphocytes. GS1, GS2, GS3, and GS4 were SCOT-deficient patients. GS1 F and GS1 M were the father and the mother of GS1, respectively. GS2F and GS2M were the father and the mother of GS2 and his sibling GS3, respectively. Kaleidoscope Prestained Standards (Bio-Rad) were used as sizemarkers.

controls, four SCOT-deficient patients (GS1, GS2, GS3, and GS4), and the parents of GS1, and lymphocytes from two controls and from GS2, his sibling GS3 and their parents (Fig. 4). A mixture of anti-[human SCOT] antibody and anti-[rat T2] antibody was used as the first antibody. The bands corresponding to T2 were clearly seen with almost the same intensity in each lane of fibroblasts or in each lane of lymphocytes, providing internal standard for amounts of applied protein. ~ 52 kDa SCOT bands in control lymphocytes were more intense than those of control fibroblasts. This is in accordance with our previous observation that lymphocytes have about two-fold SCOT activity of control fibroblasts [8].

We have reported that the causal mutation in GS1 was S283X [14]. S283X causes premature termination and is incompatible with the production of normal-sized SCOT message. A truncated 30.1 kDa SCOT protein is predicted but no such signal was detectable in this region in GS1. Each of the parents of GS1 had SCOT bands (lanes GS1F and GS1M) of much lesser intensity than controls, as expected since both carry the S283X mutation. A faint background fragment is present at the position of normal SCOT in the sample from GS1, and serves as a negative control to which to compare samples from the other

patients. Fibroblasts from GS2, GS3, and GS4 had no detectable SCOT cross reactive material (CRM).

In analysis of GS2 family using lymphocytes, GS2 and GS3 had no SCOT CRM. The parents (lanes GS2F and GS2M) had considerable SCOT protein, however, with lesser intensity than controls. This suggests that both parents are carriers of SCOT deficiency.

4. Discussion

We report the development of an antibody to human SCOT which is useful as a diagnostic tool for SCOT deficiency. The use of the antibody, as well as the human SCOT cDNAs which we recently cloned [14], will refine the diagnosis SCOT deficiency.

A previously-reported polyclonal antibody to rat SCOT [16], did not crossreact with human SCOT. In contrast antibody to human SCOT which we report here crossreact with denatured rat SCOT in immunoblots with almost the same affinity as with human SCOT, suggesting that denatured human and rat SCOT peptides share common epitopes which are masked in the native polypeptides.

To date, we have diagnosed 4 cases of SCOT deficiency ([7,8,15], and Wanders et al. unpublished). SCOT enzyme assay using fibroblasts and lymphocytes is useful for diagnosis of SCOT deficiency [8]. However, background activity in the assay is high, making it difficult to precisely evaluate residual SCOT activities in fibroblasts or lymphocytes. For example, GS1 is homozygous for S283X [14], a mutation which is predicted to obliterate the function of SCOT, but SCOT activity in fibroblasts from patient GS1 was observed to be 23% of control value [7]. One of our aims in developing antibody to human SCOT was to use it for immunotitration in precise evaluation of residual activity. Unfortunately, the antibody to denatured SCOT does not recognize the native SCOT protein.

However, as shown in Fig. 4, the antibody is useful for immunoblot analysis. None of the four SCOT-deficient patients tested had detectable CRM. By densitometry we estimate that none had greater than one-tenth the amount of SCOT present in control fibroblasts (data not shown) suggesting that their true residual SCOT activity is likely to be very low. Immunoblot analysis therefore provides complementary information useful for evaluation of the severity of the defect, in addition to enzyme assay. Any point mutation at the active centers might affect activity but not protein mass and mutations outside the active centers might affect SCOT turnover, stability etc. Thus both activity assay and immunologic protein determination are useful to the understanding of the defects. Ultimately, mutation analysis and expression of mutant cDNAs will also be necessary in order to determine true SCOT residual activity with which to correlate the clinical features of the patient.

Acknowledgements

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