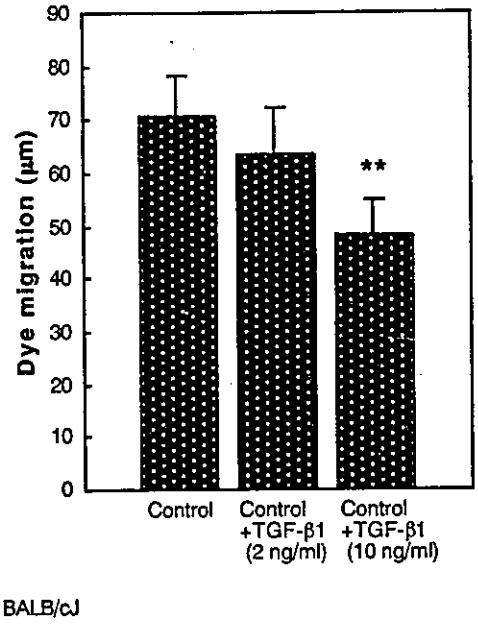
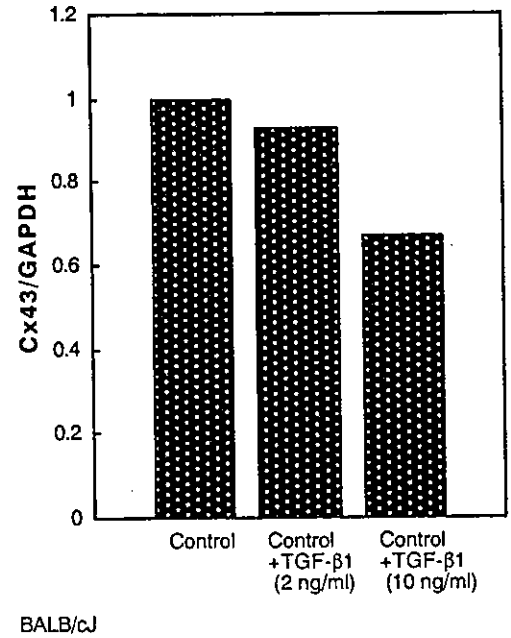


(a)



(b)

Figure 5. (A) SLDT assay. (B) National Institutes of Health image analysis quantitation of RT-PCR bands. In both figures, BALB/cJ control cells were treated with 2 and 10 ng/mL TGF-β1. GJIC was significantly inhibited and mRNA expression was significantly suppressed in BALB/cJ control cells treated with 10 ng/mL TGF-β1 compared with BALB/cJ controls. $^{**}p < 0.01$. Three dishes were used for one data point (bar) as one experiment. Results shown are representative of two independent experiments.

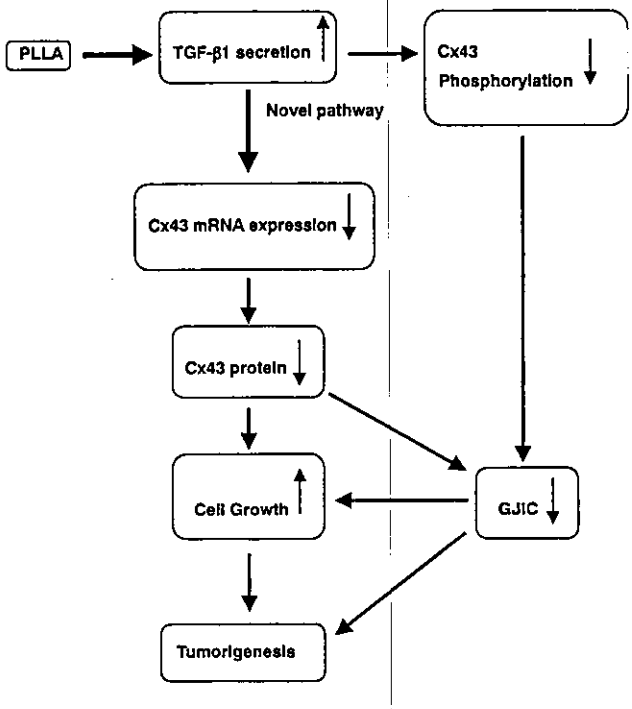


Figure 6. Schematic representation of the pathway of tumorigenesis induced by PLLA in BALB/cJ mice.

the suppression of the function of GJIC (Fig. 1) and at the same time, mRNA expression of Cx43 was suppressed in BALB/cJ mice (higher tumorigenic) but not in SJL/J mice (lower tumorigenic) [Fig. 2(A,B)]. TGF-β1 also suppressed the expression of mRNA of Cx43 and the function of GJIC in the BALB/cJ mouse cells *in vitro* [Fig. 5(A,B)]. These results indicated the novel mechanism of tumorigenesis induced by PLLA (Fig. 6).

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Proteomic Analysis of Putative Latex Allergens

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Key Words
Proteomics · Allergenomics · Latex allergy · Allergen · Two-dimensional gel electrophoresis · Immunoblotting · In-gel digestion · Mass spectrometry · Database search

Abstract
Background: Extensive analysis of allergenic proteins is generally time-consuming and labor-intensive. Accordingly, a rapid and easy procedure for allergen identification is required. As sequence information on proteins and genes is accumulated in databases, it is becoming easier to identify a candidate protein using proteomic strategies, i.e. two-dimensional gel electrophoresis, site-specific fragmentation, mass spectrometry and then database search. In this study, we evaluated the usefulness of a proteomic strategy for identifying putative allergens through its application to latex proteins. ***Methods:*** Latex proteins were separated with two-dimensional gel electrophoresis, and putative allergens were visualized by IgE immunoblotting using pooled serum from latex-sensitive patients. The IgE-interactive proteins were cut out from the negatively stained two-dimensional gel and subjected to in-gel digestion by trypsin. Then the resulting peptides were analyzed with mass spectrometry. Based on the mass spectrometric data we obtained, the allergen candidates were assigned by a database search. ***Results:*** Five previously reported allergens and five new allergen candidates were identified with the proteomic approach without isolating the individual proteins. Less than 1 mg of crude latex protein was sufficient for the entire protocol. Because plural proteins can be processed in parallel, analysis of about 50 IgE-interactive proteins was accomplished within 1 week. ***Conclusions:*** Analysis of putative allergens with proteomic strategies (allergenomics) is a promising avenue for rapid and exhaustive research. The high resolving power of two-dimensional gel electrophoresis is superior to conventional gel electrophoresis. Moreover, the notable sensitivity and speed of mass spectrometry have pronounced advantages over the N-terminal sequencing that has generally been used for protein identification.

Introduction

Latex allergy has become a serious problem in medical settings since the late 1980s [1]. Intensive research on allergenic proteins responsible for this immediate-type hypersensitivity has revealed 13 officially registered latex allergens to date. It is notable that many of the latex allergens have something to do with the defense mechanisms of the rubber tree [2–6]. Defense-related proteins of higher plants are relatively conserved in the course of evolu-

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tion and therefore share the partial sequences indispensable for their function. Such a conserved sequence is sometimes called a functional domain. For example, hevein is one of the major latex allergens and contains a chitin-binding domain that is indispensable for its antifungal activity. A homologous chitin-binding domain has already been found in several antifungal proteins from various plant species. Class I chitinase is a representative containing a hevein-like domain. Because of this shared domain, class I chitinase is expected to be allergenic for hevein-sensitized patients [4–6]. Cross-reactivity between class I chitinase and hevein was indeed confirmed by several research groups [7, 8]. However, hevein cannot explain all of the cross-reactions of latex-allergic people to a wide range of fruits and vegetables (latex-fruit syndrome). Other undiscovered minor cross-reactive latex allergens must play a role in the latex-fruit syndrome. We need a powerful methodology in order to investigate such minor but significant allergens as well as major but hidden allergenic proteins.

As sequence data of proteins and genes are accumulated in databases, it is becoming easy to analyze candidate proteins rapidly and comprehensively using an effective separation technique coupled with mass spectrometry [9, 10]. This new strategy, aiming at comprehensive analysis of total proteins (proteome) in a cell or tissue, is now referred to as 'proteomics'. For the application of proteomic strategies to allergenic proteins in particular, the term 'allergenomics' has been proposed. In conventional approaches, we needed to isolate each allergenic protein to identify and fully characterize it. This process is usually complex, time-consuming and labor-intensive. However, in allergenomics, complex protein mixtures are effectively resolved with two-dimensional gel electrophoresis or the equivalent, and IgE-interactive proteins are detected by subsequent immunoblotting using pooled patients' serum. We can easily identify putative allergens through their site-specific degradation and the subsequent mass spectrometric analysis of the fragmented peptides and database search. The overall procedure is expected to be quicker and less labor-intensive than any procedures used to date. Moreover, we can analyze numerous IgE-interactive proteins in parallel. Semiautomatic analysis of allergen candidates is also possible. From these features, allergenomics is expected to be a key technique for fast, effective and comprehensive analysis of IgE-interactive proteins.

A few examples of the application of proteomic strategies to pollen allergens have been published in recent years [11, 12]. However, the effectiveness of allergenomics has not been fully demonstrated yet. In this study, we evaluated the usefulness of allergenomics through the analysis of latex proteins. Latex allergens have been extensively studied using traditional biochemical methods [1]. Nevertheless, minor latex allergens that have been overlooked and new cross-reactive allergens pertinent to the latex-fruit syndrome remain to be scrutinized. The advantages of allergenomics in analyzing these troublesome allergens are demonstrated below.

Subjects and Methods

Subjects

Six latex-sensitive patients and four control volunteers provided their serum for this study. Latex-allergic patients (table 1) were diagnosed from their clinical history of allergy to natural rubber-containing products, positive results of a skin prick test (SPT) using crude extract of latex proteins [13] and scores from a latex-specific IgE radioallergosorbent test (CAP RAST, Pharmacia Diagnostics). When a subject with a convincing clinical history of latex allergy did not show positive reactions on the SPT, a gradual provocation test to a brand of highly allergenic latex gloves was carried out for definite diagnosis. Three of the control subjects were medical personnel who are exposed constantly to various kinds of products made from natural rubber. One control subject was a homemaker suffering from erythema nodosum but with no background of allergies. They were confirmed not to have a latex allergy from the diagnostic criteria mentioned above. Informed consent was obtained from all of the participants before collecting their blood.

Extraction of Latex Proteins

Latex proteins were extracted from nonammoniated latex (NAL) harvested from a rubber tree (*Hevea brasiliensis*, clone RRIM600) at the Rubber Research Institute of Malaysia. The NAL was immediately frozen at the site of collection and dispatched to Japan. By thawing and pressing the frozen latex, we obtained a brown liquid that oozed out from it. The liquid (about 100 ml) was supplemented with two tablets of protease inhibitor cocktail (Complete Mini, Roche Applied Science) and dialyzed against running water using Spectra/Por 3 membrane tubing (cutoff 3.5 kD; Spectrum Laboratories, Inc.) overnight. The dialyzate was then lyophilized and reconstituted in phosphate-buffered saline (pH 7.4). After condensation of the solution using Centriprep-10 (cutoff 10 kD; Millipore Co.), the concentrate was ultracentrifuged at 40,000 rpm for 24 h at 4°C (P70AT2 rotor and CP 65β ultracentrifuge, Hitachi Koki Co., Ltd.). The protein content of the supernatant was determined with a BCA Protein Assay Kit (Pierce Chemical Co.) as 13.9 mg/ml. This supernatant was submitted to later study.

Two-Dimensional Gel Electrophoresis

Latex proteins (250 μg) extracted from NAL were diluted in 250 μl of Destreak Rehydration Solution (Amersham Biosciences) supplemented with IPG buffer (0.1%, pH 3–10, nonlinear; Amersham). This solution was used for overnight rehydration of an immobilized pH gradient gel strip (Immobiline DryStrip, Amersham; pH 3–10, nonlinear, 13 cm). Then the strip was submitted to isoelectric focusing on a Multiphor II Electrophoresis Unit (Amersham) at

Table 1. Summary of latex-allergic patients who provided their blood for IgE immunoblotting

Patient No.	Age years	Gender	Occupation	Total IgE IU/ml[1]	Latex-specific IgE IU/ml (score)[2]	Latex SPT	Positive SPT	Other allergic symptoms
1	26	F	nurse	49.1	14.0 (3)	4+	Japanese horseradish	OAS to Japanese horseradish
2	34	F	housewife	1,110	4.50 (3)	4+	avocado, grapefruit	atopic dermatitis, bronchial asthma
3	22	F	dental hygienist	273	2.23 (2)	3+	tomato, spices	OAS, atopic dermatitis, pollinosis
4	25	M	medical doctor	15,500	82.0 (5)	3+	avocado	atopic dermatitis, atopic rhinitis
5	25	F	nurse	88.7	2.81 (2)	3+	–	OAS to melon
6	28	F	nurse	206	15.0 (3)	4+	–	OAS, atopic dermatitis, atopic rhinitis

OAS = Oral allergy syndrome.
[1] IgE radioimmunosorbent test (Pharmacia).
[2] CAP radioallergosorbent test (Pharmacia).

20 °C. Proteins were focused by applying programmed voltage as follows: 0–300 V in 1 min, 300–3,500 V in 1.5 h and 3,500 V for 7 h. Thereafter, the gel strip was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension of electrophoresis. Before applying to SDS-PAGE, the gel strip was incubated at room temperature in 7.5 ml of equilibration buffer (Amersham) supplemented by dithiothreitol (1%) for 30 min, then in the same equilibration buffer containing iodoacetamide (1%) instead of dithiothreitol for 30 min. The gel strip was placed on top of a slab gel (10% acrylamide, 14 × 14 cm) and fixed with agarose (0.5%). The second electrophoresis was carried out by applying a constant current of 30 mA for 30 min then 70 mA for 5 h at 20 °C using an SE 600 Ruby Electrophoresis Unit (Amersham).

Immunoblotting

Two-dimensionally separated proteins were transferred onto a polyvinylidene fluoride membrane (14 × 14 cm; Immobilon-P, Millipore) using a semidry transfer cassette (ATTO Co.) by applying a constant current of 200 mA for 1.5 h. SDS (0.1%) was added to each of the discontinuous blotting buffers containing methanol (10%) for efficient transfer of proteins with high molecular weight or a basic isoelectric point. After washing with Tris-buffered saline (TBS; pH 7.4) supplemented with Tween-20 (TBS-T; 0.1%), the membrane was soaked in a chilled and degassed 10 m*M* $NaIO_4$-50 m*M* acetate buffer (pH 4.7) and incubated at 4 °C in the dark overnight. Then the membrane was repeatedly washed with TBS-T and incubated at room temperature in a blocking buffer (Block Ace, Dai-Nippon Pharmaceutical Co.) for 5 h. Following washing with TBS-T, the membrane was incubated overnight in the pooled patients' serum (20 ml) diluted 1/20 in TBS-T at room temperature. In the control experiment, the membrane was incubated in the pooled control serum (20 ml) diluted 1/20 in TBS-T. The next day, the membrane was washed again with TBS-T and incubated at room temperature in a solution of peroxidase-labeled affinity-purified antibodies to human IgE(ε) (Kirkegaard & Perry Laboratories, Inc.) diluted 1/10,000 in TBS-T for 1 h. After repeated washing with TBS-T, IgE-recognized protein spots were visualized using an ECL Western Blotting Detection System and Hyperfilm ECL (Amersham).

In-Gel Digestion

Two-dimensionally separated proteins on the gel were visualized by negative staining [14] using a Zinc Stain Kit (Bio-Rad Laboratories). Protein spots that were judged as IgE-interactive from the immunoblotting with pooled patients' serum were cut out from the gel (highlighted spots in fig. 1A). After treatment with Zinc Destain Solution (Bio-Rad) as instructed by the manufacturer's protocol, the protein spots were submitted to tryptic digestion using a Montage In-Gel Digest96 Kit (Millipore). Digestion proceeded at 30 °C overnight following the manufacturer's recommendations except that *n*-octyl-β-*D*-glucoside (Dojindo Laboratories) was added to the digestion mixture (final 0.1%) for efficient recovery of the fragmented peptides [15].

Mass Spectrometry and Database Search

Fragmented peptides were desalted by means of a reversed-phase microcolumn (ZipTip μC_{18}, Millipore) as instructed by the manufacturer, and eluted from the column with the help of α-cyano-4-hydroxy cinnamic acid solution on a sample plate for mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed on a 4700 Proteomics Analyzer (Applied Biosystems Inc.). Positive-ion mass spectra of tryptic peptides were recorded on the reflectron mode of the instrument. The information from these spectra (peptide mass maps, fig. 2A) was used for a database search of the original proteins with the peptide mass fingerprinting (PMF) mode of a search engine (Mascot, Matrix Science). Further, tandem mass spectra (MS/MS spectra) of prominent peptide ions on the peptide mass map were recorded on the time-of-flight (TOF)-TOF mode of the instrument [16]. The information (peptide sequence tag; PST) from such tandem mass spectra (fig. 2B) was employed for a database search with the MS/MS Ion Search mode of Mascot for more precise identification of IgE-interactive proteins. National Center for Biotechnology Information (nonredundant) (NCBInr) was selected as the first database to be searched using Mascot. When there was no candidate with enough certainty, SwissProt and Expressed Sequence Tags (EST) *(H. brasiliensis)* were employed as the second and third databases to be searched, respectively. The taxonomy of the database category was set to *Viridiplantae* (Green Plants), and carbamidomethyl cysteine was selected as a fixed modification of fragmented peptides. The number of allowable missed cleavages in tryptic digestion was set at

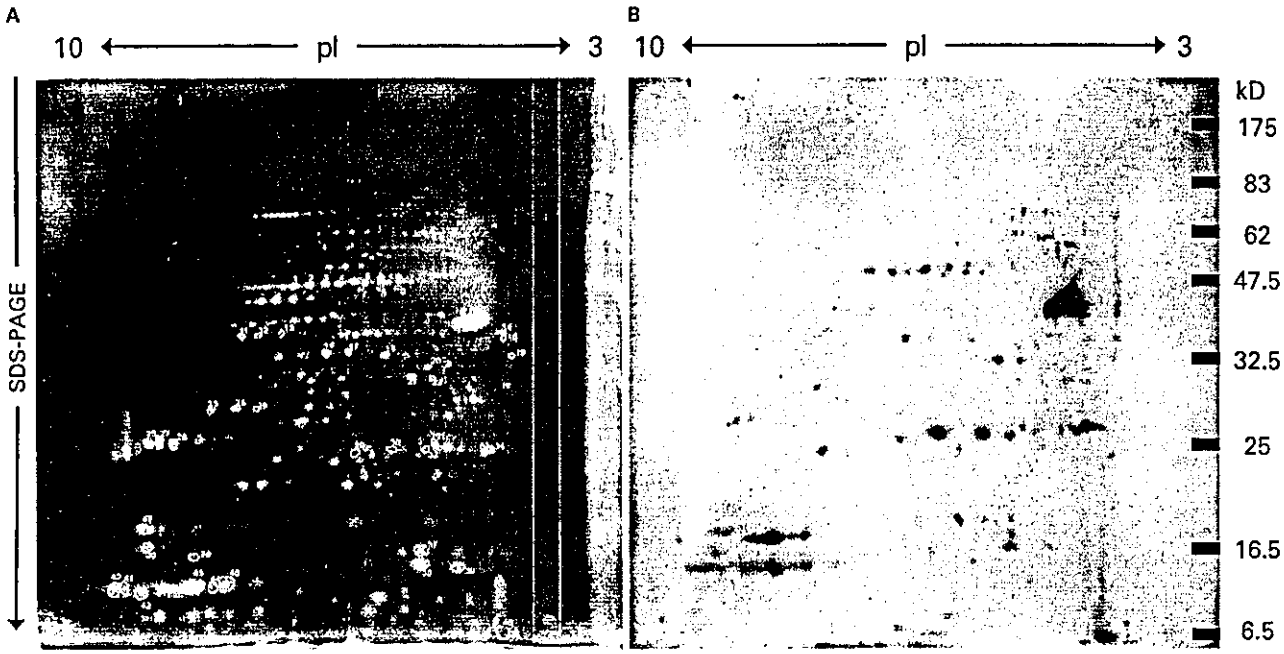

Fig. 1. Latex protein (**A**) and IgE-interactive protein (**B**) profiles on two-dimensional gels. Latex proteins extracted from NAL were first separated according to their isoelectric point (pI) on an immobilized pH gradient gel strip (pH 3–10, nonlinear). Second, the partially separated proteins were further resolved according to their molecular weight on a 10% acrylamide slab gel (SDS-PAGE). Two-dimensionally separated proteins were visualized by negative staining (**A**). IgE-interactive proteins submitted for subsequent in-gel digestion are highlighted and numbered (No. 1–47). For IgE immunoblotting (**B**), two-dimensionally separated proteins were transferred onto a membrane and treated by $NaIO_4$ for degradation of the possible carbohydrate structures. After blocking the unoccupied sites, the membrane was incubated in the pooled serum of 6 latex-sensitive patients. Spots of proteins that interacted with the IgE antibodies were then visualized by reaction with peroxidase-labeled anti-human IgE antibodies. The enzyme activity was recorded on a film using a chemiluminescent detection system. The migrating position and molecular weight of each marker protein are shown on the right side of the gel.

1. Mass error tolerance was set to plus or minus 0.5 D for PMF. In the MS/MS Ion Search mode, the mass error tolerance of a precursor ion was set to plus or minus 1.0 D, and plus or minus 0.5 D for the product ions. All the peptide masses mentioned in this article are monoisotopic masses. Sequentially homologous proteins from other organisms were searched using the Basic Local Alignment Search Tool (BLAST) search engine-based overall sequence of the top candidate picked up from the EST *(H. brasiliensis)* database.

Results

Two-Dimensional Immunoblotting of Latex Proteins

Proteins extracted from NAL were separated with two-dimensional gel electrophoresis. Its high resolution was visualized by negative staining of the gel (fig. 1A). More than 300 distinct protein spots, each with a different isoelectric point or molecular weight, were detected. This resolving power is far superior to the conventional SDS-PAGE or isoelectric focusing where proteins are separated one-dimensionally. IgE-interactive proteins were selectively detected by reaction with the pooled patients' serum following $NaIO_4$ treatment of a membrane binding the two-dimensionally separated proteins. $NaIO_4$ oxidatively degrades the carbohydrate structures of glycoproteins that tend to result in false-positive reactions of IgE antibodies by acting as monovalent antigens [17, 18]. Monovalent antigens cannot form a bridge-like structure of IgE antibodies attached to the specific receptor on a sensitized cell, and therefore they are believed not to provoke any allergic responses. We clearly detected more than 50 spots of proteins interacting with the IgE antibodies owing to the high resolving power of two-dimensional gel electrophoresis and the advanced sensitivity of the chemiluminescent detection system compared to conventional color-developing systems (fig. 1B). No significant spots of IgE-interactive proteins were detected when the pooled control serum was employed for immunoblotting (data not shown).

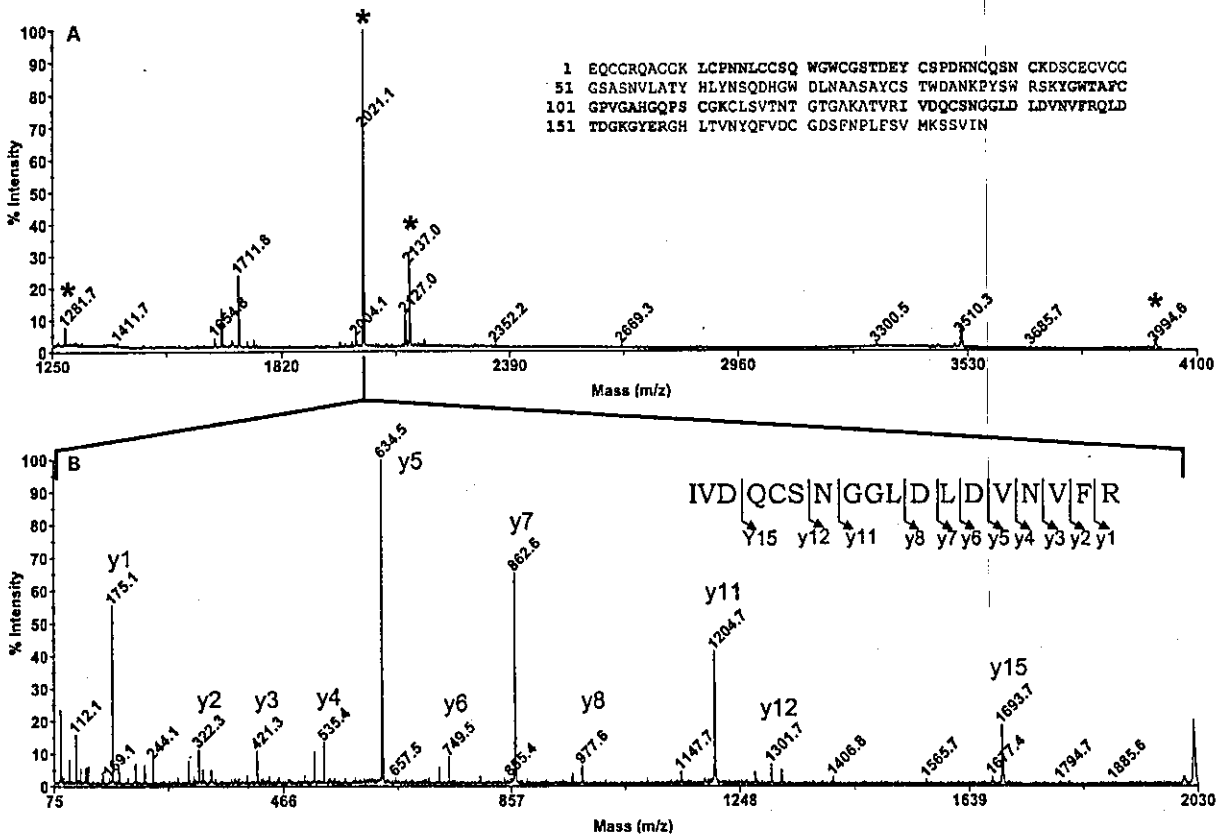

Fig. 2. Positive-ion reflectron MALDI-TOFMS spectrum (**A**) of tryptic peptides derived from an IgE-interactive protein (spot No. 38 in fig. 1A) and an MS/MS spectrum (**B**) of a prominently detected peptide ion (m/z 2021.1) on the peptide mass map. The spot of a putative allergen (No. 38) was cut out from a negatively stained two-dimensional gel (fig. 1A) and site-specifically cleaved by trypsin. The fragmented peptides were desalted using a reversed-phase microcolumn and spotted on a sample plate. α-Cyano-4-hydroxy cinnamic acid was used as a matrix for ionization. A peptide mass map (**A**) was used for PMF. This spectrum is a result of accumulation of 2,000 laser shots. The peptide ions assigned in the PMF search are labeled by asterisks, and their position is shown in boldface in the entire sequence of the top candidate (insert). The MS/MS spectrum (**B**) was recorded on the TOF-TOF mode of the instrument (4700 Proteomics Analyzer) and used for reading the PST. This MS/MS spectrum shows the accumulation of 5,000 laser shots. A partial sequence read from this spectrum is depicted as an insert with the name of the fragment ions (y series) detected. Based on a series of mass spectra including the ones shown in this figure, the original antigen (spot No. 38 in fig. 1A) was identified as a prohevein-related protein by database search (table 2).

In-Gel Digestion of Putative Allergens and Mass Spectrometry of Tryptic Peptides

Forty-seven spots of latex proteins that interacted with IgE antibodies from patients' sera were cut out from a two-dimensional gel (fig. 1A). With the help of the microtiter plate-based format of an in-gel digestion kit (Montage In-Gel Digest96 Kit), the putative allergens in the gel pieces were simultaneously digested by trypsin with less risk of keratin contamination. By adding *n*-octyl-β-*D*-glucoside to the digestion buffer, we could significantly improve the quality of mass spectra of fragmented peptides (fig. 2A). Desaltation of tryptic peptides using a reversed-phase microcolumn (ZipTip μC_{18}) was also helpful for improving the signal-to-noise ratio of the mass spectra (fig. 2A). We used a MALDI-TOF-TOF instrument for mass spectrometric analysis of tryptic peptides. Though the quality of MS/MS spectra is generally inferior to the spectra obtained on an electrospray ionization-based tandem mass spectrometer, the operation is less labor-intensive and very quick. The sensitivity of the mass spectrome-

Table 2. Summary of IgE-interactive proteins identified by allergenomics with a Mascot search

Spot No.	Name of the top candidate	Method used	Database used	Total score	Sequence coverage, %	Accession No.	Calculated Mr, kD	Calculated pI	Observed Mr, kD	Observed pI
1	UDP-glucose pyrophosphorylase	PST	NCBInr	46	2	gi|12585472	51.6	5.92	49.0	6.6
3	UDP-glucose pyrophosphorylase	PST	NCBInr	24	2	gi|12585472	51.6	5.92	49.0	6.4
4	UDP-glucose pyrophosphorylase	PST	NCBInr	58	6	gi|8099155	50.9	6.10	49.0	6.3
5	enolase 2 (Hev b 9)	PST	NCBInr	32	5	gi|14423687	48.1	5.92	49.0	6.3
7	enolase 1 (Hev b 9)	PMF	NCBInr	52	22	gi|14423688	48.0	5.57	47.5	6.0
	enolase 1 (Hev b 9)	PST	NCBInr	38	5	gi|14423688	48.0	5.57		
10	patatin-like latex allergen 2 (Hev b 7)	PMF	NCBInr	156	37	gi|7442023	43.0	5.00	40.0–43.5	4.6–5.5
	patatin-like latex allergen 1 (Hev b 7)	PST	NCBInr	68	13	gi|7442022	43.1	5.12		
21	isoflavone reductase[1]	PST	EST(latex)	201	15	gi|29054556	27.0	5.32	32.5	5.6
22	isoflavone reductase[1]	PST	EST(latex)	69	15	gi|29054556	27.0	5.32	32.5	5.4
26	hevamine A	PMF	NCBInr	151	54	gi|1311006	29.9	8.44	29.0	9.4
	hevamine A	PST	NCBInr	156	19	gi|1311006	29.9	8.44		
27	hevamine A	PMF	NCBInr	151	65	gi|1311006	29.9	8.44	29.0	9.3
	hevamine A	PST	NCBInr	240	32	gi|1311006	29.9	8.44		
28	hevamine A	PMF	NCBInr	137	54	gi|1311006	29.9	8.44	29.0	9.1
	hevamine A	PST	NCBInr	113	13	gi|1311006	29.9	8.44		
33	class I chitinase[1] (Hev b 11-related)	PST	EST(latex)	87	7	gi|29054149	23.5	8.28	26.5	4.2
34	class I chitinase[1] (Hev b 11-related)	PST	EST(latex)	121	15	gi|29054149	23.5	8.28	26.5	4.0
36	hevein precursor (Hev b 6 -related)	PST	NCBInr	36	8	gi|123062	22.7	5.63	16.5	8.6
37	prohevein (Hev b 6)	PST	NCBInr	69	20	gi|2832430	20.9	5.64	17.0	5.6
38	prohevein (Hev b 6)	PMF	NCBInr	62	43	gi|2832430	20.9	5.64	15.0	5.6
	prohevein (Hev b 6)	PST	NCBInr	198	20	gi|2832430	20.9	5.64		
39	prohevein (Hev b 6)	PST	NCBInr	127	20	gi|2832430	20.9	5.64	15.0	5.3
40	rotamase	PST	SwissProt	21	6	Q39613	18.5	8.36	11.0	9.8
41	rotamase	PMF	SwissProt	38	27	Q39613	18.5	8.36	11.0	9.6
	rotamase	PST	NCBInr	50	6	gi|3334157	18.5	8.36		
42	rotamase	PST	NCBInr	98	8	gi|118104	18.6	8.91	10.5	9.3
43	prohevein (Hev b 6)	PMF	NCBInr	44	32	gi|2832430	20.9	5.64	11.0	8.7
	prohevein (Hev b 6)	PST	NCBInr	201	26	gi|2832430	20.9	5.64		
44	pseudo-hevein (Hev b 6-related)	PMF	NCBInr	49	33	gi|6562381	21.0	7.98	11.0	8.4
	prohevein (Hev b 6)	PST	NCBInr	114	20	gi|2832430	20.9	5.64		
45	pseudo-hevein (Hev b 6-related)	PMF	NCBInr	39	33	gi|6562381	21.0	7.98	11.0	8.0
	prohevein (Hev b 6)	PST	NCBInr	196	20	gi|2832430	20.9	5.64		
46	thioredoxin h	PST	NCBInr	48	13	gi|14485509	13.2	4.83	6.5	3.9
47	citrate-binding protein precursor	PST	NCBInr	72	10	gi|32363139	27.5	9.09	17.0	9.4

Mr = Molecular weight; pI = isoelectric point.
[1] Sequentially homologous protein was searched using BLAST.

try is also notable. We could easily obtain positive-ion peptide mass maps (fig. 2A) and MS/MS spectra of prominent ions (fig. 2B) from as small as several hundred femtomoles of tryptic peptides on a sample plate.

Identification of Putative Allergens by Database Search

Based on a peptide mass map of the tryptic peptides and MS/MS spectra of the intense peptide ions, the proteins in question were identified by database search. The identified putative allergens are listed in table 2, with the total score and sequence coverage rate showing the certainty of the protein candidate. Thanks to the high resolution of two-dimensional gel electrophoresis and the remarkable sensitivity of MALDI-TOFMS, we could identify five new allergen candidates (UDP-glucose pyrophosphorylase, isoflavone reductase, rotamase, thioredoxin and citrate-binding protein) and five previously reported latex allergens or their relatives (Hev b 9, Hev b 7, Hev b 11, Hev b 6 and hevamine) without isolating each protein

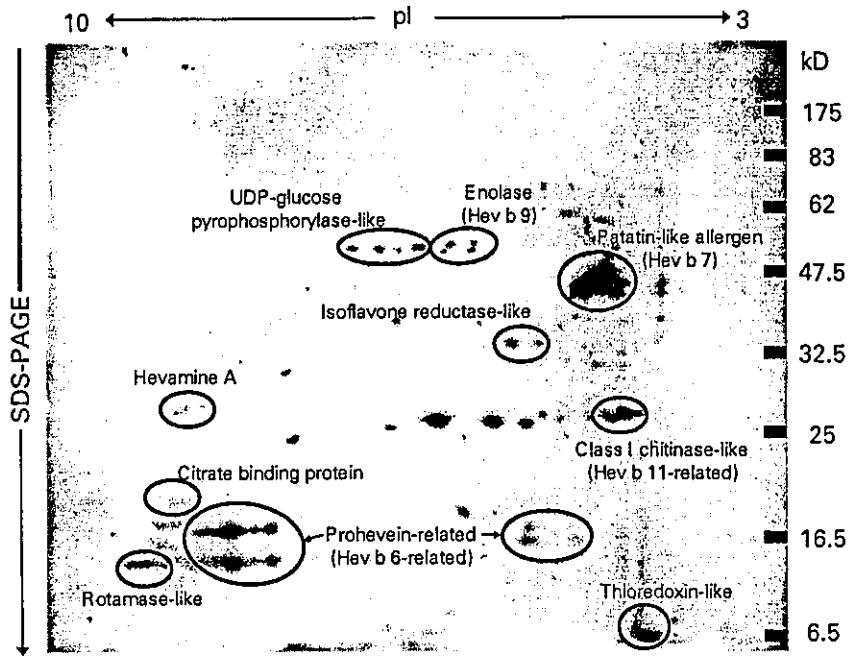

Fig. 3. Putative latex allergens identified by a proteomic strategy. The names of the top candidates are shown on an IgE immunoblot (same as in fig. 1B). Spots enclosed by an ellipse are IgE-interactive proteins assigned to the same or closely related entries (isoallergens) by database search. More information on each antigenic protein is presented in table 2.

(table 2, fig. 3). A few allergens were identified by database search with the PMF mode of Mascot, but the searching based on PST was much more powerful for protein identification. From the MS/MS spectra of tryptic peptides derived from a protein, we could obtain information on the partial sequences (fig. 2B) that would be valuable for designing the oligonucleotide probes as well as for the database search. The BLAST search was also effective in finding a protein candidate that is sequentially comparable to a DNA fragment registered in the EST database *(H. brasiliensis)*. The entire protocol, from two-dimensional gel electrophoresis to the database search, was accomplished quickly. We were able to analyze about 50 IgE-interactive latex proteins within 1 week.

Discussion

In this study, we evaluated the usefulness of a proteomic strategy for analyzing putative allergens (allergenomics) through application to latex proteins. Latex proteins were first separated two-dimensionally according to their isoelectric point and molecular weight under denaturing conditions. Two-dimensional gel electrophoresis appeared to be remarkably superior to SDS-PAGE with regard to its high resolution. In conventional SDS-PAGE, proteins were separated based solely on their molecular weight under a denaturing condition. Therefore, a seemingly single band on an SDS-PAGE gel frequently contains several protein entities. This poor resolution might bring about erroneous identification of IgE-interactive proteins. Additionally, faint structural differences of isoallergens are hardly reflected on SDS-PAGE gel and subsequent IgE immunoblot. In contrast, we can detect slight structural differences of isoallergens as distinct spots on a two-dimensional gel and subsequent IgE immunoblot. Hev b 7 isoallergens are one such example (fig. 3, table 2, spot No. 10). Historically, the reproducibility of two-dimensional gel electrophoresis has been a critical problem in applying this technique to research of complex protein mixtures. However, the appearance of commercially available immobilized pH gradient gels has solved a large part of this difficulty. The effectiveness of two-dimensional gel electrophoresis for analysis of allergenic proteins has already been demonstrated in previous studies [19–23]. Nevertheless, this technique has not attracted the attention of many researchers, due to the lack of a convenient identification method for the resolved proteins. N-terminal sequence analysis of a purified protein has been the first choice for identification [10, 20], but this technique is time-consuming and requires a relatively large quantity of purified sample. Putative allergens avail-

able only in minute amounts were therefore not readily amenable to this approach.

As the sequence data of proteins and genes accumulate in databases, the situation is changing. Candidate proteins are becoming easier to identify after site-specific fragmentation and mass spectrometric analysis of the component peptides [9, 10]. Mass spectrometry is so sensitive that we can analyze putative allergens on quantities as small as the nanogram or several hundred-femtomole level. Intensive research of such a small quantity of proteins was impossible with traditional Edman degradation-based N-terminal sequencing. The speed of mass spectrometric analysis is also advantageous. We can obtain thousands of mass spectra and MS/MS spectra within a day using a MALDI-TOFMS instrument. Semiautomated analysis of fragmented peptides from spotting on a sample plate to output of the mass spectra is also possible. By combining with high-resolution two-dimensional gel electrophoresis, the mass spectrometric approach will certainly become the most powerful means for fast and exhaustive analysis of IgE-interactive proteins [24].

The efficiency of allergenomics was substantiated in this study through the analysis of latex proteins. We were able to identify five new allergen candidates as well as five previously reported allergens without any difficulties (fig. 3, table 2). The required starting material was less than 1 mg. In addition, the entire analysis was completed within 1 week; two-dimensional gel electrophoresis and subsequent IgE immunoblotting usually takes not more than 5 days. Referring to the immunoblotting result with pooled patients' serum, IgE-interactive proteins were cut out from a two-dimensional gel and digested by trypsin overnight. A more rapid digestion protocol for proteins is also applicable [25]. The proteins in question were identified the next day from mass spectrometric analysis of the resulting fragments and subsequent database search. These analytical steps for each IgE-interactive protein proceed in parallel with the help of a microtiter plate-based tryptic digestion regime. In this way, allergenomics was concretely verified as a quick and effective strategy for exhaustive analysis of IgE-interactive proteins from various species.

We should point out at this stage that IgE-interactive proteins or antigens identified with allergenomics are just putative allergens or allergen candidates. IgE-binding activity of a protein is one of the prerequisites for it to be an allergen and does not necessary indicate its actual allergenicity. Some IgE-interactive proteins are indeed asymptomatic but others are symptomatic [17, 18]. Therefore, the actual allergenicity of putative allergens must finally be confirmed with other techniques like SPT or histamine release test. This situation is common to almost all the allergen identification procedures reported to date where allergen candidates are screened based on their IgE-binding activity.

Even though allergenomics is very promising, as already illustrated, some of the latex antigens were not identified by database search, as shown in figure 3, despite their clear detection in IgE immunoblotting. There are two possible reasons for this failure. One possibility is that the quantity of IgE-interactive protein was too small to gain meaningful data regardless of the high sensitivity of the mass spectrometry. Another possibility is that data on the sequence of the antigen in question had not been registered in the databases used. The genome of the rubber tree *(H. brasiliensis)* has not been sequenced thoroughly. Therefore, we cannot identify some of the latex proteins by the proteomic strategy in principle. If we analyze proteins from rice or *Arabidopsis*, this is not the case because the genome projects of these species have already been finished. Even when the sequence of an IgE-interactive protein is not included in databases, we can search sequentially homologous proteins from other species using the partial sequences of tryptic peptides or cDNA fragments. Moreover, the gene coding the putative allergen can be fully sequenced by cloning the corresponding cDNA using oligonucleotide probes designed from the revealed partial sequences. These prospects for further research also suggest the versatility of allergenomics.

Acknowledgement

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CHANGE OF THE CELLULAR FUNCTION BY CONNEXIN GENE TRANSFECTION IN A HEPATOMA CELL LINE

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Abstract

Connexin 32 (Cx32) is the main gap junction protein in hepatocytes and plays an important role in the regulation of liver gap junctional communication (GJIC). In this study, the human Cx32 gene was transfected in a hepatoma cell line (HepG2) that is aberrant expression of Cx32 and deficient in GJIC. Cx32-transfected HepG2 showed the increased GJIC comparing with HepG2 and the vector-transfected HepG2. Furthermore, the liver functions of ammonia removal activity of HepG2 were remarkably enhanced with Cx32 gene transfection. It may be expected to improve the cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an efficacious bioartificial liver.

Introduction

A cell-based biohybrid artificial liver (BAL) is a promising approach to support patients with acute liver failure[1]. To overcome worldwide shortage of donor organs and avoid xenozoonosis risk, a hepatoma cell line HepG2 derived form the human-origin cell has growth characteristic and are less severe antigenecity, and then has already been used for developing the BAL[2]. Although HepG2 keeps liver-specific functions well among hepatoma cell lines, the activities of the liver-specific functions in HepG2 were far lower comparing with these of primary hepatocytes[3]. On the other hand, gap junction intercellular communication (GJIC) is considered to play an essential role in the control of proliferation, differentiation and homeostasis of various cells. In the liver, hepatocytes are coupled to each others by gap junctions and GJIC is necessary for liver homeostasis growth control and signal transfer, especially related to glycogen

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mobilization and neoplastic transformation[4]. However, HepG2 is aberrant expression of connexin protein and is deficient in GJIC. In the liver, connexin32 (Cx32) is the major gap junction protein expressed in hepatocytes, therefore, the aim of this study is focused on enhancing GJIC and improving liver-specific functions of HepG2 by Cx32 gene transfection.

Key word: Bioartificial liver, Connexin, Hepatoma cell

2. Materials and Methods

2.1. Cell culture

The human hepatoma cell lines HepG2 from the Riken cell bank (Tokyo, Japan) was cultured at 37℃ under 5% CO_2 / 95% humidified air using Minimum Essential Medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.1mM non-essential amino acids (NEAA) (Gibco), 10% fetal bovine serum (FBS) (Intergen Co., NY.) and 100U/ml Penicillin-Streptomycin (Gibco).

2.2. Plasmid construction and transfection

The connexin gene fragments amplified by polymerase chain reaction were isolated and inserted into the pTARGET™ mammalian expression system. HepG2 cells were transfected with the Cx/pTARGET™ plasmid DNA using FuGENE6 transfection reagent according to manufacturer's instruction with minor modification, cells transfected with empty vector as a control. After continuously culturing for two days, transfectants were selected by adding geneticin (Life Technologies, Inc., Frederick, MD) in the culture medium for one week. Individual transfected clones were prepared by limiting dilution cloning in 96-well plates, and then cultured as same as the HepG2.

2.3. Immunocytochemical stainings

Immunocytochemical staining of Cx32 protein was performed using the VECTASTAIN ABC kit in manufacturer's instruction with some modification. Briefly, cells grown on the glass cover slips were fixed in cold pure acetone for 5 min. The acetone-fixed specimens were blocked in diluted normal blocking serum in Dulbecco's phosphate buffered saline (PBS) at room temperature for 30min, and incubated with polyclonal rabbit anti-connexin32 (Zymed Laboratories Inc., San Francisco, CA) over night at 4 ℃ . Protein-antibody complexes were visualized by the biotin/streptoavidin/peroxidase method with diaminobenzidine tetrahydrochloride (DAB) as the chromogen (Vextor Laboratories, Burlingame, USA). All slides were viewed with a Nikon microscope (Nikon, Japan).

2.4. Scrape-loading dye transfer (SLDT) assay for measurement of GJIC

The SLDT technique was adapted after the method of El-Fouly et al. [5]. Briefly, when the cells grew into confluent monolayer cells in 35-cm dishes, cell dishes were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA) in PBS (+) solution and were scraped immediately with a sharp blade after rinsing with PBS (+). Then incubating for 5 min at 37℃, cells were washed with PBS (+) and monitored using fluorescence microscope. The distance of the dye spreading was measured from the cell layer at the scrape to the edge of the dye front that was visually detectable.

2.5. Liver-specific function assay

The functions of the HepG2 and Cx32 transfected cells were evaluated by measuring ammonia removal and albumin secretion. For the ammonia removal activities of these cells, the cells were cultured in MEM medium with 5mM ammonium chloride. After the exchange of the medium containing ammonium, the concentration of ammonia in the medium was measured at 0 and 24hrs, respectively, using the indophenol method (an ammonia assay kit, Wako Pure Chem., Japan).

2.6. Statistical analysis

Student's t-test was used to compare the samples. Statisticall significance was represented by $p<0.05$. Date were indicated as the mean±S.D (Standard Daviation). Three cultures were run for each case, and all experiments were repeated at least twice.

3. Results and Discussion

3.1. Functional GJIC in HepG2 enhanced by Cx32 gene transfection

HepG2 cells were transfected with Cx32/pTARGET™ plasmid DNA using FuGENE6 transfection reagent, and the transfectants were obtained by selection with geneticin. Enhanced expression of Cx32 mRNA was confirmed by RT-PCR (date not shown). The abilities of GJIC in Cx32 plasmid DNA transfectants were investigated by the scrape-loading dye transfer technique. Lucifer yellow, a molecular probe with low molecular weight, can diffuses in the neighboring cells through the gap junction, but not transmits from intact plasma membranes. Therefore, the transfer distances of lucifer yellow reflect the functional GJIC in the cells, and the longer distance shows the higher functional GJIC in the cells. The transfer distance of lucifer yellow in Cx32 gene transfected cells was longer than those of HepG2 and empty vector transfected cells. Thus, the distance in Cx32 gene transfected cells was 2.8 times and 2 times as long as that of HepG2 and empty vector transfected cells, respectively. It could be concluded

that the functional GJIC in HepG2 was significantly enhanced by the Cx32 gene transfection.

3.2. Localization of Cx32 protein before and after Cx32 gene transfection

In order to confirm the contribution of Cx32 proteins for the formation of functional GJIC after the Cx32 gene transfection, the localizations of Cx32 protein in the cells were further observed by immunocytochemical staining (Fig.1). The results demonstrated that the Cx32 protein expressed in HepG2, Cx32 gene transfected cells

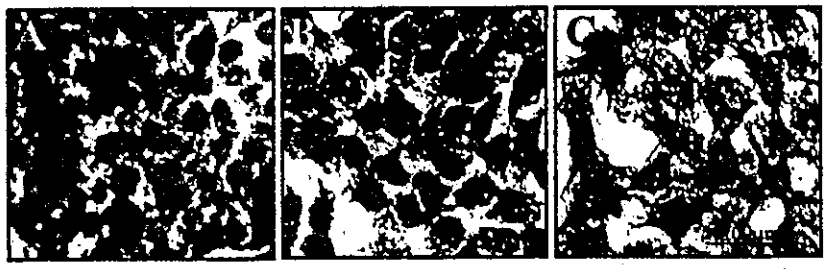

Fig.1. Immunocytochemical staining for Cx32 in HeG2 (A), empty vector transfected cells (B) and Cx32 gene transfected cells (C).

and empty vector transfected cells, but the localizations of Cx32 protein were obviously different among them. Thus, the Cx32 protein was localized in the cell borders and formed many small gap junction plaques in the neighboring cells transfected with Cx32 gene (Fig.1.C), however, the Cx32 protein was limited in the cytoplasm and hardly detected the gap junction plaques in the HepG2 (Fig.1.A) and empty vector transfected cells (Fig.1.B). Furthermore, the morphologies of the cells showed that Cx32 gene transfected cells grew as a monolayer with the spreading cell shape, whereas the HepG2 grew as the clusters with the spherical cell shape. Although the precise role of the cellular morphology in gap junctional channel formation between the cells is not clear at the present, the results in our study could be concluded that the traffic of Cx32 protein to the cell membrane in HepG2 was enhanced by Cx32 gene transfection and then increased the GJIC in Cx32 gene transfected cells.

3.3. Liver-specific function in HepG2 improved by Cx32 gene transfection

For determining the effect of Cx32 gene transfection on the liver-specific function in HepG2, the ammonia removal activity were continuously monitored in the HepG2, empty vector transfected cells and Cx32 gene transfected cells, respectively. Ammonia clearance, which represents the detoxification potentiality of liver, was significantly higher in the Cx32 gene transfected cells than HepG2 and empty vector transfected cells

during the 14 days. These results showed that the ammoniun metabolic activity in HepG2 related with the functional gap junctional channel composed of Cx32 proteins. It was considered that the small molecular ammonium was effectively eliminated through the gap junctional channels improved by Cx32 gene transfection in HepG2.

In conclusion, transfection of Cx32 gene increased the functional GJIC in HepG2 and enhance the activity of detoxification in the Cx32 gene transfected HepG2. It may be expected to improve the cellular function of the hepatoma cell line by Cx32 gene transfection and serve to develop an excellent biohybrid-artificial liver.

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Synthesis of C_{60} derivatives for photoaffinity labeling

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Abstract—In order to study the interaction of fullerenes with biological molecules, a novel photoaffinity labeling agent derived from C_{60} was designed and synthesized. As photosensitive functional groups, azide group, and aziridine group are utilized. A convenient synthetic route via fulleropyrrolidine **2** was employed to obtain compounds labeling agents **5** and **9**.

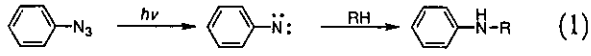
The biological activities of fullerenes have attracted considerable attention due to their potential medicinal applications.[1–3] Their novel and unexploited properties stem from their bulky hydrophobic shape and their photosensitivity[4–7] and radical-generating[8–11]/-quenching[12,13] activities enabled by highly conjugated π-electron system.

As a most remarkable activity, direct inhibition of enzymes by C_{60} has been reported. The first example, HIV-1 protease inhibition by a water soluble fullerene derivative, was reported in 1993[14–16] by Wudl, Wilkins, et al. Independently, Toniollo et al. has reported C_{60}-peptide conjugates and identified activity of these compounds against HIV-1 protease and chemotatic activity against human monocytes.[17] Separately, we have developed new procedures for solubilizing C_{60} in water[18] and assayed unfunctionalized C_{60} for direct enzymatic inhibtion. These studies led to the discovery that aqueous solutions of C_{60} inhibit glutathione-*S*-transferase (GST).[19]

The ability of C_{60}, which is large (7 Å id) hydrophobic molecules, to bind to biological compounds, was initially surprising and several groups have attempted to identify and calculate the binding sites. Based on a computer simulated docking study, Wudl, Wilkins, et al. speculated that the C_{60} core was enclosed in the cylindrical active site, which consists primarily of hydrophobic amino acid residues, of HIV-1 protease. In our own work, we calculated that C_{60} binds to GST at a cleft between two subunits of the enzyme, although the specific residues, which make up the active site are unclear.[20]

In order to clarify the more detailed binding site of C_{60}, two solutions are possible as follows. One is to isolate pure enzyme–fullerene complex and determine the structure by NMR or crystallographic methods. Another potential method for identifying the active site area is photoaffinity labeling, which is particularly useful for identifying the active site in solution under physiological conditions.

We now report the design and synthesis of the first C_{60}-derived photoaffinity labeling reagents. Our synthetic route to photoaffinity reagents **5** and **9** provide a concise, flexible route to fullerenes functionalized with photoreactive pendant groups such as phenylazide and phenyldiazirine, which generate aryl nitrene and aryl carbene, respectively (Eqs. 1 and 2).[21]



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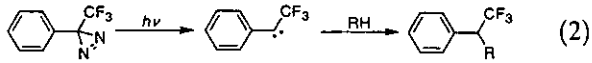

(2)

In order to develop an efficient and flexible synthetic method, which would allow the late-stage introduction of a variety of photoaffinity labels, we chose to utilize dimethylfulleropyrrolidine (**2**). This C_{60} derivative

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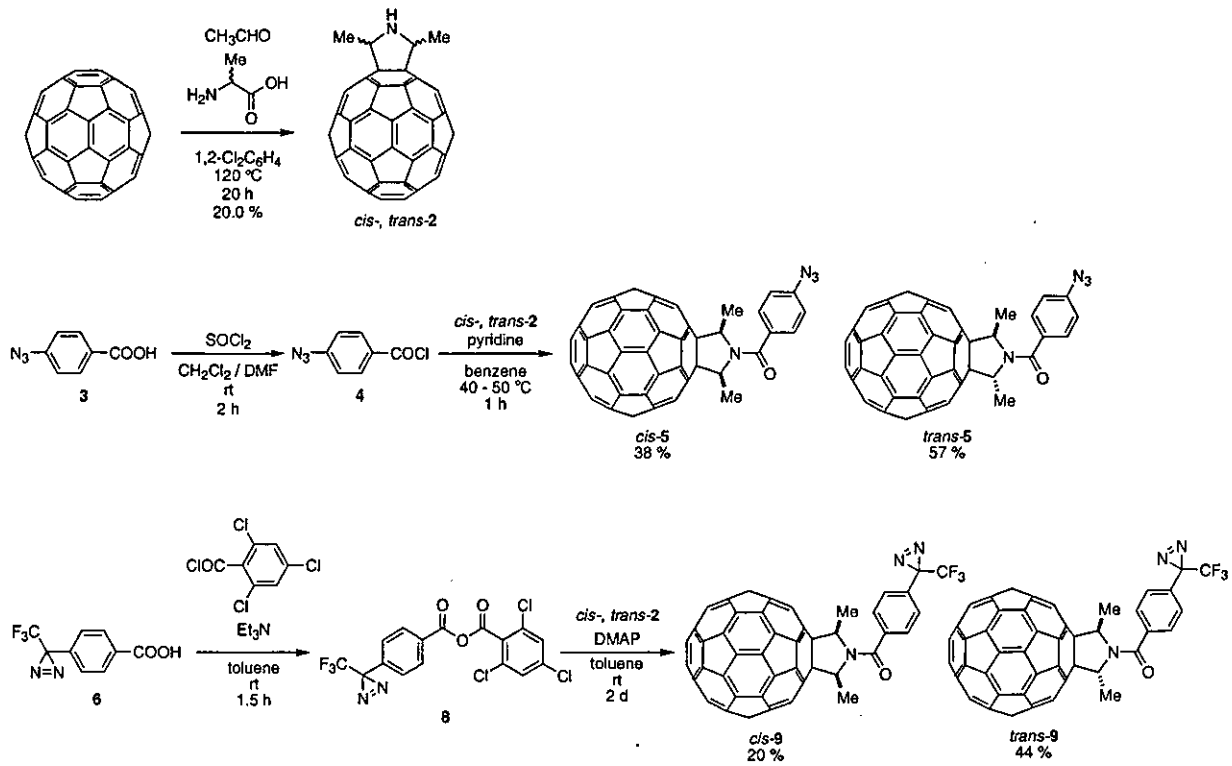

Scheme 1. Synthesis of C_{60} derivatives with phenylazide (**5**) and phenylaziridine (**9**) group.

is readily prepared by the method of Prato and co-workers[22] and Wilson and co-workers.[23,24] This route provides a convenient approach to C_{60} derivatives with a secondary amine as an ideal site for the incorporation of further functionalization.

The synthesis of phenylazide derivative of fullerene was achieved as shown in Scheme 1. Dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture) was prepared by 1,3-dipolar cycloaddition[25] and then acylated with acid chloride **4** to give *cis*- and *trans*-C_{60}-phenylazide derivatives **5**, which can be easily separated by silica gel column chromatography.[26]

To synthesize the C_{60}-phenyldiazirine derivative **9**, we first attempted the reaction of fulleropyrrolidine **2** with an acid chloride, but this reaction did not give useful amounts of the desired product. Despite attempts to activate the acyl moiety by a succinimide group using 4-(3-trifluoromethylazirino)benzoic succinimide, product formation was not observed. In sharp contrast, however, the use of Yamaguchi reagent **8** to couple **6** and **2** gave good yields of *cis*- and *trans*-C_{60}-phenyldiazirine derivatives **9**.[27] These stereoisomers are readily separated by silica gel chromatography. Compounds **5** and **9** were characterized by spectroscopic methods.[28] The *cis*- and *trans*-stereochemistry of each compounds were determined according to the reported studies.[23,24]

In addition to the potential utility of fullerene-derived photoaffinity labels for elucidating the active site of C_{60} binding to enzymes such as GST and HIV-1 protease, the ability to selectively tag a protein or enzyme with fullerene may offer a new approach to the detection of biological molecules with high sensitivity. For example, an acidic isozyme of GST is specified as cancer expressing marker in liver cancers.[29,30] The ability to selectively tag such diagnostic enzymes with C_{60}, which has unique and useful chemical and photophysical properties, may offer a novel and rapid detection method for identifying trace amounts of enzyme present in a biological sample. These and other applications of the reported photoaffinity labeling reagents currently in progress.

In conclusion, we have described a concise and flexible route to fullerene-derived photoaffinity labels with potential utility in enzyme tagging and the elucidation of the binding sites of protein to C_{60}.

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25. To a solution of C_{60} (36 mg, 0.05 mmol) and D,L-alanine (9.2 mg, 0.10 mmol) in 1,2-dichlorobenzene (10 mL), acetaldehyde (11 mg, 0.25 mmol) was added and the mixture stirred at 120 °C for 20 h. The reaction process was checked by HPLC [silica gel column, solvent: benzene–EtOAc (10:1)]. The reaction mixture was purified by silica gel column chromatography (hexane–benzene–EtOAc) to give brown solid **2** (9.0 mg, 0.011 mmol, $y = 22\%$) as a *cis*- and *trans*-mixture.
26. To a solution of 4-azidobenzoic acid **3** (1.84 g, 11 mmol) in CH_2Cl_2 (5 mL), $SOCl_2$ (4.0 mL, 6.5 g, 55 mmol) in CH_2Cl_2 (5 mL) was added under argon atmosphere. Subsequently, dry DMF (1.5 mL) was added dropwise under Ar. After stirring for 2 h under Ar, the generation of acid chloride **4** was checked by TLC [solvent: hexane–EtOAc (1:1)] and then reaction mixture was filtered and concentrated in vacuo. To a solution of dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture, 20 mg, 0.025 mmol) in benzene (10 mL), acid chloride **4** (100 mg, 0.55 mmol) and pyridine 1 mL were added and the mixture stirred at 50 °C for 1 h. The reaction process was checked by TLC [benzene–EtOAc (1:1)], and then small amount of Et_3N was added. The products (*cis*- and *trans*-isomers) were separated by silica gel column chromatography (hexane–benzene–EtOAc) to give *cis*-**5** (8.9 mg, 9.5 μmol, $y = 38\%$) and *trans*-**5** (13.3 mg, 14.2 μmol, $y = 57\%$).
27. To a solution of 4-(3-trifluoromethylazirino)benzoic acid **6**, (9.7 mg, 0.042 mmol) with Et_3N (10 μL) in toluene (2 mL), 2,4,6-trichlorobenzoyl chloride (10 μL) was added and stirred under Ar at room temperature for 1.5 h. The reaction process was monitored by TLC [hexane–EtOAc (1:1)]. Subsequently, dimethyl fulleropyrrolidine **2** (10 mg, 12.6 μmol), DMAP 7 mg in toluene (4 mL) was added and then stirred under Ar at room temperature in dark condition for 2 days. The reaction process was monitored by TLC [benzene–EtOAc (1:1)] and then reaction mixture was purified by silica gel column chromatography (hexane–benzene–CH_2Cl_2) to give *cis*-**9** (2.5 mg, 2.5 μmol, $y = 20\%$) and *trans*-**9** (5.6 mg, 5.6 μmol, $y = 44\%$).
28. Selected spectroscopic data for *cis*-**5**: ^{1}H NMR ($CDCl_3$, 300 MHz): 2.28 (d, $J = 6.9$, 6H), 6.14 (q, $J = 6.9$, 2H), 7.23 (d, $J = 8.7$, 2H), 7.81 (d, $J = 8.7$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720. *trans*-**5**: 2.21(d, $J = 6.0$, 6H), 5.74 (q, $J = 6.6$, 2H), 7.22 (d, $J = 8.4$, 2H), 7.99 (d, $J = 8.4$, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): 19.9 (CH), 65.3 (CH3), 119.5 (CH), 130.2 (CH), 133.3–154.6 (C_{60}), 173.1 (CO); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720; FT-IR (KBr): 2122 (N_3), 1670 (CO), 1600, 1260, 1182, 842, 756, 527 cm^{-1}. *cis*-**9**: ^{1}H NMR ($CDCl_3$, 300 MHz): 2.27 (d, $J = 6.7$, 6H), 6.08 (q, $J = 6.7$, 2H), 7.40 (d, $J = 8.5$, 2H), 7.81 (d, $J = 8.5$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720. *trans*-**9**: 2.23 (d, $J = 6.9$, 6H), 5.17 (q, $J = 6.9$, 2H), 7.40 (d, $J = 8.3$, 2H), 7.81 (d, $J = 8.3$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720.
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DIFFERENT EXPRESSION OF GAP JUNCTIONAL PROTEIN CONNEXIN43 IN TWO STRAINS OF MICE AFTER ONE-MONTH IMPLANTATION OF POLY-L-LACTIC ACID

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Abstract. The implantation of a biomaterial often induces host inflammatory responses. Some adverse effects by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments. PLLA produced tumorigenicity in rats after long-term implantation. The purpose of this study was to determine the in vitro effect of PLAO3 (high-molecular weights of PLLA) and PU8 (PTMO/MDI/BD) on the function of the normal human dermal fibroblasts (NHDF) and the in vivo effect of PLAO3 on the function of the cells originated from the subcutaneous tissue in the two female mouse strains, BALB/cJ and SJL/J. The results with Scrape-loading and dye transfer (SLDT) assay, Western Blot and RT-PCR analysis clearly demonstrated that gap-junctional intercellular communication (GJIC) and the expression of Cx43 were significantly suppressed in PLAO3-implanted group of BALB/cJ mice in compared to the control mice. While, no significant difference was found in GJIC and the expression of mRNA level but a little bit difference was observed in the Cx43 protein expression between the SJL/J implanted and the control mice. We considered that the PLAO3 suppressed irreversibly gap junctional protein connexin43 at the earlier stage after implantation and the suppression of connexin43 gene-expression might play a vital role in the inhibition of GJIC and thus promotes the tumorigenesis.

Keywords: Poly-L-lactic acid, GJIC, Connexin43.

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