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分担研究報告書

トログリタゾンにより肝障害を起こした患者血清中にアルドラーゼBに対する自己抗体の発見

Detection of Autoantibody to Aldosase B in Sera from Patients with Troglitazone-induced Liver Dysfunction

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研究要旨

抗糖尿病薬トログリタゾンは、一部の患者に薬物誘導性突発性肝障害を起こすことにより2000年に販売を取り下げた薬である。こうした患者の血清中にトログリタゾン誘発性肝障害に関連する自己抗体の産制の有無を検討した。40歳と70歳の２名の女性患者が、トログリタゾン１日投与量400 mg/dayで、それぞれ23.5週と16週でALTの顕著な上昇を認めた。イムノブロットおよび２次元電気泳動法を駆使して、患者血清中にアルドラーゼＢに対する強い抗体を両患者に認めた。この抗体は、薬物投与を中止後も、高いタイターを保ち続けた。この抗体がトログリタゾン投与患者特異的なものか否かについて、慢性肝障害患者血清40検体、肝硬変患者血清40検体および健常人血清80検体についてELISAを用いて検討した。その結果、いずれの患者でもアルドラーゼＢに対する自己抗体が認められ、健常人に比べて前者で$P<0.05$、後者で$P<0.001$という有意を示した。一方、急性の肝障害患者では認められなかった。この結果は、アルドラーゼＢが肝障害を憎悪する原因ではなく、肝障害のマーカーの１つとして有用であることが示唆された。

Troglitazone is a thiazolidinedione antidiabetic agent with insulin-sensitizing activities that was withdrawn from the market in 2000 due to its association with idiosyncratic hepatotoxicity. To address the suspected autoantibody production associated with troglitazone, autoantibodies in sera from patients with type II diabetes mellitus with troglitazone-induced liver dysfunction were determined. Two female patients (47- and 70-year old) ceased taking troglitazone (400 mg/day) after 23.5 and 16 weeks, respectively, due to increased serum ALT. Using 2-dimensional electrophoresis and amino acid sequence analyses, aldolase B was identified as an autoantigen that reacted with antibodies in sera from both patients. The titer of anti-aldolase B remained high for several weeks after stopping troglitazone administration. The mean reactivity of autoantibodies to aldolase B determined by ELISA with sera of patients with chronic hepatitis (n = 40) and liver cirrhosis (n = 40) was significantly higher ($p < 0.05$ and $p < 0.001$, respectively) than with sera of healthy subjects (n = 80). These findings suggest that liver injury may cause the appearance of autoantibodies to aldolase B which may then aggravate the hepatitis. In addition, the anti-aldolase B titer might indicate the severity of liver dysfunction.

A. 研究目的

Adverse drug reactions can be classified into two basic types, reactions that occur directly and can be predicted from the pharmacology of the drug and, in contrast, idiosyncratic reactions which are induced dose-independently and are infrequent and unpredictable. Many idiosyncratic drug reactions have an immunological (hypersensitivity) basis, whereas some are due to a metabolic abnormality of the host (Ju and Uetrecht, 2002; Pirmohamed et al., 1998; Pohl et al., 1988). The liver is an important target for the toxic effects of drugs because of its essential role in the metabolism of xenobiotic substances. Idiosyncratic drug-induced hepatitis has been assumed to be mediated by immunogens formed by covalent interaction of a reactive drug metabolite with cellular macromolecules (Ju and Uetrecht, 2002; Park et al., 1998). The bioactivated immunogens may not only lead to an immune response directed against the haptenic epitope and the neoantigen, but also against autoantigenic determinants, which is characterized by the formation of autoantibodies (Pohl et al., 1988). A number of hepatotoxic drugs have been reported to produce autoantibodies. For instance, anti-protein disulfide isomerase, anti-microsomal carboxyesterase, anti-calreticulin, anti-ERp72, anti-GRP78, anti-GRP94 and anti-CYP2E1 in halothane hepatitis (Bourdi et al., 1996; Gut et al., 1993; Kenna et al., 1993; Pumford et al., 1993), anti-CYP2C9 in tienilic acid-induced hepatitis (Homberg et al., 1984; Robin et al., 1996), anti-CYP1A2 in dihydralazine-induced hepatitis (Bourdi et al., 1990), and anti-CYPs in aromatic anticonvulsant-induced hypersensitivities (Leeder et al., 1992). However, it is not known whether the autoantibodies are the cause or consequence of the progression of hepatotoxicity. Studies to clarify the possible involvement of autoantibodies in drug-induced hepatitis have been limited, since the appearance of autoantibodies can be seen usually only in human (Descotes, 2000).

Troglitazone (Noscal®, Sankyo, Tokyo, Japan or Rezulin®, Parke-Davis, Morris Plains, NJ) was an early member of the thiazolidinedione chemicals developed for type II diabetes. It has a novel mechanism of action on lowering the blood glucose level by increasing glucose uptake by skeletal muscles, decreasing hepatic glucose production, and sensitizing target tissues to insulin (Fujiwara et al., 1995, 1988; Ciaraldi et al., 1990). However, a rare type of hepatic injury has been reported to be associated with troglitazone therapy. During clinical trials, 1.9% of patients experienced increases in ALT levels greater than 3 times the upper normal limit (Watkins and Whitcomb, 1998). Fulminant hepatic failure in some patients was reported to occur after long-term troglitazone treatment (more than 4 weeks) (Gitlin et al., 1998; Kuramoto et al., 1998; Neuschwander-Tetri et al., 1998; Shibuya et al., 1998). The hepatic toxicity of troglitazone was not observed in any experimental animals tested including monkey, which has a similar metabolic profile to human (Rothwell et al., 2002; Watanabe et al., 1999). Although the mechanism by which troglitazone causes liver dysfunction in certain individuals is not yet clear, it is thought to be idiosyncratic.

There is no report so far of whether a metabolic idiosyncrasy or immunological idiosyncrasy causes this hepatotoxicity.

In the present study, aldolase B was identified as an autoimmune antigen which reacted against antibodies in sera of patients with troglitazone-induced liver dysfunction. The titer of the aldolase B autoantibody remained high for several weeks after stopping troglitazone administration. In addition, the formation of the aldolase B autoantibodies was also investigated in patients with chronic hepatitis and liver cirrhosis as compared with healthy subjects.

B. 研究方法

B-1. Materials

Biotinylated anti-human IgG, biotinylated anti-rabbit IgG, and a VECTASTAIN ABC kit were purchased from Vector Labolatories, Inc. (Burlingame, CA). Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standard of low molecular weight range and prestained isoelectric point (pI) marker were from Bio-Rad (Hercules, CA). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) and 3,3',5,5'-tretramethylbenzidine (TMB) liquid substrate system were from Sigma (St. Louis, MO). HRP conjugated anti-human IgA, IgG, IgM, kappa, lambda was from DakoCytomation (Glostrup, Denmark). PVDF membrane (Immobilon-P) was from Millipore (Bedford, UK). Ampholine was from Amersham Biosciences (Buckinghamshire, UK). Purified human aldolase B protein was previously prepared by Haimoto et al. (1989). Recombinant human aldolase B was a generous gift from Prof. Dean R Tolan (Boston University, Boston, MA). Other chemicals were of the highest grade commercially available.

B-2. Patients

This study was approved by the Ethics Committee of Kanazawa University, Nagoya First Red-Cross Hospital, and Fukui Prefectural Hospital, Japan. The two patients (A and B) gave written informed consent. Serum ALT was periodically measured throughout the time of monitoring. Patient A was a 47-year old Japanese female with diabetes mellitus. She had been prescribed insulin (36 U/day) for 3 years. Because of inadequate control of the blood sugar level, administration of insulin was stopped and troglitazone therapy (400 mg/day) was started in 1998. Sixteen weeks after the start of troglitazone therapy, the serum ALT level started to increase (32 IU/L). Since the serum ALT level had prominently increased to 229 IU/L at 23.5 weeks, troglitazone therapy was stopped (Fig. 1A). The serum ALT levels gradually decreased (183 IU/L, week 24; 113 IU/L, week 26; 24 IU/L, week 30; 12 IU/L, week 75). Patient B was a 70-year old Japanese female with diabetes mellitus, hyperlipidemia, and essential hypertension. The patient had been prescribed glibenclamide (10 mg/day), pravastatin (10 mg/day), and celiprolol (100 mg/day). In 1998, because of inadequate control of the blood sugar level, troglitazone therapy was started at 400 mg/day. Eight weeks after the start of troglitazone therapy, the serum ALT level started to increase (24 IU/L). Since the serum ALT level continued to increase (44 IU/L, week 12; 99 IU/L, week 16), the troglitazone therapy was stopped

(Fig. 1B). Although the serum ALT level had reached 205 IU/L at 20 weeks, the level subsequently decreased to normal (13 IU/L) at week 28.

Serum samples of 80 in-patients with liver dysfunction, 22 males and 18 females with chronic hepatitis, and 18 males and 22 females with liver cirrhosis, were obtained from Kanazawa University Hospital, Kanazawa, Japan. Serum samples of 80 healthy subjects, 45 males and 35 females, were obtained from the Red Cross Blood Supply Center, Kanazawa, Japan. The sera from healthy subjects were negative for serological tests of recent infection with hepatitis A and B viruses, cytomegalovirus and Epstein-Bar virus. All samples were stored at -20°C until analysis.

B-3. Preparation of liver subcellular fractions

For Western blotting, human liver samples were obtained from autopsy. The use of the human liver for the study was approved by the Ethics Committee of Dokkyo University School of Medicine (Tochigi, Japan). Liver tissues were rapidly frozen in liquid nitrogen immediately after excision and were stored at -80°C. Human liver samples from two individuals (54-year old male and 60-year old female, ischemia cardionecrosis) were homogenized with 3 volumes of 0.1 M Tris-HCl (pH 7.4), 0.1 M KCl and 1 mM EDTA. Nuclei fractions were isolated by centrifugation at 600 g for 10 min. The supernatant was centrifuged at 9,000 g for 20 min to isolate the mitochondoria fraction. The supernatant was centrifuged at 105,000 g for 60 min to prepare the microsome and cytosol fractions. The prepared fractions of nuclei, mitochondria, and microsomes were washed two times using the same experimental procedures. The protein concentration was measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard. The prepared subcellular fractions were stored at -80°C until use.

B-4. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were carried out. In brief, human liver homogenates (20 μg), nuclei (10 μg), mitochondria (10 μg), microsomes (10 μg), and cytosol (10 μg) were applied to 7.5% polyacrylamide gel. Proteins were transferred to PVDF membrane. The membrane was incubated with serum from healthy subjects, patient A or B (diluted 1:100) as the first antibody at 37°C for 60 min. Subsequently, the membrane was incubated with biotinylated anti-human immunoglobulin (diluted 1:2000) at 37°C for 30 min and incubated with avidin-biotin complex (VECTASTAIN ABC kits) at 37°C for 30 min. Anti-sera to the human aldolase B raised in rabbit as described previously (Haimoto et al., 1989) was used (final concentration: 0.1 μg/ml) at 37°C for 30 min. The membrane was incubated with biotinylated anti-rabbit IgG (diluted 1:2000) at 37°C for 20 min and then with avidin-biotin complex (VECTASTAIN ABC kit) at 37°C for 20 min. 3,3'-diaminobenzidinetetrahydrochloride (DAB) was used as a substrate for peroxidase.

B-5. Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis was carried out according to the method described by O' Farrell (1975) with slight modifications. Isoelectric focusing (IEF) gel was prepared from a mixture containing 8 M urea, 2% Nonidet P-40, 2% Ampholines carrier ampholites (0.5% pH 6.0-8.0 range, 1.5% pH 7.0-9.0 range), and 4% acrylamide. Prefocusing was carried out at 200 V for 15 min, 300 V for 20 min, and then 400 V for 20 min. Samples up to 50 µl in volume were subjected to electrophoresis at 400 V for 12-16 h and at 800 V for 1 h using 0.02 M NaOH at the anode and 0.01 M H_3PO_4 at the cathode. The second dimensional separation was carried out using the 7.5% SDS-PAGE as described above.

B-6. Amino acid sequence analysis

After the 2-DE, the separated proteins were visualized by staining with Coomassie brilliant blue G-250 (CBB). The corresponding spots of interest were compared with the profile from the Western blotting with the patient A serum sample. The stained 40 kDa spots with pIs of 7.4 and 7.6 were excised and subjected to trypsin digestion. The analysis of peptides was carried out at APRO Science Inc. (Tokushima, Japan) by nanoflow ESI on a Q-TOF mass spectrometer.

B-7. Enzyme-linked immunosorbent assay (ELISA)

The sera of patient A (serum 2), the healthy subjects (n = 80), and those with chronic hepatitis (n = 40) and liver cirrhosis (n = 40) were subjected to ELISA. Recombinant human aldolase B purified from *E. coli* DH5_ expressing cells was used as an antigen. To avoid non-specific reactions with the bacterial antigens, all human sera were pre-absorbed with *E. coli* DH5a lysate 1:2 (v/v) for 4 h at room temperature with agitation and were centrifuged at 10,000 rpm for 15 min at 4°C.

Hundred microlitres of human recombinant aldolase B (10 µg/ml) in carbonate/bicarbonate buffer, pH 9.6 were coated onto each well of flat-bottomed microtiter plates (Corning, NY) and incubated overnight at 4°C. The wells were washed three times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked with 150 µl of solution containing 50 mM Tris-HCl, pH 8.0, 0.14 M NaCl and 1% BSA, at 37°C for 2 h. After washing three times, 100 µl of sera (1:100) in the solution containing 50 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 0.1% BSA and 0.05% Tween 20, were applied to each well and incubated at 37°C for 1 h. Each plate included serum from patient A as a positive control. The wells were washed three times before adding HRP conjugated anti-human IgA, IgG, IgM, kappa, lambda (1:8000) in the same solution as for the serum dilution. The wells were incubated at 37°C for 1 h, washed five times and 100 µl of tetramethyl benzidine (TMB) substrate solution were added to each well. After 30 min of incubation, the TMB reaction was stopped by adding 100 µl of 2 M H_2SO_4. The optical density was read at 450 nm by using a microtiter plate reader (Biotrak II, Amersham Biosciences). Controlled wells that were coated without aldolase B were also used for each serum sample. All assays

were performed in triplicate and the specific binding of antibody to aldolase B in sera was calculated by subtracting the average absorbance of the control wells from the average absorbance of the aldolase B coated wells.

B-8. Statistical analysis

Data from ELISA were analyzed by Kruskal-Wallis nonparametric analysis of variance (ANOVA) followed by Dunn's multiple comparison test. $p < 0.05$ was considered significant.

C. 研究結果

Western blotting was performed with human liver subcellular fractions using sera from healthy subjects and patients A and B. In order to investigate the existence of troglitazone-induced autoantibodies and to elucidate the subcellular localization of antigen proteins, human liver subcellular fractions from two different individuals were subjected to SDS-PAGE. Western blotting was performed using sera 1 of patients A and B (Fig. 1) as the first antibody. Immunostained bands of 55 kDa and 49 kDa molecular weight were observed in the serum of a healthy subject. The 55 kDa band was more intense than the 49 kDa band (Fig. 2). These bands could be detected in the sera of all healthy subjects examined (n = 8, data not shown). The serum of patient A recognized an intense band of 40 kDa and a faint band of 45 kDa in the cytosolic fraction, whereas a faint band of 40 kDa was detected with the serum of patient B (Fig. 2). The serum of a healthy control could not detect these 40 kDa and 45 kDa bands. Other minor bands in patient A and B sera showed low reproducibility. Thus, this study was focused on the identification of the 40 kDa antigenic protein in further experiments.

In the serum of patient A, the immunoglobulin subtypes of antibody detecting the 55 kDa, 49 kDa, 45 kDa and 40 kDa proteins were IgG, IgM, IgG and IgG, respectively (data not shown). In the serum of patient B, the 55 kDa, 49 kDa and 40 kDa proteins were IgG, IgM and IgM, respectively (data not shown).

C-1. Identification of the 40 kDa antigenic protein

Two of the three immunoreactive spots with pIs of 7.6 and 7.4 in Fig. 3 were excised from the protein-stained 2-D gel and subjected to trypsin digestion. Amino acid sequencing was carried out with nanoflow ESI on a Q-TOF mass spectrometer. A 15-amino acid sequence (GGKAANKEATQEAFM) was identified from the trypsin-treated fragment of both spots. This sequence was perfectly matched with the amino acid sequence from 315 to 329 of human aldolase B.

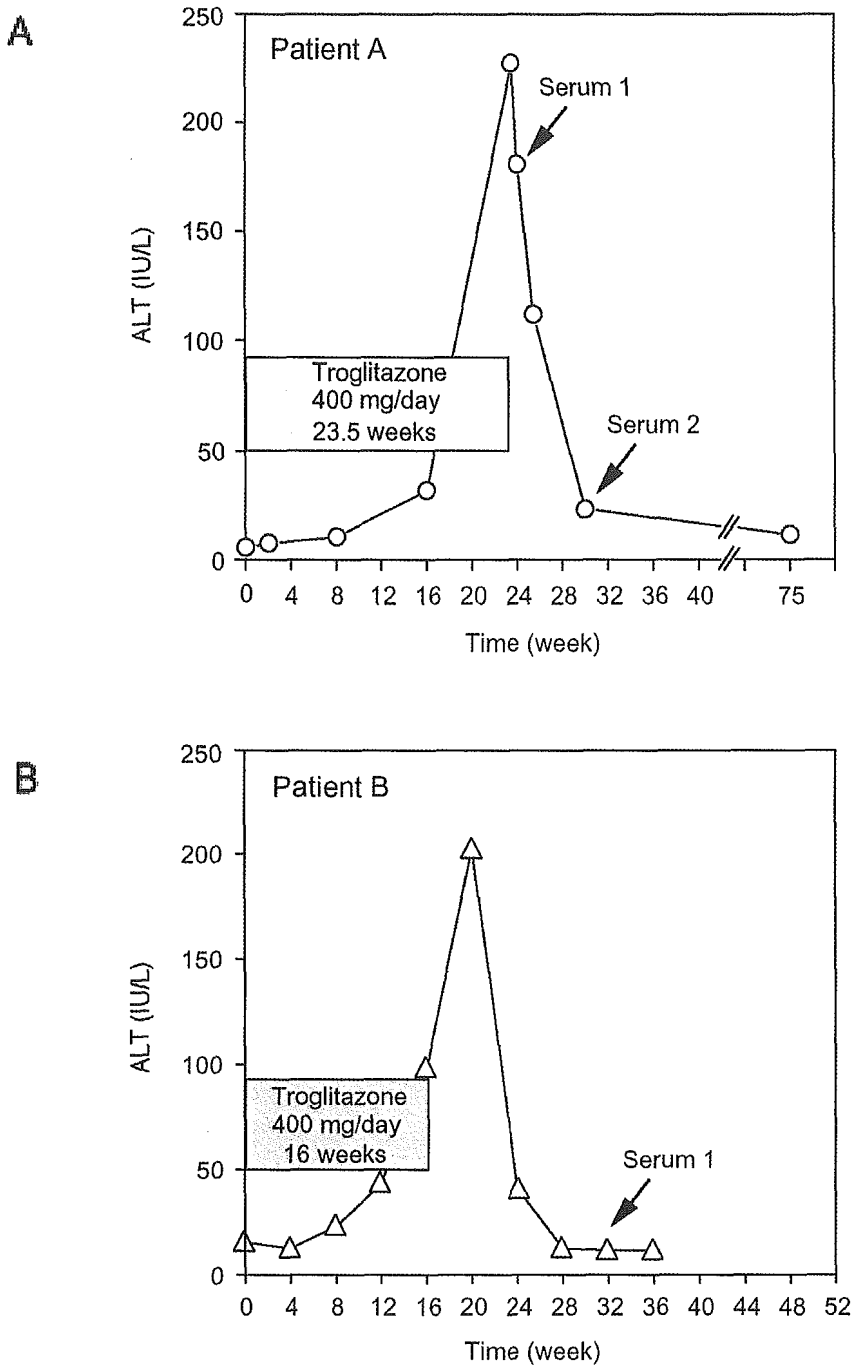

Fig. 1. Changes of serum ALT in patients with troglitazone-induced liver dysfunction. (A) Patient A (female, 47-year old) and (B) patient B (female, 70-year old) were administered troglitazone (400 mg/day) for 23.5 and 16 weeks, respectively. During the time of monitoring, the serum ALT of these patients was periodically measured at Fukui Prefectural Hospital and Nagoya First Red-Cross Hospital, Japan. Serum1 and 2 of patient A and serum 1 of patient B were used in the further study.

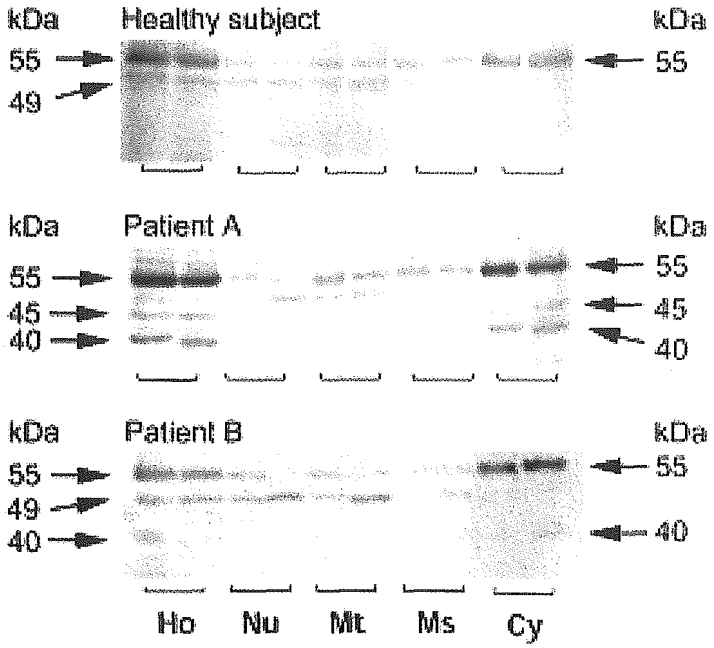

Fig. 2. Western blotting for human liver subcellular fractions using sera from healthy controls and patients A and B. Human liver homogenates (Ho), nuclei (Nu), mitochondria (Mt), microsomes (Ms), and cytosol (Cy) from two different individuals were subjected to SDS-PAGE. Western blotting was performed using sera from a healthy subject (1:100), patient A (serum 1, 1:100) and patient B (serum 1, 1:100) as the first antibody as described in Experimental Procedures.

C-2. Two-dimensional electrophoresis of human liver cytosol and Western blotting with the serum of patient A as the first antibody

To identify the 40 kDa antigenic protein, 2-DE was carried out to analyze the liver cytosolic proteins. The separated proteins were stained with CBB (Fig. 3A). A relatively small number of protein spots were detectable due to the narrow pH range (pH 6-9) employed for the IEF. Western blotting using serum 1 of patient A as the first antibody indicated the presence of three 40 kDa antigenic proteins, whose estimated pIs were 7.6, 7.4 and 7.2 (Fig. 3B). The other immunostained spots seen on the membrane were assumed to be due to non-specific or non-immunological reactions with the serum. Spots of 55 kDa and 49 kDa were also observed on the immunostained membrane with the sera from the healthy subjects (data not shown).

C-3. Confirmation of aldolase B by Western blotting

To confirm that the 40 kDa protein was aldolase B, the following experiments were performed. To examine the possibility that the serum of patient A contained an antibody to aldolase B, purified human aldolase B protein (40 kDa) of 3 μg, 1 μg, and 0.25 μg were applied to SDS-PAGE and subsequently transferred onto PVDF membrane and immunostained with the serum of patient A as the first antibody. The serum 1 of patient A recognized the aldolase B in a concentration-dependent manner (Fig. 4A). The other serum of

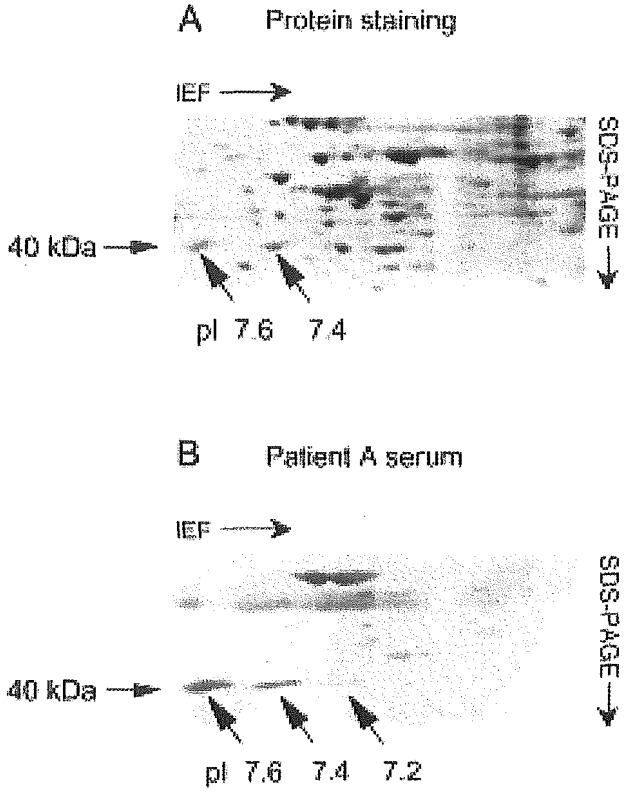

Fig. 3. Two-dimensional electrophoresis and Western blotting using serum from patient A. Human liver cytosol (230 μg) was separated by 2-DE. (A) The gel was stained with CBB. (B) Western blotting was performed using the serum 1 from patient A (1:100) as the first antibody as described in Experimental Procedures. The approximate pIs on the 2-D gel are indicated.

patient A (serum 2) and that of patient B (serum 1) showed the same immunological recognition of the purified aldolase B protein as that of serum 1 from patient A (data not shown).

To examine the possibility that the spots recognized by Western blotting with the serum of patient A were derived from aldolase B, anti-human aldolase B antibody was applied instead of the serum of patient A to the liver cytosol prepared by 2-DE. Three distinct spots with approximate pIs of 7.6 and 7.4, and 7.2, appeared (Fig. 4B). No other major spot was recognized by the anti-human aldolase B antibody. These spots showed the same pIs and molecular weights as the spots demonstrated by the serum of patient A.

To evaluate the titer of anti-aldolase B autoantibody in the sera of patients A and B the PVDF membrane was employed to transfer the purified human aldolase B protein (1 μg). Immuoblot experiments were performed with the diluted sera from the patients. The sera 1 and 2 (23.5-week after stopping troglitazone therapy) of patient A showed titers of 1:3,200 and 1:6,400, respectively (data not shown). The titer of serum 1 (16-week after stopping troglitazone therapy) of patient B was 1:1,600 (data not shown).

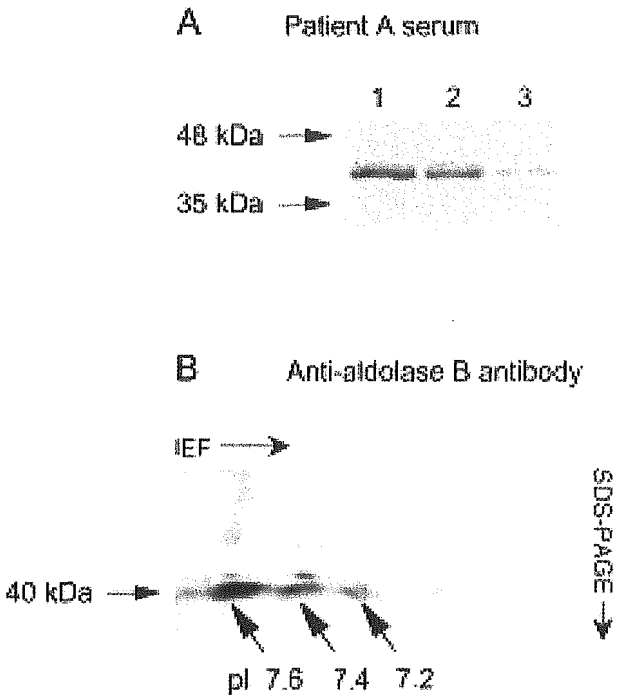

Fig. 4. Western blotting to confirm the occurrence of anti-aldolase B antibody. (A) Purified aldolase B protein at 3 μg (lane 1), 1 μg (lane 2), and 0.25 μg (lane 3) was subjected to SDS-PAGE. Western blotting was performed using serum 1of patient A (1:100) as the first antibody. (B) Human liver cytosol (230 μg) was separated by 2-DE. Western blotting was performed using anti-human aldolase B antibody as the first antibody as described in Experimental Procedures.

C-4 Determination of anti-aldolase B autoantibodies in sera of patients with liver diseases

The sera of the patients with liver diseases were examined by ELISA to determine whether the anti-aldolase B antibody appeared in association with the liver diseases. Fig. 5A shows the individual anti-aldolase B antibody in sera from the healthy subjects (n = 80) and chronic hepatitis (n = 40) and liver cirrhosis (n = 40) patients. Anti-aldolase B autoantibodies in the chronic hepatitis and liver cirrhosis patients were significantly higher than those in the healthy subjects ($p < 0.05$ and $p < 0.001$, respectively). Using serum 2 of patient A as a reference, all sera having an OD_{450} higher than that of patient A as well as negative sera from healthy subjects and those with chronic hepatitis, N10 and 39, respectively, were subjected to Western blotting. Except the negative sera, all sera which showed positive reactivity by ELISA also showed a single positive band of aldolase B by Western blotting (Fig. 5B).

D. 考察

Idiosyncratic adverse reactions are difficult to study because of their rare occurrence, dose-independence and lack of reproducibility in animal models. There are two classes of reactions, metabolic and immunologic idiosyncrasy. The later class is thought to be responsible for most adverse drug reactions (Ju and Uetrecht, 2002; Pirmohamed et al., 1998; Pohl et al., 1988).

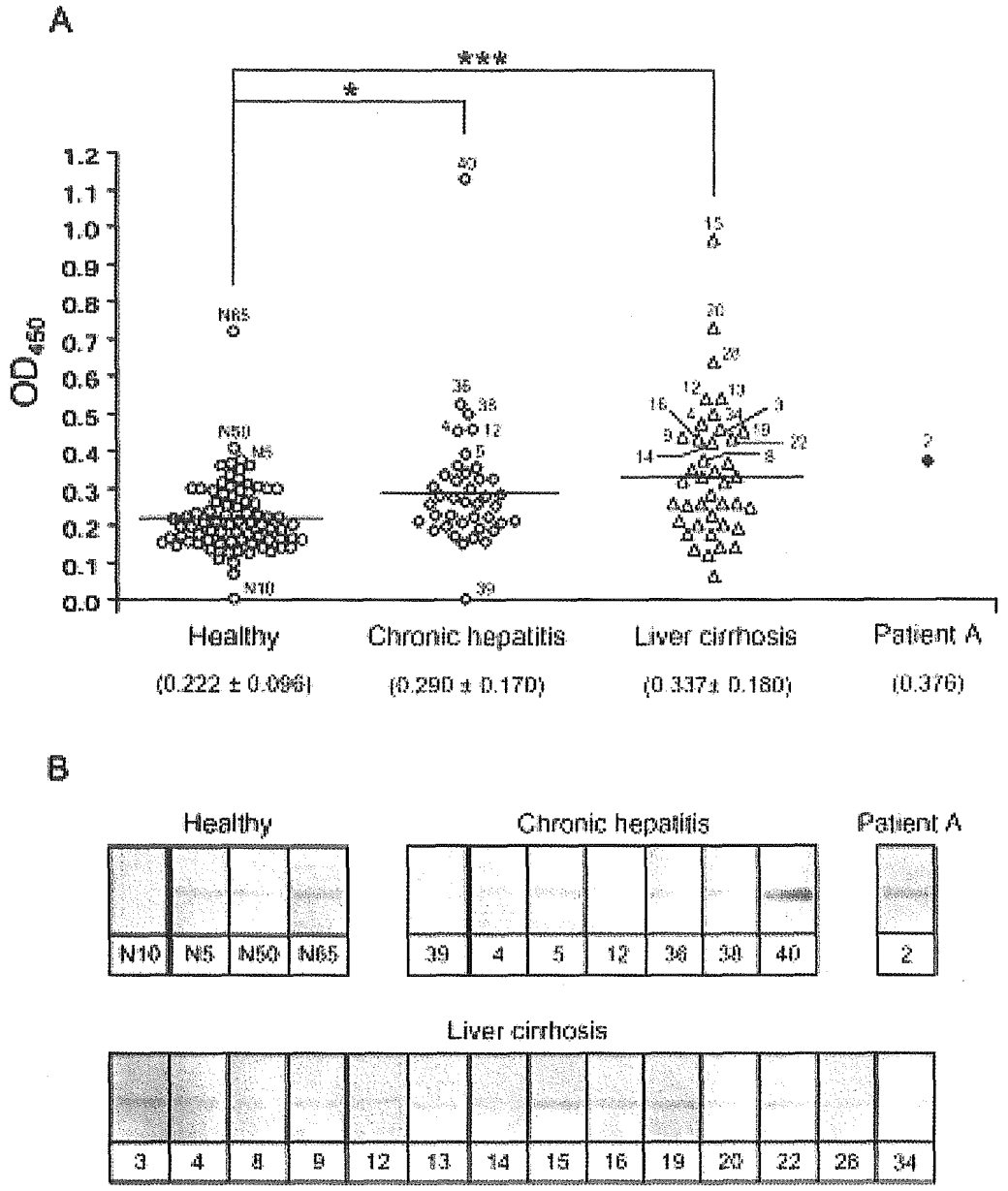

Fig. 5. Identification of autoantibodies reacting with aldolase B in sera from patients with liver diseases. (A) Sera of healthy subjects (n = 80), chronic hepatitis (n = 40) and liver cirrhosis (n = 40) as well as patient A (positive control) were investigated for anti-aldolase B autoantibodies by ELISA as described in Experimental Procedures. The horizontal bar represents the average value of each group. The means ± SD are indicated in parentheses. * $p < 0.05$, *** $p < 0.001$ compared with the control. (B) Western blotting of anti-aldolase B autoantibody positive sera. The sera which showed an OD_{450} higher than that of patient A, as well as negative sera from a healthy subject and chronic hepatitis, N10 and 39, respectively, were subjected to Western blotting. The numbers correspond to the subjects indicated in (A).

In the present study, troglitazone-induced liver dysfunction was investigated in terms of the autoantibody formation. Two patients (A and B) who received troglitazone 400 mg/day showed increases of serum ALT (Figs. 1A and B), which indicated an abnormality of liver function. The sera of both patients reacted prominently against troglitazone-induced neoantigens in the cytosol subfraction (Fig. 2) which were identified as aldolase B (EC 4.1.2.13). Schapira et al. (1977) reported the microheterogeneity of human liver aldolase B, which shows 5 bands by IEF according to tetramers formed by two types of aldolase B chains, β and β'. The β' chain is the result of a post-transcriptional modification by the deamidation of β chain. In this study, the spots of the 2-DE at pI 7.6, 7.4, and 7.2 might be consistent with the β^4, $\beta^3\beta'$ and $\beta^2\beta'^2$ chains, respectively (Fig. 3). In mammal, 3 isoenzymes of aldolase (A, B and C) have been classified (Penhoet et al., 1966; Rutter, 1964). They are distinguished by their different activity towards the two substrates of fructose 1,6-diphospate and fructose 1-phosphate (Rutter et al., 1961). Aldolase B is predominantly localized in liver and kidney, while aldolase A and C are mainly localized in muscle and in brain, respectively (Penhoet et al., 1966; Rutter et al., 1964).

The mechanism by which autoantibodies are formed is still incompletely understood. Hepatic microsomal cytochrome P450 (CYP) isoforms such as CYP1A2, CYP2A6, CYP2B6, and CYP2E1 have been extensively studied as autoantigens in many liver diseases including viral, autoimmune and drug-induced hepatitis (Park et al., 1998; Manns et al., 1997). Although how these CYPs become autoimmune targets is still unknown, the findings of these studies reflected the major metabolic role of CYPs in the liver. However, in this finding no prominent band of CYPs could be detected in the microsomal subfraction of troglitazone-treated patient sera that was distinct from those in the sera of healthy subjects.

There is evidence that the metabolic activation of some drugs can result in the formation of chemically reactive metabolites that bind to macromolecules in the cell, unless adequately detoxified, leading to several pathological effects including hypersensitivities (Park et al., 1998). Thus, an imbalance between metabolic activation and detoxification in some individuals may lead to idiosyncratic adverse drug reactions. Troglitazone is metabolized by many enzymes in the liver including CYP3A4 and CYP2C8 which generated a quinone-type metabolite, troglitazone quinone (Tettey et al., 2001; Yamazaki et al., 1999). Based on the general involvement of quinones in cytotoxicity, troglitazone quinone has been proposed to have an association with troglitazone-induced hepatotoxicity (Neuschwander-Tetri et al., 1998). Aldolase B, which is an enzyme predominantly localized in the liver (Penhoet et al., 1966), may be one of the target proteins that interact with reactive species generated by troglitazone and trigger the immune response. Based on the detoxification enzyme in Phase II, which is responsible for eliminating toxic metabolites before the generation of neoantigens, a recent report has shown that the double null genotype of

GSTM1 and *GSTT1* is associated with an abnormal elevation of liver enzymes caused by troglitazone treatment (Watanabe et al., 2003). This may suggest that idiosyncratic drug reactions might be the consequence of a complex genetic basis involving numerous processes.

In order to investigate whether the formation of anti-aldolase B was specific for troglitazone-induced hepatotoxicity, the sera of patients with liver diseases, chronic hepatitis and liver cirrhosis, were also examined. According to the etiology, chronic hepatitis results from several causes such as autoimmune reactions, viral hepatitis B, C and D, drugs, Wilson's disease, α-1-antitrypsin disease as well as unknown causes, whereas liver cirrhosis is considered to be the advanced stage of hepatitis (Batts and Ludwig, 1995; Jevon, 2001). As shown in Fig. 5, the mean reactivity of autoantibodies to aldolase B in the patient sera with liver diseases was also significantly higher than that in the healthy subjects. The average ALT level in chronic hepatitis and liver cirrhosis was 39.50 ± 15.72 IU/L (n = 40) and 52.35 ± 28.70 IU/L (n = 40), respectively (data not shown) which correlated with the titer of autoantibodies to aldolase B. Brown et al. (1987) reported that the presence of autoantibodies to aldolase in patients with hepatitis A, B, non-A/non-B, and those who were hepatitis B surface antigen positive or had autoimmune hepatitis was higher than in healthy subjects. In that study, rabbit muscle aldolase, which is referred to as aldolase A (Penhoet et al., 1966), was used. According to the high homology between aldolase A and B, only the amino acid sequence 357-362 is recognized as a non-conserved residue that enables the classification of the mammalian aldolase (Rutter, 1964). The amino acid sequence 315-329 of aldolase B was identified in this study. Therefore, these findings were remarkably consistent with those of a previous report in the hepatic virus-infected patients (Brown et al., 1987). Herein, the data of autoantibodies to aldolase B detected in troglitazone-induced hepatitis as well as those in liver cirrhosis also demonstrated.

At present, the exact mechanism involved in the formation of autoantibodies is unknown. There is a possibility that, after cellular injury, intracellular antigens might be seen as non-self by the immune system and result in the stimulation of an immune response toward cryptic epitopes on the antigens (Lanzavecchia, 1995). Accumulating evidence suggests that liver damage may cause the production of autoantibodies to aldolase B and that the severity of hepatitis may be estimated by the antibody titer.

E. 結論

Aldolase B was identified as an autoimmune antigen in patients with troglitazone-induced liver dysfunction. This finding is the first evidence that troglitazone-induced hepatitis may have an immunological basis. In addition, the elevation of anti-aldolase B autoantibody in sera is likely a common phenomenon associated with hepatitis. This autoantibody may play an essential role in aggravating the liver dysfunction. Further studies will be needed to clarify the mechanisms of idiosyncratic adverse drug reactions.

F. 研究発表

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G. 知的財産権の出願・登録

なし

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分担研究報告書

肝毒性化合物５種類の投与における網羅的遺伝子発現変動解析

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研究要旨

遺伝子発現を検討する手段として DNA マイクロアレイは、トキシコゲノミクスの研究分野で主たる研究方法になってきており、開発候補化合物の副作用予測や化合物のリスクアセスメントなどに活用されようとしている。本研究では、肝障害性というフェノタイプと網羅的遺伝子発現プロファイルの関係について、DNA マイクロアレイを用いて検討した。６週齢雄性 SD ラットを用い、アセトアミノフェン、ブロモベンゼン、四塩化炭素、ジメチルニトロソアミンとチオアセタミドをそれぞれ単回投与した。血清学的な生化学値は、投与後の肝障害性を示す時間を知るために測定した。肝 mRNA は、1097 の薬物反応性遺伝子を網羅した DNA マイクロアレイを用いて検討した。このアレイにはチトクロム P450 を初めとする Phase I の酵素の遺伝子、Phase II, 核内受容体、シグナル伝達遺伝子、トランスポーターなどが搭載されている。個々の化合物はそれぞれ特徴的な遺伝子発現のプロファイルを示した。アセトアミノフェンは他の化合物とは異なるクラスターに分類された。遺伝子発現プロファイルと毒性を示す時間を考慮して、10 個の発現が誘導される遺伝子と、10 個の発現が抑制される遺伝子と共通して pick up することが出来た。これらは肝障害に共通するマーカー遺伝子と考えられた。Quality-Threshold（QT）クラスター解析により、個々の化合物による特徴的な遺伝子の発現変化を見出すことができた。興味深いことに、QT クラスター解析における平均遺伝子変化は、生化学値と一致した変動を示した。さらに、この変動は、肝障害性の大小に係わらず同じパターンを示した。こうした解析を考慮し、本研究では、17 の遺伝子を肝障害性に関係する遺伝子として示すことができた。すなわちこれらの遺伝子の発現プロファイルから、肝毒性の程度に関係なく毒性発現時間を推測することが可能になった。本研究における解析手法は医薬品開発における肝障害性を予測する有用な手段となりうるものである。

A. 研究目的

薬物性肝障害の発現予測は、医薬品の開発において非常に重要な項目の一つである。本章では、古くから典型的な肝毒性化合物と言われているアセトアミノフェン、四塩化炭素、ブロモベンゼン、チオアセタミド、ジメチルニトロソアミンの５つの化合物について、それぞれの化合物を投与したラットから調製した total RNA を用い、DNA マイクロアレイにより、網羅的遺伝子発現変動解析を行った。また、得られたデータに対して様々な解析方法を適用し、肝毒性化合物処置状態における共通な遺伝子変動について検討を行った。

B. 研究方法

B-1. 実験材料および試薬

APAP、BB、CT、DMN、TA および臓器固定用中性緩衝ホルマリン液は和光純薬工業 (Osaka, Japan) より購入した。ISOGEN は日本ジーン (Tokyo, Japan) より購入した。吸光光度計は日立の分光光度計 U-2001 (Tokyo, Japan) を使用した。その他の試薬類は市販品の特級、生化学用または高速液体クロマトグラフィー用のものを用いた。

B-2. ラットへの化合物の投与

Sprague-Dawley 系ラット (雄性、6 週齢 200～220 g; 日本 SLC, Shizuoka, Japan) に、それぞれの化合物を単回腹腔内投与 (2 mL/kg 体重) した。投与時間は各肝毒性化合物において 6 時間、12 時間、24 時間および 48 時間、各対照群において 48 時間とした。各群 4 匹のラットを使用した。投与に使用した化合物の溶媒と投与量について、アセトアミノフェン (以下 A または APAP) は 500 mg/kg をコーン油に懸濁させ、ブロモベンゼン (B または BB) と四塩化炭素 (C または CT) はそれぞれ 2.5 mmol/kg および 1 mL/kg をコーン油に溶かし、ジメチルニトロソアミン (D または DMN) とチオアセタミド (T または TA) はそれぞれ 20 mg/kg および 400 mg/kg を生理食塩水に溶解して投与した。対照群としてコーン油 (NT C) と生理食塩水 (NT S) を使用した。

B-3. Total RNA の調製

ISOGEN のマニュアルに修正を加え、以下の方法で肝臓から total RNA を調製した。約 0.1 g の肝臓に、ISOGEN 1 mL を加えてホモジナイズ後、サンプルチューブに分注し、クロロホルム 0.2 mL を加え、激しく 15 秒間攪拌し、室温で 3 分間放置した後、10,000 g (15,000 rpm)、4°C で 15 分間遠心分離した。上清 400 μL を別のチューブに採取し、0.5 mL のイソプロパノールを加えて室温にて 8 分間放置した後、10,000 g (15,000 rpm)、4°C で 10 分間遠心分離した。沈殿を 70%エタノールで洗浄した。この沈殿を乾燥させた後、DEPC 処理精製水に溶解させ、260 nm における吸光度を測定することにより定量した。