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LKM-1 Sera from Autoimmune Hepatitis Patients that Recognize ERp57, Carboxylesterase 1 and CYP2D6

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Summary: Liver kidney microsomal antibody type 1 (LKM-1) is a diagnostic marker for autoimmune hepatitis type 2 (AIH-2). Characterization of LKM autoantibodies of patients with AIH-2 demonstrated that a proportion of LKM sera contains autoantibodies which recognize one or more small linear epitopes on cytochrome P450, CYP2D6, an enzyme of drug metabolism pathways. The identification and epitope mapping of antigens involved in autoimmune diseases are important in understanding the mechanisms triggering autoimmunity and providing guidance for designing immunomodulatory therapy. In this study, several proteins recognized by LKM-1-positive sera in rat and human hepatic microsomes were analyzed by MALDI-TOF-MS after separation with ion-exchange chromatography or two-dimensional polyacrylamide gel electrophoresis. We identified these proteins as ERp57 and carboxylesterase 1 (CES1) as well as CYP2D6. Epitopes in ERp57 and CES1 recognized by LKM-1-positive serum were investigated by enzyme-linked immunosorbent assay (ELISA) with protease-digested peptides of ERp57 and CES1. The peptides comprising amino acids 105-129 of ERp57 and 558-566 of CES1 were specifically recognized by the serum. The epitopes in EPp57 and CES1 recognized by LKM-1-positive sera were homologous with those in hepatitis C virus (HCV). Viral infection of such as HCV may thus possibly trigger autoimmune hepatitis.

Keywords: liver kidney microsomal (LKM) antibody; CYP2D6; ERp57; carboxylesterase

Introduction

Liver kidney microsomal antibody type 1 (LKM-1) is a diagnostic marker of autoimmune hepatitis type 2 (AIH-2).[1,2] The major autoantigen to anti-LKM-1 has been identified as a cytochrome P450 (P450), CYP2D6, an enzyme of drug metabolism pathways.[3–5] Another LKM antibody, LKM-2, also recognizes P450, CYP2C9.[6] A possible mechanism for the involvement of CYP2C9 with LKM-2 is suggested. Tienilic acid metabolized by CYP2C9 binds to CYP2C9 covalently and accumulation of the product causes this P450 to become antigenic and induce hepatitis.[7,8] Anti-LKM-1-associated hepatitis is regarded as an autoimmune disease because of the absence of infectious or toxic causes of liver damage. However, LKM-1 is also present in up to 10% of patients with chronic hepatitis C virus (HCV) infections.[3,9] Under these pathological conditions, LKM-1 gives an identical pattern of immunofluorescence, recognizing the same antigenic target, the hepatic P450 CYP2D6. However, the mechanism behind the formation of LKM-1 has not been elucidated.

A proportion of LKM-1 positive sera from patients with AIH-2 contains autoantibodies which recognize one or more small linear epitopes on CYP2D6. The identification and epitope mapping of antigens involved in autoim-

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Abbreviations: LKM, liver-kidney microsomal; AIH, autoimmune hepatitis; CHC, chronic hepatitis C; HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay; CYP or P450, cytochrome P450; TFA, trifluoroacetic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDHA, succinate dehydrogenase A; CES, carboxylesterase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ER, endoplasmic reticulum

mune diseases are important for understanding the mechanisms triggering autoimmunity and providing basis for immunomodulatory treatment. Major epitopes recognized by LKM-1-positive sera are reported to span amino acids 257–269 and 321–351 CYP2D6.[10–12] Additional epitopes are also located at 181–214, 246–281, 373–389, and 410–429.[4] The epitope at 193–212 shares similarity with the RNA-dependent polymerase (HCV-NS5) of HCV and alkaline exonuclease of CMV.[13]

This study, identifies new antigens other than CYP2D6 recognized by LKM-1-positive sera. We detected three proteins of 48 kDa, 57 kDa and 65 kDa by Western blotting with LKM-1 sera. Manns *et al*. found three polypeptides of 50, 55, and 64 kDa using LKM-1 sera and indentified the 50 kDa protein as CYP2D6.[5] However, 55 kDa and 64 kDa proteins have not been identified. We identified two new antigens, protein disulfide isomerase (ERp57) and carboxylesterase 1 (CES1). ERp57 interacts with major hitocompatibility complex (MHC) class I, which presents short peptides to $CD8^+$ T lymphocytes, allowing detection and elimination of infected cells.[14–16] In ERp57-deficient B cells, there is lack of optimal peptide loading and absence of MHC class I molecules.[17] ERp57 plays an important role in the immune response of cells but its function in the peptide-loading complex remains unclear. CES is a serine esterase involved in drug metabolism. In sera of patients with hepatitis caused by a volatile anesthetic, halothane, an antibody against TFA-bind (a metabolite of halothane) CES was detected.[18–20] The mechanism by which this antibody is produced is not clear. Recently Kobayashi *et al*.[21] reported that interleukin-17 has an important role in halothane-induced liver injury. In general, viral infection induces cytokines such as interleukins. Viruses have been implicated as causative agents in autoimmune disorders and this raises the possibility that HCV contributes to the autoimmune response to CYP2D6.[3,22] The appearance of autoantibodies following an infection is well known and thought to arise through molecular mimicry between the host and pathogen.[4] In this study, the epitopes in EPp57 and carboxylesterase recognized by LKM-1-positive sera were analyzed.

Materials and Methods

Materials: Human microsomes were purchased from TCubed (Phenix, CA). The Isogen for isolating RNA was obtained from Nippon Gene (Toyama, Japan). RevertAid™ M-MuLV Reverse Transcriptase was purchased from MBI Fermentas (Vilnius Lithuania). KOD-Plus-DNA polymerase was provided by Toyobo (Osaka, Japan). The Vectastain ABC kit was from Vector Laboratories (Burlingame, CA). Horseradish peroxidase-Goat Anti-Human IgG conjugate was obtained from ZYMED Laboratories (San Francisco, CA). 4-chloro–1-naphthol was purchased from Bio-Rad Laboratories (Hercules, CA). The ELISA plate (amino-type Sumilon) was from Sumitomo Bakelite Co (Tokyo, Japan). Peptides were synthesized by Sigma Genosys (Tokyo, Japan).

Patient sera: Sera were collected from patients admitted to the Osaka City University Medical School Hospital. The sera were taken with informed consent from the patients and their families. The entire study protocol was approved by the Institutional Human Investigational Committee of Osaka City University. In this study, we used sera from two patients diagnosed as AIH-2 based on the criteria proposed by the Intractable Hepatitis Study Group of the Ministry of Health and Welfare of Japan. Their sera were LKM-1-positive by the indirect fluorescence method with sections of livers and kidneys and were HCV-negative based on analysis of antibodies. Epitope analysis of one serum (1) was completed but another serum (2) was used up during the analysis.

Western blot analysis of human microsomes: Human or rat hepatic microsomes (25 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad) and incubated with LKM-1-positive sera at a dilution of 1:1000 overnight at 4°C. Proteins were visualized using the Vectastain ABC kit.

Separation of rat hepatic microsomes by ion-exchange column chromatography and mass-spectral analysis: Rat hepatic microsomes (2 mg protein) were solubilized with sucrose monolaurate (0.5%) in 20 mM Tris-HCl, pH 7.5 and separated by a cation-exchange column (bed volume 2 ml, DE52, Whatman, Maidstone, UK). Proteins were eluted in a stepwise manner with 50 mM, 100 mM, 150 mM and 200 mM NaCl in 20 mM Tris-HCl, pH 7.5 (5 ml). The elution of antigens was detected by Western blotting with LKM-1 serum. Each fraction was concentrated and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were visualized by CBB staining. The proteins were eluted from the gels by digestion with trypsin in 5% acetnitrile and 0.1% TFA. Each digested protein sample was analyzed by MALDI-TOF-MS (Ultra flex, Bruker Daltonics, Billerica, MA).

Two dimensional (2D)-polyacrylamide gel electrophoresis: First, the human hepatic microsomes were precipitated by acetone (final concentration, 80%) to remove lipids. The pellets (70 μg of protein) were solubilized with 0.1 M DTT, 0.25% IPG buffer, 0.1 M acrylamide and Tris-HCl buffer (pH 8.8) containing 7 M urea, 2 M thiourea and 4% CHAPS in a final volume of 125 μL. After centrifugation at 10,000 × g for 30 min, the supernatant was mixed with bromophenolblue. As a first step, isoelectric focusing (IEF) was performed using an immobiline DryStrip (pH 3–11, 7 cm) (Amersham Biosciences) according to the manufacturer's instructions. The DryStrip after IEF was immersed in buffer A (50 mM Tris-HCl (pH 8.8) containing 30% glycerol, 2%

SDS, and 4 mM DTT) for 15 min and buffer B (50 mM Tris-HCl (pH 8.8) containing 30% glycerol, 2% SDS, and 0.1 M acrylamide) for 10 min. It was then laid on a 10% polyacrylamide gel and the second step, SDS-polyacrylamide gel electrophoresis, was performed. The proteins were visualized by the negative staining method of Matsui *et al.*[23] or by immunoblotting with LKM-1 sera. The proteins were eluted from the gels by digestion with trypsin in 5% acetnitrile and 0.1% TFA. The digested protein sample was analyzed by MALDI-TOF-MS (Ultraflex II, Bruker Daltonics, Bremen, Germany).

Expression and purification of human ERp57, HSP70, SDHA, and CES1: Total RNA was extracted from Hep3B cells using total RNA isolation reagent (Isogen, Nippon gene, Toyama, Japan). After deoxyribonuclease treatment (Nippon Gene) to remove contaminating genomic DNA, total RNA was reverse transcribed using an RNA PCR kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The reaction mixture, containing reverse transcriptase, avian myeloblastosis virus, RT-PCR buffer, deoxy-NTPs, random 9-mer primer, RNase inhibitor and total RNA, was incubated at 55°C for 60 min. RT was terminated by heating to 99°C for 5 min. The primers used for amplification of full-length cDNAs were 5′-TTTGGATCCATG CGCCTC-CGCCGCCTAGC-3′ (forward; underline, BamHI site; double underline, start codon) and 5′-CCCAAGCTTTTA GAGATCCTCCTGTGCCT-3′ (reverse; underline, HindIII site; double underline, terminal codon) for human ERp57 (GenBank accession No. NM005313); 5′-ATA GGATCCATGTCCAAGGGACCTGCAGT-3′ (forward; underline, BamHI site; double underline, start codon) and 5′-GCGCGTCGACTTAATCAACCTCTTCAATGG-3′ (reverse; underline, SalI site; double underline, terminal codon) for human HSP70 (NM006597); 5′-ATT GGATCCATGTCG-GGGGTCCGGGGCCT-3′ (forward; underline, BamHI; double underline, start codon) and 5′-CCC AAGCTTT GAGACAAGA-TGTGGTGATG–3′ (reverse; underline, HindIII site, terminal codon is upstream of this primer) for human SDHA (NM004168); and 5′-ACATGCATGCATGTGGCTCCGTGCCTTTAT-CC-3′ (forward; underline, SphI site; double underline, start codon) and 5′-CGGGGTACCTCACAGCTC-TATG-TGTTCTGT-3′ (reverse; under line KpnI; double underline, terminal codon) for human CES1 (NM001266). PCR was carried out for 35 cycles using KOD-Plus-DNA polymerase as follows: denaturation at 94°C for 2 min, annealing at 62°C (for ERp57 and SDHA), 60°C (for HSP70) or 54°C (for CES1) for 30 sec and extension at 68°C. After digestion with each restriction enzyme, cDNAs were subcloned into the pBluescript vector (Stratagene, La Jolla, CA). Full-length cDNAs of ERp57, SDHA, HSP70, and CES1 were cut out from pBluescript with restriction enzymes and subcloned into the histidine-tagged expression vector pQE-80L (QIAGEN, Valencia, CA). The resulting plasmids containing ERp57 and SDHA cDNAs were transfected into *Escherichia coli* DH5α (Toyobo, Osaka, Japan) and the plasmid containing CES1 cDNA was transfected into *Escherichia coli* BL21 (Takara, Shiga, Japan). ERp57, SDHA, HSP70 and CES1, each possessing six histidines (His-tag) at the N-terminal were expressed in *E. coli* cells and purified on a Ni NTA-agarose column (Qiagen). The expression and purification of ERp57, SDHA, HSP70 and CES1 were done according to the manufacturer's recommendations.

Digestion of ERp57 and CES by protease: Purified ERp57 or CES1 (500 μg) was digested with lysyl endopeptidase (0.12 units, Wako Pure Chemical Co., Tokyo, Japan) in 100 mM Tris-HCl (pH 9.0) at 37°C overnight. Digested peptides were separated by HPLC on a reversed-phase column (5C18 HG, 4.6×150 mm, Wako). Chromatography was done at a flow rate of 1.0 ml/min with a linear gradient from an aqueous solution containing 0.5% TFA to a solution containing 100% acetonitrile and 0.5% TFA for 90 min. Fractions were collected every minute and lyophilized. Peptides separated by HPLC and synthesized were dissolved in PBS. The solution was divided into two portions and used for ELISA and N-terminal sequence analysis (PPSQ-21A; Shimadzu, Kyoto, Japan).

ELISA: The ELISA plate, with amino groups on the surface of each well, was treated with 2.5% glutaraldehyde for 2 h at room temperature. After washing with H_2O, peptide solutions were added to the wells followed by standing at 4°C overnight. After washing with PBS, 3% skim milk was added followed by standing at 37°C for 1 h. Sera from patients were diluted with 10% Block ace (Dainippon Pharmaceutical Co., Osaka, Japan), added to the wells followed by standing at room temperature for 2.5 h. After washing with PBS, the secondary antibody conjugated by peroxidase, anti-human IgG, was added. *o*-Phenylene diamine was used as substrate for the peroxidase. Immunoreactivity was measured by a plate reader (Model 550, Bio-Rad) at 415 nm. The synthetic peptides (5 or 10 μg) were fixed on the ELISA plate using 2 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 5 mM N-hydroxysuccinimide in 0.1 M MES, pH 6.0, containing 0.5 M NaCl for 2 h at room temperature. The remaining EDC was quenched with 10% 2-mercaptoetanol. ELISA was performed with this plate as described above.

Results

Identification of antigens in rat hepatic microsomes with LKM-1 positive sera: The specificity of LKM-1-positive sera for human and rat hepatic microsomes was investigated. The microsomes were separated by SDS-polyacrylamide gel elecrophoresis and subjected to immunoblotting using LKM-1-positive sera (**Figs. 1A** and **1E**). Two patient sera (serum 1 and 2) used in this

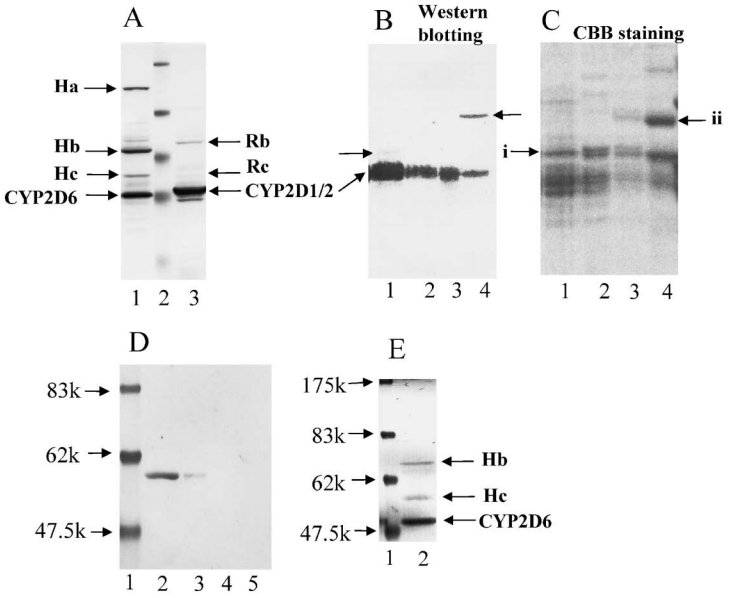

Fig. 1. Recognition of antigens in human and rat hepatic microsomes by LKM-1-positive sera and identification of the antigen in rat hepatic microsomes

(A) Immunoblot of human and rat hepatic microsomes with LKM-1-positive serum. Patient serum (1) was used at dilution of 1:1000. Human and rat hepatic microsomes (25 μg of protein) were separated with a 10% polyacrylamide gel. Lane 1, human hepatic microsomes; lane 2, marker proteins (175 k, 83 k, 62 k, 47.5 k, and 32.5 k); and lane 3, rat hepatic microsomes. The bands Ha, Hb, and Hc; and Rb and Rc indicate major proteins of human and rat, respectively, recognized by LKM-1 serum. (B) and (C) Analysis of fractions of rat hepatic microsomes separated on an ion-exchange column. Solubilized rat hepatic microsomes were applied onto a cation-exchange column (DE-52). Proteins were eluted in a stepwise manner with 50 mM, 100 mM, 150 mM, and 200 mM NaCl in 20 mM Tris-HCl, pH 7.5 (5 ml). The eluted fractions (10 μl) were subjected to Western blotting with LKM-1 serum. Lanes 1, 2, 3, and 4 indicate fractions eluted by 50 mM, 100 mM, 150 mM, and 200 mM NaCl, respectively. The fractions were concentrated and subjected to SDS-polyacrylamide gel electrophoresis and CBB staining. The bands i and ii were picked out, subjected to MALDI-TOF-MS, and indentified as ERp57 and HSP70, respectively. (D) Immunoblotting of human ERp57 and HSP70 expressed in *E. coli* with LKM-1-positive Serum. Human ERp57 and HSP70 including a His-tag were expressed in *E. coli* and purified with a Ni-NTA agarose column. Purified proteins were subjected to Western blotting with LKM-1 serum. Lane 1, marker proteins; lane 2, ERp57 (0.5 μg); lane 3, ERp57 (0.05 μg); lane 4, HSP70 (0.5 μg); and lane 5, HSP70 (0.05 μg). (E) Immunoblot of human hepatic microsomes with LKM-1-positive serum (2). Conditions are the same as in (A). lane 1, maker and lane 2, human hepatic microsomes.

study both gave similar results. Several bands including CYP2D6 and CYP2D1/2 were detected by the LKM-1 antibody. The molecular weights of bands in human hepatic microsomes on immunoblotting were calculated as 48 kD (CYP2D6), 57 kD (Hc), 65 kD (Hb), and 103 kD (Ha), respectively. The corresponding bands (CYP2D1/2, Rb, and Rc) were also detected in rat hepatic microsomes. First, we used rat hepatic microsomes, which are easy to handle and can be obtained in large amounts. The microsomes were separated by a cation-exchange column and proteins were eluted stepwise with change in NaCl concentration. Each fraction was subjected to immunoblotting with LKM-1 serum and CBB staining (**Figs. 1B** and **1C**, respectively). The bands Rc and Rb were eluted in the fractions with 50 and 200 mM NaCl, respectively. The corresponding bands (i and ii) were eluted from the gel by digestion of trypsin and analyzed by MALDI-TOF-MS. A database search with the peptide mass finger-print method identified bands i and ii as rat ERp57 and rat HSP70. Homology percentages between human and rat ERp57 and between human and rat HSP70 were 93% and 85%, respectively. Human ERp57 and HSP70 cDNAs were isolated and the proteins were expressed with His-Tag in *E. coli*. Purified proteins were subjected to immunoblotting. The molecular weights of ERp57 (NM005313) and HSP70 (NM006597), calculated from the amino acid sequence, were 56,781 and 70,897, respectively. Human ERp57 was recognized by LKM-1 serum but not HSP70 (**Fig. 1D**).

Identification of antigens in human hepatic microsomes with LKM-1-positive serum: By two-dimensional polyacrylamide gel electrophoresis using human hepatic microsomes we detected spots by negative staining and immunoblotting using LKM-1-positive serum (**Figs. 2A** and **2B**, respectively). Several spots were found by immunostaining. The proteins in two major spots (iii and iv) were eluted from the gel by trypsin digestion and analyzed by MALDI-TOF-MS. A database search with the peptide mass finger-print method identified the two spots as succinate dehydrogenase complex subunit A (SDHA) and carboxylesterase 1 (CES1), respectively. The molecular weights of SDHA (NM004168) and CES1

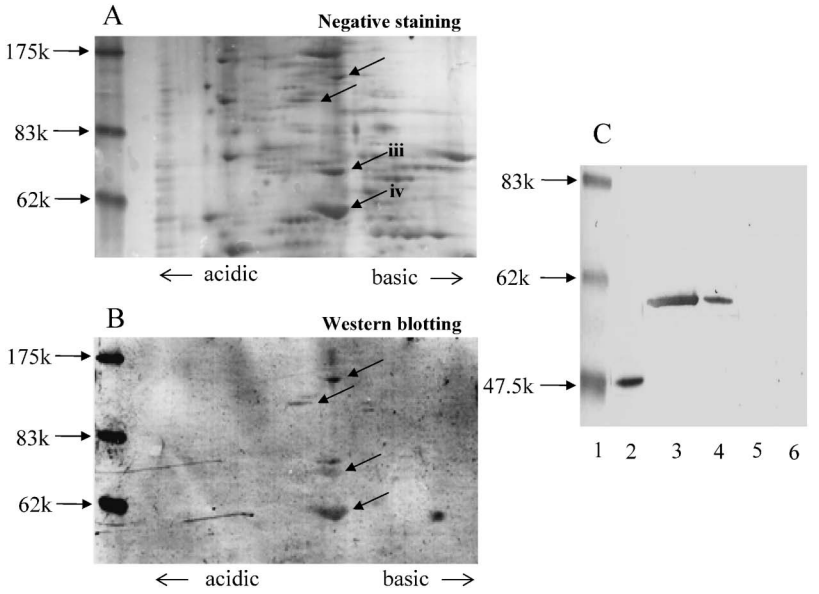

Fig. 2. Two-dimensional electrophoresis of human hepatic microsomes and identification of the LKM-1 antigen in human hepatic microsomes

(A) and (B) The human hepatic microsomes were separated by two-dimensional polyacrylamide gel electrophoresis. Hepatic microsomes (240 μg) were treated with acetone. The pellets (70 μg of protein) were solubilized and subjected to electrofocussing (pH 3–11). Next, proteins were further separated by SDS-polyacrylamide gel electrophoresis. The proteins were visualized with negative staining and subjected to immunoblotting with LKM-1 serum. Arrows indicate spots that reacted with LKM-1. The major spots iii and iv were removed, subjected to MALDI-TOF-MS, and identified as SDHA and CES1, respectively. (C) Immunoblotting of human SDHA and CES1 expressed in *E. coli* with LKM-1 serum. Human SDHA and CES1 including a His-tag were expressed in *E. coli.* and purified with a Ni-NTA agarose column. Purified proteins were subjected to Western blotting with LKM-1 serum. Lane 1, marker proteins; lane 2, CYP2D6 (0.5 μg); lane 3, CES1 (0.5 μg); lane 4, CES1 (0.05 μg); lane 5, SDHA (0.5 μg); and lane 6, SDHA (0.5 μg).

(NM001266) were 72,691 and 62,392, respectively and their isoelectric points, 7.03 and 6.15, respectively. We investigated the specificity of LKM-1-positive sera for CES1 and SDHA. Human CYP2D6, CES1 and SDHA cDNAs were isolated and the proteins were expressed with a His tag in *E. coli*. The purified human CYP2D6, CES1 and SDHA were subjected to Western blotting (**Fig. 2C**). The LKM-1-positive serum reacted with CES1 but not with SDHA, although the apparent molecular weight of CES1 (calculated as 59 k) is smaller than that of CES1 (62 k) as speculated from amino acid sequence.

Immunoreactivity of LKM-1-positive serum with peptides from ERp57: To identify epitopes of the LKM-1 antibody, we performed ELISA using digested peptides of ERp57 (**Fig. 3**). ERp57, which had a linker peptide including a His-tag in its N-terminal region, was expressed in *E. coli*. Purified ERp57 was digested by lysyl endopeptidase. The peptide fragments were separated by reversed-phase HPLC. Each fraction was collected and coated onto ELISA plates with glutaraldehyde. The reactivity of LKM-1 serum was evaluated by ELISA. The serum reacted with fr. 39 only in ELISA with ERp57. The N-Terminal amino acid sequence of the peptide in fr.39 was analyzed with a peptide sequencer and identified as IFRDGEEAGAYDGPRTADGIVSHLK which corresponded to amino acids 105–129 in human ERp57 (NM005313).

Immunoreactivity of LKM-1-positive serum with peptides from CES: Human CES1 was digested by the same method as ERp57. The peptides were separated by HPLC and collected. ELISA was conducted with these fractions (**Fig. 4**). LKM-1 serum reacted with fr. 25 (and fr. 26). The corresponding fraction from HPLC was analyzed by a protein sequencer and the peptide sequence was identified as PPQTEHIEL. This peptide contains the 558–566th amino acid (C-terminus) of CES1 (GenBank Accession No. NM001266).

Homology of amino acid sequence in epitopes of ERp57 and CES1 with those of other proteins: A homology search was performed with the amino acid sequences of the epitopes ($ERp57_{105-129}$ and $CES_{558-566}$) in the GenBank data base (**Fig. 5**). Within the epitopes of $ERp57_{105-129}$, the motif "YDGPR" was found identical with that of HCV $polyprotein_{474-478}$. The motif "HIEL" within $CES1_{558-566}$ was identical with that of HCV NS3/4A $protein_{559-662}$. To confirm the reactivity of the motif sequences with LKM-1 sera, peptides were synthesized and analyzed with ELISA (**Fig. 6**). The motif peptides reacted with LKM-1 sera dose-dependently. A viral sequence may thus sossibly act as a molecular mimic to induce the breakdown of tolerance against ERp57 or CES1.

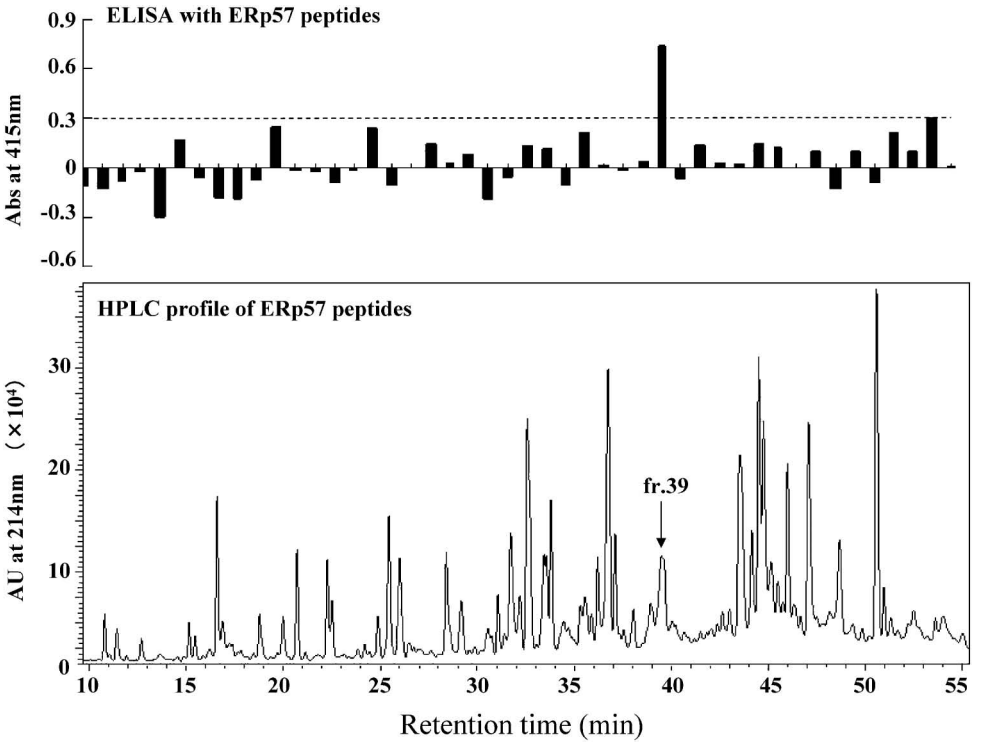

Fig. 3. Chromatographic profiles of peptide fragments of ERp57 digested with lysyl endopeptidase and ELISA with LKM-1-positive serum

Purified human ERp57 (500 μg) was digested with lysyl endopeptidase and the digested peptides were separated by HPLC with a reverse-phased column. The chromatography was done at a flow rate of 1.0 ml/min with a linear gradient of 0 to 100% acetonitrile containing 0.5% TFA for 90 min. Fractions were collected every minute. Each fraction was analyzed with ELISA (upper panel). The immunoreactivity of these fractions with LKM-1 serum is expressed as absorbance at 415 nm. The positive fraction (fr. 39) is indicated by an arrow in the chromatogram with a fraction number. The protein in the fraction was identified as IFRDGEEAGAYDGPRTADGIVSHLK with a protein sequencer.

Discussion

Anti-LKM-1 reacting with human cytochrome P450, CYP2D6, is regarded as a serological marker of type II autoimmune chronic active hepatitis.[3–5] 50-kD protein in rat hepatic microsomes and 48-kD protein in human hepatic microsomes have been recognized as LKM-1-reacting protein. Manns *et al.*[5] identified this protein as CYP2D6. They also found 55-kD and 64-kD proteins with which LKM-1 reacted, although they did not identify them.[5] We recognized two proteins other than CYP2D6 using LKM-1-positive sera of Japanese AIH-2 patients in this study. Their molecular weights calculated from mobility in SDS-polyacrylamide gel electrophoresis were 57 kD and 65 kD. They may possibly correspond to the 55-kD and 64 kD proteins reported by Manns *et al.*[5] The 57-kD protein was isolated from rat hepatic microsomes by ion-exchange chromatography and identified as a protein disulfide isomerase, ERp57. Yokoi et al. identified ERp57 as a protein with which autoantibodies of Long Evans Cinnamon (LEC) rats react.[24] LEC rats show hepatitis about three to four months after birth with a high rate of mortality.[25] LEC rats which died of fulminant hepatitis possess autoimmune antibodies to liver microsomal proteins.[26] The antibody recognizes a protein with a molecular weight of 56 kDa, which is an ERp57. The accumulation of copper in the liver of the LEC rats is linked with the development of hepatic damage, although the mechanism of this damage is not clear.[27,28] DNA damage by free radicals produced by copper ion has been suggested.[29] Autoantibodies against ERp57 have also been detected in the sera of patients with sulfonamide antimicrobial delayed onset hypersensitivity syndrome reactions.[30] The roles of these antibodies in the pathogenesis of these reactions are still unknown. Autoantibodies against CES as well as ERp57 were detected in the sera of patients with halothane-induced hepatitis.[19] In the case of this hepatitis, the hapten antigen, a TFA-protein complex, is thought to produce autoantibodies which cause hepatitis.[31] Halothane is metabolized by a cytochrome P450, CYP2E1 and active metabolites bind to CYP2E1 but no antibodies against CYP2E1 were recognized.[32,33] It has been proposed that covalent binding or cellular stress alters the structures of membranes or proteins, triggering immunological abnormality. CES is present at the lumen site of ER mem-

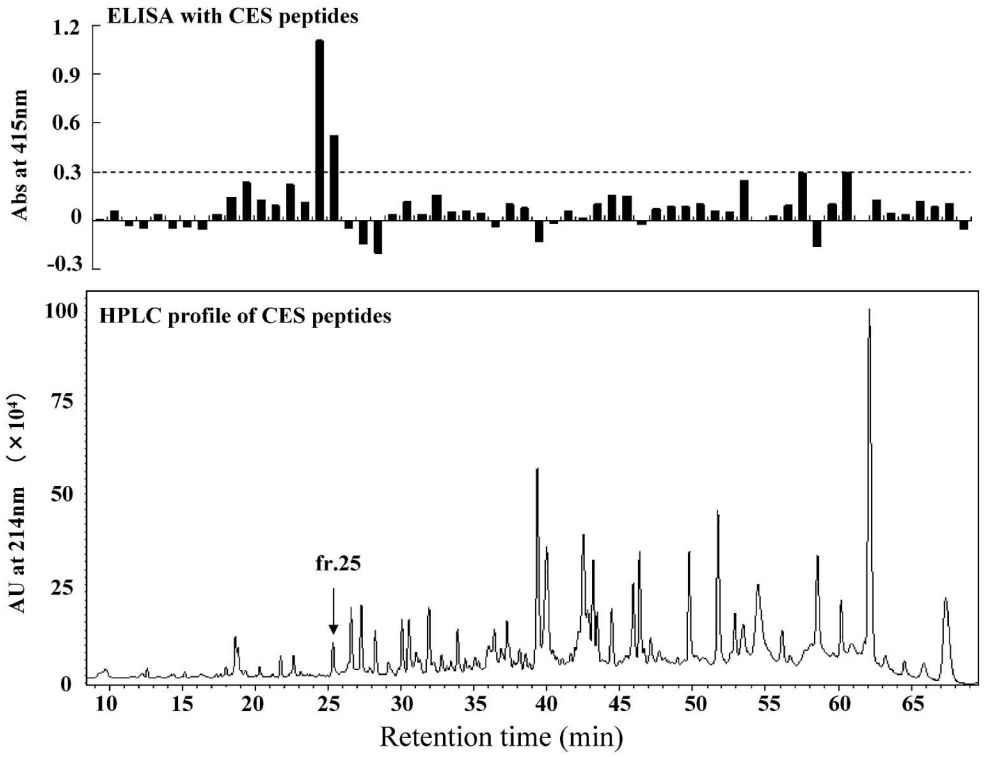

Fig. 4. Chromatographic profiles of peptide fragments of CES1 digested with lysyl endopeptidase and ELISA with LKM-1-positive serum

Purified human CES1 (500 μg) was digested with lysyl endopeptidase and the digested peptides were separated by HPLC with a reverse-phased column. The conditions are the same as in the legend of **Figure 3**. The positive fraction (fr. 25) is indicated by an arrow in the chromatogram with a fraction number. The protein in the fraction was identified as PPQTEHIEL with a protein sequencer.

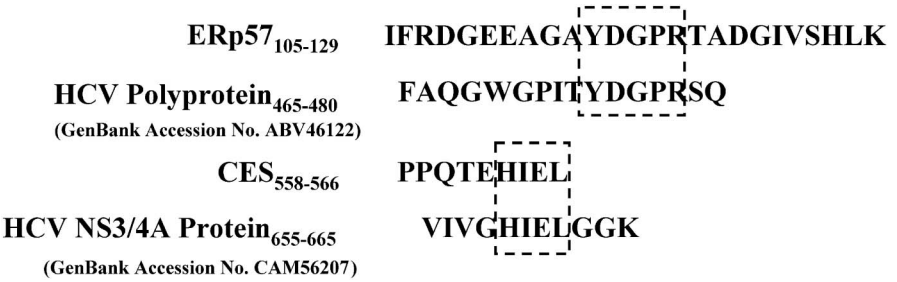

Fig. 5. Homology of amino acid sequences in ERp57$_{105-129}$ and CES1$_{558-566}$ and HCV proteins

Amino acids are in the standard single letter code. Identical residues in CES1, ERp57 and the homologous viral peptides are within the box.

branes. However, the possibility of the presence of CES in the plasma membranes has been reported.[34] CYP2D6 is also reported to exist in the plasma membranes and attack of LKM-1 antibody on CYP2D6 may occur on the surface of cells causing cell injury.[35] These findings suggest that autoantibodies against CYP2D6 and CES may be involved in the pathogenesis of liver damage.

Epitope mapping is useful for understanding the mechanism of production of autoantibodies. Therefore, we analyzed epitopes of antigens identified in this study. The homologous amino acid sequences of epitopes in ERp57 and CES1 were not found in CYP2D6, suggesting that the antibodies against these proteins are produced independently. Interestingly, the epitopes in both ERp57 and CES had homology with proteins of HCV although the sera used in this study were HCV-negative. In CYP2D6, amino acid sequences of some epitopes are homologous with those of HCV and CMV, suggesting that infection is linked to autoimmune hepatitis.[13] Furthemore, infection of adenovirus expressing CYP2D6 to mice causes persistent autoimmune liver disease.[36] Antibodies against ERp57 and CES1 were detected both in AIH-2 and drug-induced hepatitis but the epitopes recognized by these antibodies may differ although further

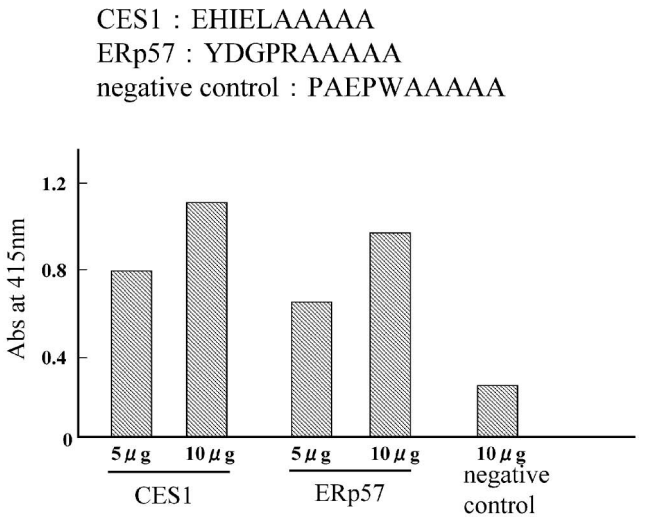

Fig. 6. Immunoreactivity of motif sequence peptides with LKM-1-positive serum
The peptides from ERp-57 and CES1 homologous with those of HCV proteins were synthesized and their reactivity with LKM-1 was investigated by ELISA. The peptides include five alanine residues as linkes to be fixed on the ELISA plate. Peptides (5 and 10 μg) were used for this fixing to the ELISA plate. The negative control is the peptide $CES1_{70-74}$.

study is necessary. The epitope peptides of CYP2D6, ERp57 and CES1 may be new diagnostic markers for these hepatitis.

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