Production and Characterization of a Monoclonal Antibody to Capture Proteins Tagged with Lithocholic Acid

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Reactive metabolic-modified proteins have been proposed to play an important role in the mechanism(s) of the hepatotoxicity and colon cancer of lithocholic acid (LCA). To identify cellular proteins chemically modified with LCA, we have generated a monoclonal antibody that recognizes the 3α-hydroxy-5β-steroid moiety of LCA. The spleen cells from a BALB/c mouse, which was immunized with an immunogen in which the side chain of LCA was coupled to bovine serum albumin (BSA) via a succinic acid spacer, was fused with SP2/0 myeloma cells to generate antibody-secreting hybridoma clones. The resulting monoclonal antibody (γ2b, 8) was specific to LCA-N5=BOC-lysine as well as the amidated and nonamided forms of LCA. The immunoblot enabled the detection of LCA residues anchored on BSA and lysozyme. The antibody will be useful for monitoring the generation, localization, and capture of proteins tagged with LCA, which may be the cause of LCA-induced toxicity.

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Introduction

In 1977, lithocholic acid (LCA), a hydrophobic bile acid, was firstly isolated as a tissue-bound form from pathological specimens of human livers by Nair et al.,1,2 and its concentration was reported to be elevated in the livers of rat treated with a carcinogen, methyloxazobenzethanol.3 Furthermore, the tissue-bound LCA was detected in normal and neoplastic human mammary tissues and in the neoplasms of the uterus, kidney, lung and the colon.4,5 In 1984, Yanagisawa et al. reported that they could not substantiate the presence of the tissue-bound LCA in either cirrhotic or non cirrhotic liver tissues.6 However, taking into account that LCA and deoxycholic acid (DCA) act as promoters of cancer,7-10 and that reactive metabolic-modified proteins have been proposed to play an important roles in the mechanism(s) of the hepatotoxicity and colon cancer of theses bile acids,11-19 it may be a reasonable hypothesis that the formation of tissue-bound LCA is related to possible carcinoenesis of this bile acid. Therefore, the identification of the cellular proteins chemically modified with LCA is essential as a step in elucidating the mechanism of LCA-induced cytoxicity.

To date, there has been a rapid increase in the use of mass spectrometry (MS) in biological science. Biological MS is based on the micro-scale immunoaffinity capture of the target antigen, followed by mass-specific identification and quantification with greater selectivity, accuracy and efficiency. A major obstacle for the identification of native protein complexes by MS is the isolation of sufficient amounts of purified materials from cells. The traditional approach for the isolation of protein complexes employs antibodies that are directed against a protein of interest, the “bait”, to enable the isolation of interaction of partners, the “prey”. This method has been successfully used for many years due to its simplicity and fast implementation. One of the major advantages of this approach is its ability to capture proteins in their native environment and concentration, thus reducing the possibility of experimental artifacts. Both monoclonal and polyclonal antibodies have been used with success. In general, monoclonal antibodies are preferred for immunoprecipitation experiments, since they generally have better specificity than polyclonal antibodies.20 However, polyclonal antibodies are also widely used, since they are more easily produced. From these points of views, we have generated rabbit polyclonal antibodies with high specificity for the 3α-hydroxy-5β-steroid moiety of LCA for identifying LCA-bound proteins in the liver of bile duct-ligated rat.21 It is difficult to obtain conventional polyclonal antibodies in large amounts and with identical properties, because the proportion of antibody subpopulations that are components of a polyclonal antibody varies due to individual differences between animals. Monoclonal antibodies produced by B cell hybridoma technology have the advantage of an almost unlimited supply with identical and uniform specificity. Additionally, many successful results have recently been published dealing with the generation of genetically engineered
antibodies or antibody-like molecules,\textsuperscript{22-24} some of which have been used for the large-scale production of immunosorbents.\textsuperscript{25,26} The antibody-secreting hybridomas are also useful for cloning variable region genes of an antibody, which can be a starting material for preparing such an engineered antibody. We therefore generated a monoclonal antibody, which specifically recognizes the 3α-hydroxy-5β-steroidal moiety of LCA. The resulting antibody was characterized in detail by a competitive enzyme-linked immunosorbent assay (ELISA), and we consequently succeeded to produce an antibody that is applicable for detecting LCA residues anchored on proteins.

**Experimental**

**Materials**

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and ursodeoxycholic acid (UDCA) was kindly donated by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). Glycine and taurine conjugated LCA were stock samples in our laboratory. 3α-Hydroxy-24-hemisuccinoyloxy-5β-cholane (LCA-HS), N-α-(t-butyxycarbonyl)-L-lysine-ε-L-lysine-N\textsuperscript{ε}-BOC-lysine (LCA-N\textsuperscript{ε}-BOC-lysine), LCA-HS-bovine serum albumin (BSA) conjugate as an immunogen, alkaline phosphatase (ALP)-labeled N-(3α-hydroxy-5β-cholane-24-onyl)-6-aminohexanoic acid (LCA-HA) as a probe for ELISA, and LCA-lysosome and LCA-HA-BSA adducts as model modified proteins were previously prepared in our laboratory.\textsuperscript{21} Freund’s complete adjuvant (FCA) and incomplete adjuvant (FIA) were purchased from Rockland (Gilbertsville, PA, USA). RPMI 1640 medium and fetal bovine serum were obtained from GIBCO (Grand Island, NY, USA). Polyethylene glycol (PEG) 4000 and the ORIGEN Hybridoma Cloning Factor (HCF) were purchased from Merck Co. (Darmstadt, Germany) and IGEN Inc. (Rockville, MD, USA), respectively. Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG antibody was purchased from BIOSOURCE (Camarillo, CA, USA). AffiniPure rabbit anti-mouse IgG (H+L) antibody (the second antibody in the following ELISA) was obtained from ZYMED Laboratories (South San Francisco, CA, USA). HyTrapTM protein G HP (1 ml) column was purchased from GE Healthcare (Uppsala, Sweden). EIA/RIA plates were purchased from Costar (Cambridge, MA, USA). Zip-Tip C\textsubscript{18} was purchased from Millipore (Billerica, MA, USA). Coomassie brilliant blue R-250 was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Nitrocellulose membrane (0.45 μm) was obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All other reagents were of analytical reagent grade and water from a Millipore Ultra Water Purification System (Milli-Q Synthesis A10) was used to prepare the mobile phase and the aqueous solutions described below.

**Medium**

Medium A, RPMI 1640 medium supplemented with 10 mM HEPES buffer (pH 7.3) and penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), kanamycin sulfate (60 μg/ml), glucose (0.2%, w/v), pyruvic acid (1 mM) and glutamic acid (2 mM); medium B, medium A supplemented with fetal bovine serum (10%, v/v); HAT medium, medium A supplemented with hypoxanthine (1 mM), aminopterin (16 μM) and thymidine (16 μM); HT medium, the same as a HAT medium, but does not contain aminopterin.

**Buffers**

The buffers used for this work were as follows: buffer A, 50 mM Na\textsubscript{2}HPO\textsubscript{4}-NaH\textsubscript{2}PO\textsubscript{4} (pH 7.3); buffer B, buffer A containing gelatin (0.1%, w/v), NaCl (0.9%, w/v) and Na\textsubscript{2}S (0.1%, w/v); buffer C, buffer A containing NaCl (0.9%, w/v); buffer D, buffer C containing Tween 20 (0.05%, v/v).

**Cell**

Sp2/0 myeloma cells were kindly donated by Prof. Kunihiko Ito, School of Pharmaceutical Sciences, University of Shizuoka.

**Immunization, cell fusion and cloning**

Five female BALB/c mice (6 - 8 weeks of age) purchased from Japan SLC (Hamamatsu, Japan) were immunized with the LCA-HS-BSA conjugate at approx. 10-day intervals. The conjugate (50 μg) was injected with the emulsion (250 μl) of FIA and sterile saline (1:1, v/v) on day zero. On days 10 and 20, mice were boosted with the immunogen mixed with FCA. Five days after the third booster injection, blood was collected from the retrobulbar plexus, and the binding ability of the serum antibodies to the corresponding bile acid was determined by the ELISA procedure (see below). On day 27, a mouse selected as a spleen donor was injected intraperitoneally with the conjugate (20 μg) dissolved in a sterile saline (200 μl), and the spleen cells were prepared three days later. The immune spleen cells (approx. 3.38 × 10\textsuperscript{6} cells) and myeloma cells (approx. 2.03 × 10\textsuperscript{7} cells) were fused with PEG 4000 (50%) in medium A, and the fused cells were suspended in the HAT medium. After washing with medium A, the fused cells were suspended in medium B. This cell suspension was seeded in 96-well cluster dishes (100 μl/well) and cultured overnight in a humidified atmosphere of CO\textsubscript{2}/air (5:95) at 37°C; then, the HAT medium (100 μl) was added to each well. Three and 6 days after the fusion, a portion of the culture supernatant (approx. 100 μl) was replaced by a fresh HAT medium. After an additional 2 - 3 days, a small portion of hybridoma supernatants was taken from each well and submitted to screening by ELISA (described below). The antibody secreting hybridomas were expanded in the HT medium and then cloned twice by limiting dilution in medium B supplemented with HCF (10%). Cloned hybridomas were grown in 25 cm\textsuperscript{2} culture flasks in medium B (5 ml) until confluence was reached. The cell suspensions were centrifuged (1000 × g for 15 min) at 4°C, and monoclonal antibodies contained in the resulting supernatant were used for isotyping, or ELISA for investigating their binding characteristics.

**Screening antibody-secreting hybridomas**

The 96-well EIA/RIA plates were coated with 100 μl/well of the second antibody diluted at 1:1000 with buffer A (100 μl) by overnight incubation at 4°C. After washing three times with buffer C, the wells were blocked with 5% skimmed milk in buffer C (300 μl) at 37°C for 1 h. The wells were washed three times with buffer C, to which diluted hybridoma supernatants with buffer B (100 μl) were then added. After incubation at 37°C for 1 h with continuous shaking, the solutions were aspirated off, and the wells were washed three times with buffer D. The ALP-labeled LCA-HA (200 ng) dissolved in buffer B (100 μl) was then added and incubated as described above. After washing in the same manner, a solution of substrate (100 μl) containing 1 mM p-nitrophenyl phosphate disodium salt and Mg\textsubscript{2+} (0.01%, w/v) in 50 mM carbonate buffer (pH 10) was added and incubated at 37°C for 1 h. This enzyme reaction was terminated by the addition of 0.1 M NaOH (100 μl), and the absorbance at 415 nm was measured using an MPR A4i microplate reader (TOSOH, Tokyo).
Characterization of monoclonal antibody

For determining the optimal dilution of the hybridoma supernatant, a serially diluted supernatant diluted with buffer B (100 μl) was added to the wells in the second antibody coated microtitrte plates, which were then incubated at 37°C for 1 h. After washing the wells as described above, the enzyme-labeled antigen (200 ng) dissolved in buffer B (100 μl) was added and incubated at room temperature for 1 h. The wells were washed again and the bound enzyme activity was measured as mentioned above.

A dose response curve was constructed as follows: a solution of enzyme-labeled antigen (200 ng) dissolved in buffer B (100 μl) and a series of LCA-N⁴-BOC-lysine (1 - 5000 ng) in buffer B (100 μl) was added to the wells, which were vortex-mixed for 30 s. In this assay a blank containing buffer B was included. The hybridoma supernatant diluted with buffer B (100 μl) was added to the mixture, vortexed, and incubated at 37°C for 1 h. The wells were then washed, and the bound enzyme activity was measured as described above (enzyme reaction = 1 h). The ratios of the bound enzyme activities to those at 0 dose of LCA-N⁴-BOC-lysine were represented as B/B₀.

In a cross-reaction study, a competitive antigen-antibody reaction was performed by replacing LCA-N⁴-BOC-lysine with various related compounds. The cross-reactivity of each analog was expressed as the relative amount (ng) required to reduce the initial bound enzyme activity by half, where the mass of LCA-N⁴-BOC-lysine was arbitrarily taken as 100%.²²

Preparation of IgG fraction

Pristane (0.5 ml) was injected intraperitoneally into male BALB/c mice (five mice; seven weeks of age). After 13 days, these mice were injected intraperitoneally with hybridomas (1.1 x 10⁷ cells) in a sterilized saline. After 10 days, ascites fluids were drained and centrifuged (3000 rpm for 5 min). To the obtained supernatant was added 33% saturated (NH₄)₂SO₄, and the whole mixture was stirred at 4°C for 1 h. After centrifugation (10000 rpm for 30 min) at 4°C, the precipitate dissolved in buffer C (1.6 ml) was submitted to precipitation by 33% saturated (NH₄)₂SO₄, followed by centrifugation in the manner described above. The resulting supernatant, after having been kept dialyzed against cold running PBS for 48 h at 4°C, was applied to a HiTrap Protein G HP column according to the manufacturer’s instruction and the obtained IgG fraction was used for detecting the LCA residues on the protein in following experiments.

LC/ESI-MS analysis of LCA-lysozyme adduct

LCA-lysozyme adduct (220 μg) was reduced with dithiothreitol (2.2 μg) in 182 μl of 0.1 M Tris-HCl buffer (pH 8.1) containing 4 M urea and 1 mM EDTA for 15 min at 50°C, and carboxymethylated with sodium iodoacetate (2.6 μg) at room temperature in the dark for 1 h under flushing with a stream of N₂. A solution of trypsin (2 μg) in 50 mM NH₄HCO₃ (50 μl) was added to the reaction mixture, and the resulting mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of acetic acid (20 μl). After desalting the mixture with Zip-Tip C₁₈, 10 μl of the mixture was submitted to liquid chromatography (LC)/electrospray ionization (ESI)-linear ion-trap MS analysis using a Finnigan LTQ ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ESI source and coupled to a Paradigm MS4 pump (Michrom Bioresources, Inc., Auburn, CA, USA) and an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). The ionization conditions for verifying the structures of the peptides were as follows: ion source voltage, 2.4 kV; capillary temperature, 200°C; capillary voltage, 31 V; tube lens offset voltage, 205 V. For tandem MS (MS²) analysis, helium gas was used as the collision gas and the normalized collision energy was set at 35%. The LC separations were conducted on a reversed-phase (RP) micro column, Magic C18 (5 μm, 50 x 0.2 mm i.d.) from CTC Analytics by a linear gradient elution: 80% solvent A (H₂O/acetonitrile/formic acid; 98:2:0.1, v/v/v) to 20% solvent A against solvent B (H₂O/acetonitrile/formic acid; 10:90:0.1, v/v/v) over 20 min at a flow rate of 3 μl/min. The trap cartridge was from CTC Analytics.

SDS-PAGE and immunoblotting analysis of LCA-lysozyme and LCA-HA-BSA adducts

SDS-PAGE and immunoblotting were performed with NA-1013 mini gel slab electrophoresis equipment and an NA-1510B double cassette mini transfer instrument (Nihon Eido, Tokyo, Japan), respectively.

For SDS-PAGE, 5 μg of protein was diluted to 1:1 with 125 mM Tris-HCl buffer (pH 6.8) containing 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.02% (w/v) pyronin Y, heated at 100°C for 5 min, and resolved on 15% acrylamide gels. Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue. Proteins resolved by polyacrylamide gels were transblotted to a 0.45-μm nitrocellulose membrane at 40 V for 1 h in 25 mM Tris-192 mM glycine buffer (pH 8.3). After transferring, the blots were blocked by shaking overnight at 4°C in buffer C containing 5% skim milk. The blotted membranes were incubated with 1:1000 diluted anti-LCA monoclonal antibody for 3 h at room temperature with shaking. The blots were rinsed for three 5-min washes in buffer D and two 5-min washes in buffer C. The immunoblots were incubated for 1 h with 1:2000 diluted rabbit anti-mouse IgG (H + L) diluted with buffer C. After washing as mentioned above, the immunoblots were incubated for 1 h with 1:2000 diluted HRP labeled goat anti-rabbit IgG. The nitrocellulose membranes were again washed extensively as described above and the immunoreactive proteins were visualized with 0.05% (w/v) 3,3’-diaminobenzidine containing 0.03% (v/v) H₂O₂ in buffer C for 5 min.

Results and Discussion

Cell fusion and monoclonal antibody production

Among the common bile acids in human body fluids, only LCA has been isolated so far as a tissue-bound form. It is expected that LCA would be covalently linked to the lysine residues in a protein via its carboxyl group,²¹,²² thus exposing a partial structural containing the A and B rings of the steroid molecule out from the tissue matrix. Based on this speculation, we previously designed two immunogens in which the terminal carbon of the side chain of LCA was coupled to BSA via a 6-aminoaxanoic acid and a succinic acid spacer, resulting in the generation of specific polyclonal antibodies that recognize the steroidal moiety of LCA anchored on proteins (Fig. 1 contains the structures of LCA-HA-BSA and LCA-HS-BSA conjugates).²¹ Since the titer of the antiserum obtained from the LCA-HS-BSA conjugate was higher than that of the LCA-HA-BSA conjugate, BALB/c mice were immunized with the LCA-HS-BSA conjugate. The hapten-carrier conjugate was designed for obtaining an antibody, which specifically recognizes the target LCA residues anchored on an endogenous protein with such an orientation as mentioned above. The titer of the serum antibody against the corresponding bile acid was checked by a competitive ELISA system using ALP labeled LCA-HA as a
Fig. 1 Structures of bile acid derivatives in this study. Five major endogenous bile acids and amidated lithocholic acid derivatives: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; GLCA, glycodeoxycholic acid; TLCA, taurolithocholic acid; LCA-HS, LCA-hemisuccinate derivative; LCA-\(N^b\)-BOC-Lys, \(N\)-\(\alpha\)-(2-butoxy-carbonyl)-l-lysine-\(\varepsilon\)-LCA; LCA-HS-BSA, bovine serum albumin conjugate of LCA-HS; LCA-HA-BSA, bovine serum albumin conjugate of LCA-HA; LCA-lysozyme, LCA-lysozyme adduct.

Fig. 2 Comparison of the immune response between BALB/c mice after repeated immunization with the LCA-HS-BSA conjugate. Serum was collected after the third immunization, and 10-fold diluted serum was submitted to the ELISA, whose procedure is described in Experimental.

probe. LCA-HA was used for the labeling of ALP, because the spacer of this compound consists of six carbons identical to the lysine residue; the antibody recognizes the bridge portion of haptenic derivatives, and thus this compound was required for enzyme labeling. Significantly increased titer was observed for one of the mice (Fig. 2), and then this mouse was given a final immunization for the cell-fusion experiment.

It has been reported that mice immunized with the immunogen, in which the carboxyl group at C-24 was directly coupled to BSA, exhibited a poor response. They speculated that the highly hydrophobic nature of LCA might have prevented the contact of the hapten portion in the LCA-BSA conjugate with the responsible B-cell receptor. However, our study showed a high response toward one of the mice. This may be attributed to individual differences between mice and/or immunogenic differences toward the B-cell receptor between immunogens.

Characterization of monoclonal antibody
The heavy- and light-chain isotypes of the resulting monoclonal antibody were determined to be \(\gamma_2b\) and \(\kappa\); respectively. The binding characteristics of the antibody were investigated by the competitive ELISA system. The optimum dilution of the antibody (culture supernatant) in this ELISA was arbitrarily determined to be 1:1000, at which the enzyme activity corresponding to a 0.5 absorbance unit (1-h incubation) was bound. The absorption of nonspecific binding was below 1% of the \(B_0\) value in this ELISA system. For the purpose of capturing the LCA residues on tissue proteins, the antibody should be capable of binding to LCA-\(N^b\)-BOC-lysine. Figure 3 demonstrates that the monoclonal antibody reacts with the LCA-\(N^b\)-BOC-lysine in a dose-response manner, and that 1 – 5000 ng of LCA-\(N^b\)-BOC-lysine can be determined by the ELISA system. The midpoint, which is the analyte dose required to inhibit the bound enzymic activity, was 231 ng.

The specificity of this monoclonal antibody was examined by a cross-reaction study with eight related compounds, taking the reactivity of LCA-\(N^b\)-BOC-lysine as 100% (Table 1). The bile acids having an additional hydroxyl group on the steroidal moiety (UDCA, CDCA, and DCA) were all discriminated (<5.9%). CA possessing three hydroxyl groups at the C-3, C-7, and C-12 positions also exhibited only a negligible cross-reactivity (<0.1%). These results demonstrate that the monoclonal antibody is specific to the LCA structure, and well

![Graph showing typical dose-response curve for LCA-\(N^b\)-BOC-lysine in the competitive ELISA using the monoclonal antibody and the ALP-labeled LCA-HA.](image)

![Graph showing absorbance at 456 nm for different mouse samples.](image)

Table 1 Binding ability of the monoclonal antibody as determined by competitive ELISA

<table>
<thead>
<tr>
<th>Bile acid derivative</th>
<th>Cross-reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA-(N^b)-BOC-lysine</td>
<td>100</td>
</tr>
<tr>
<td>LCA</td>
<td>146.5</td>
</tr>
<tr>
<td>GLCA</td>
<td>109.1</td>
</tr>
<tr>
<td>TLCA</td>
<td>214.2</td>
</tr>
<tr>
<td>LCA-HS</td>
<td>444.3</td>
</tr>
<tr>
<td>UDCA</td>
<td>5.5</td>
</tr>
<tr>
<td>CDCA</td>
<td>2.1</td>
</tr>
<tr>
<td>DCA</td>
<td>5.9</td>
</tr>
<tr>
<td>CA</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

a. Calculated by the 50% displacement method.
Table 2 Calculated and observed ions of trypsin digests of reduced and alkylated LCA-lysozyme adduct

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time/min</th>
<th>Amino acid sequence From</th>
<th>Amino acid sequence To</th>
<th>Sequence</th>
<th>m/z [M+H]+</th>
<th>Parent ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.53</td>
<td>6</td>
<td>14</td>
<td>C'ELAAAM'K'R</td>
<td>533.77</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>2</td>
<td>3.54</td>
<td>15</td>
<td>21</td>
<td>HGDLNYR</td>
<td>437.71</td>
<td>[M+2H]2+</td>
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<tr>
<td>3</td>
<td>3.64</td>
<td>34</td>
<td>45</td>
<td>FESNFQATQR</td>
<td>714.82</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>4</td>
<td>4.35</td>
<td>115</td>
<td>125</td>
<td>C'KGTDVQAWIR</td>
<td>667.83</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>5</td>
<td>4.56</td>
<td>117</td>
<td>125</td>
<td>GTDVQAWIR</td>
<td>1045.54</td>
<td>[M+H]+</td>
</tr>
<tr>
<td>6</td>
<td>4.61</td>
<td>46</td>
<td>61</td>
<td>NTDGSTDGILQINSR</td>
<td>877.41</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>7</td>
<td>5.17</td>
<td>51</td>
<td>61</td>
<td>NTDGSTDGILQINSR</td>
<td>877.41</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>8</td>
<td>6.53</td>
<td>22</td>
<td>33</td>
<td>GYSLGNWVCAK</td>
<td>663.81</td>
<td>[M+2H]2+</td>
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<tr>
<td>9</td>
<td>9.29</td>
<td>74</td>
<td>96</td>
<td>NLCNIPCSALLSSDITASVN</td>
<td>1256.08</td>
<td>[M+2H]2+</td>
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<tr>
<td>10</td>
<td>13.41</td>
<td>6</td>
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<td>C'ELAAAM'K'R</td>
<td>713.92</td>
<td>[M+2H]2+</td>
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<td>11</td>
<td>15.02</td>
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<td>125</td>
<td>C'KGTDVQAWIR</td>
<td>847.98</td>
<td>[M+2H]2+</td>
</tr>
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c. Carboxymethylated. b. Oxidated. c. Binding site of LCA.

Recognizing the addition, substitution, and modification of the hydroxyl groups. The antibody showed a stronger reactivity to free LCA (146.5%), GLCA (109.1%), TLCA (214.2%), and haptenic derivative (444.3%) than LCA-N8-BOC-lysine, indicating that the lysine portion is hardly recognized by the monoclonal antibody. This binding property, however, does not hinder the application of this antibody to the immunochromatographic analysis of the covalently linked LCA residues, because the LCA molecule should be, if any, washed out from biological samples before the antibody is added to capture the LCA moiety.

Immunoblot detection of LCA-protein adducts

An immunoblotting technique has been used as a powerful tool to identify protein adducts by detecting the hapten moiety of protein adducts formed during the exposure of cellular proteins to electrophilic species (haptons). We therefore evaluated the utility of the antibody in the detection of LCA residues anchored on proteins by immunoblotting. For this purpose, LCA-lysozyme and LCA-HA-BSA adducts, which were prepared by the transacylation reaction of LCA-adenylate with lysozyme and by the activated ester method via LCA-HA p-nitrophenyl ester, were used as model modified proteins. The molar ratio of LCA-HA/BSA has been determined to be 28 by the titration of free amino groups on BSA using trinitrobenzensulfonic acid, thus exposing the steroidal moiety of LCA out from the adduct.

On the other hand, the binding sites of LCA in the LCA-lysozyme adduct have been determined to be Lys-1, Lys-13, Lys-97, and Lys-116 by matrix-assisted laser desorption ionization-time of flight mass spectrometry. However, the sequence analysis of the LCA modified peptides was not accomplished. We then tried to identify the binding site of the LCA by LC/ESI-linear ion-trap MS of peptide mixtures obtained by proteolytic digestion with trypsin after reduction with dithiothreitol and alklylation with iodoacetic acid of the LCA-lysozyme adduct. A total ion chromatogram in scanning product ions showed several protonated ions, [M+H]1+ and [M+2H]2+ of the peptide fragments. As listed in Table 2, the ions at m/z 534.11, 438.08, 715.12, 668.14, 1045.54, 877.72, 877.70, 664.23, and 1256.57 were peptides corresponding to residues 6 - 14, 15 - 21, 34 - 45, 115 - 125, 117 - 125, 46 - 61, 22 - 33, and 74 - 96. The ions at m/z 713.09 and 847.22 were shifted by one molecule of LCA from the unmodified peptides corresponding to residues 6 - 14 and 115 - 125, suggesting that the site of covalent binding was the K residue. Collision-induced dissociation mass spectra of these ions evidently indicated the ions b- and y-series, which were definitely identical to the peptides bound-LCA. As a result, the covalent LCA-lysozyme adduct bound through Lys-13 and Lys-116 (Fig. 4).

Accordingly, the above-mentioned LCA-protein adducts were separated by SDS-PAGE, followed by blotting to a nitrocellulose membrane. The blotted membrane was incubated with the monoclonal antibody and consecutively incubated with rabbit anti-mouse IgG (H+L) and HRP labeled goat anti-rabbit IgG antibody, followed by staining with 3,3'-diaminobenzidine and H2O2. The combined use of these antibodies in immunoblotting has greatly contributed toward overcoming the sensitive and specific detection of the LCA-protein adducts. As shown in Fig. 5, the results of immunoblots showed a strongly stained band in the lane loaded with LCA-HA-BSA and LCA-lysozyme adducts, although no significantly stained bands in the lane loaded with BSA and lysozyme were observed. These results indicate that the binding to LCA anchored on proteins attributed to the immunorecognition of the steroidal moiety of LCA.

In summary, we successfully generated monoclonal antibodies to capture LCA protein adducts with no cross reactivity toward native proteins, providing a powerful tool to facilitate our investigation on the identification and structure elucidation of the protein-bound LCA.

Conclusion

In our previous paper, we showed that the Rab and Ras proteins chemically modified with LCA were formed in liver of bile duct-ligated rats by means of immunoprecipitation using polyclonal antibody, followed by the technique of 2-DE, combined with the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and computer-assisted programs. However, it is unclear why LCA binds only to these GTP binding proteins in vivo. The monoclonal antibody prepared in this study would be useful to define the precise chemical form of LCA that binds to other proteins, and to compare the binding of the acyl-adenylates and acyl-CoA thioester derivatives of other bile acids to see whether such binding to Rab and Ras is specific for LCA. Further studies on the characterization of the LCA modified proteins by immunoprecipitation followed by LC/MS analysis are now being conducted in our laboratory.
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