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UV B-irradiation enhances the racemization and isomerizaiton of aspartyl residues and production of N$^{\varepsilon}$-carboxymethyl lysine (CML) in keratin of skin☆

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ABSTRACT

UV-B irradiation is one of the risk factors in age-related diseases. We have reported that biologically uncommon D-β-Asp residues accumulate in proteins from sun-exposed elderly human skin. A previous study also reported that carboxymethyl lysine (CML; one of the advanced glycation end products (AGEs)) which is produced by the oxidation of glucose and peroxidation of lipid, also increases upon UV B irradiation. The formation of D-β-Asp and CML were reported as the alteration of proteins in UV B irradiated skin, independently. In this study, in order to clarify the relationship between the formation of D-β-Asp and CML, immunohistochemical analysis using anti-D-β-Asp containing peptide antibodies and anti-CML antibodies was performed in UV B irradiated mice. Immunohistochemical analyses clearly indicated that an anti-D-β-Asp containing peptide antibody and anti-CML antibody reacted at a common area in UV B irradiated skin. Western blot analyses of the proteins isolated from UV B irradiated skin demonstrated that proteins of 50–70 kDa were immunoreactive towards antibodies for both D-β-Asp containing peptide and CML. These proteins were identified by proteomic analysis as members of the keratin families including keratin-1, keratin-6B, keratin-10, and keratin-14.

1. Introduction

Biologically uncommon D-aspartyl (Asp) residues have been reported in proteins of various tissues such as tooth [1], eye lens [2,3], retina [4], conjunctivae [4], cornea [4], skin [5], aorta [6], and brain [7] in elderly humans. Aspartic acid is the most easily racemizable amino acid and D-Asp may be formed by racemization in metabolically inactive tissues during the chronological aging process. In previous studies, we identified a biologically uncommon D-isomer at Asp-58 and Asp-151 in αA-crystallin [2] and also at Asp-36 and Asp-62 in αB-crystallin [3] from aged human lenses. D-Asp formation was also accompanied by isomerization from the natural α-Asp to the biologically uncommon β-Asp (isoaspartate) through a succinimide intermediate. Therefore, four isomers, namely normal L-α-Asp, biologically uncommon L-β-Asp, D-α-Asp, and D-β-Asp are formed in protein spontaneously during aging [8]. Since D-β-Asp was the major isomer of the uncommon species we prepared an antibody which reacts specifically with D-β-Asp-containing peptides thus allowing the detection of the location of D-β-Asp-containing protein in any tissue [9]. Consequently, we detected D-β-Asp-containing protein in the sun-exposed skin of elderly donors but not in sun-exposed skin of young donors or the sun-protected skin of elderly donors [5]. This result strongly indicates that UV irradiation enhances D-β-Asp formation in protein upon aging.

A previous study using an anti-carboxymethyl lysine (CML) antibody [10] reported that advanced glycation end products (AGEs) also increased in UV B irradiated skin. AGEs are formed by metal-catalyzed oxidation of glucose or Amadori products and are thought to be involved in aging or age-related diseases such as diabetes [11], atherosclerosis [12], Alzheimer's disease [13], and cataract [14,15]. These findings echo the detection of D-β-Asp in age-related disease. The AGEs products are very complicated and include pyrraline [16], pentosidine [17] and carboxymethyl lysine (CML) [18].

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CML is formed by oxidative cleavage of Amadori products or Schiff bases or by modification with glyoxysal generated through auto-oxidation of glucose and also by lipid peroxidation [19,20]. CML has been proposed as a potential marker of oxidatively damaged tissues or proteins *in vivo*. Mizutari [10] reported that CML modification occurred in photo-induced degenerated areas of skin. The result suggests that UV induced oxidation accelerates the formation of CML in photo-aged skin. Both D-β-Asp and CML formation have been observed in similar tissues with aging or upon UV B-irradiation but as yet any correlation between the two is unclear. In this study, in order to clarify the relationship between the formation of D-β-Asp and CML, immunohistochemical analysis using anti-D-β-Asp antibodies and anti-CML antibodies was performed in UV B irradiated mouse skin. Furthermore, those proteins that undergo D-β-Asp and CML formation by UV B irradiation were identified by MALDI-TOF/MS/MS as members of the keratin family of proteins.

2. Materials and methods

2.1. Human skin

In the present study, informed consent was obtained from all donors. This study was conducted according to the principles of the Declaration of Helsinki, and was approved by the Medical Ethical Committee of the National Defense Medical College. Normal-appearing skins of sun-exposed and sun-protected areas were obtained from the face region of 80-year-old donors and the inguinal region of 70-year-old donors.

2.2. Irradiation of mouse skin with UVB

Normal back skin of HR-1 mice (5 week-old) received UVB irradiation at a total dose of 100 mJ/cm^2 or 200 mJ/cm^2. Skin samples were taken by punch biopsy before and 3 days after irradiation, embedded in paraffin, then subjected to immunohistochemical examination using the antibodies. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kyoto University Animal Experimentation Regulation.

2.3. Antibody against D-β-Asp containing peptide

The preparation and characterization of the antibody used are described in our previous paper [9]. The polyclonal antibody against the peptide Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr (designated peptide 3R), containing three repeats of position 149–153 of human αA-crystallin optic isomer, was purified from rabbit serum by affinity chromatography using peptide 3R and bovine αA-crystallin as ligands. The anti peptide 3R antibody clearly distinguished the configuration of the Asp-residue, such that it reacted very strongly with the D-β-Asp containing peptide but did not react with the L-α-Asp, D-α-Asp and L-β-Asp-containing peptides [9]. The antibody clearly recognized the presence of D-β-Asp containing αA-crystallin in aged human lenses [9].

2.4. Anti-CML antibody

Monoclonal anti-CML antibody (Trans Genic Inc., Kobe, Japan) was used for immunohistochemistry. Polyclonal anti-CML antibody which was prepared by Horiuchi et al. [21] was used in western blotting.

2.5. Immunohistochemistry

Skin specimens were embedded in paraffin and cut into 2–4 μm thick sections. The sections were deparaffinized with xylene and were washed twice with 100% ethanol for 3 min, with 90% ethanol for 3 min, with 80% ethanol for 3 min, and then washed with distilled water at room temperature. Endogeneous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide in 70% methanol for 20 min. The sections were washed three times with phosphate buffered saline (PBS) and were blocked with 5% skimmed milk/PBS for 1 h at room temperature. The sections were incubated with the antibodies against peptide 3R or CML at 1:100 dilution for 17 h at 4 °C, then incubated with EnVision$^+$Polymer anti-rabbit IgG antibody (Dako, Glostrup, Denmark) at 1:500 dilution for antibodies for peptide 3R or EnVision$^+$Polymer anti-mouse IgG antibody (Dako, Glostrup, Denmark) 1:500 dilution for anti CML antibody for 1 h at room temperature. The location of antigen–antibody complexes was detected using 3,3′-diaminobenzidine (DAB) substrate kit (Dako, Glostrup, Denmark).

For double immunofluorescence labeling, skin sections with a histological change of actinic elastosis (70-year old facial skin) were incubated with polyclonal antibody for peptide 3R (1:100) and monoclonal antibody for CML (1:100) for 17 h at 4 °C. Bound antibodies were visualized with fluorescein-conjugated (Alexafluor 594) anti-rabbit IgG antibody (1:500) and fluorescein-conjugated (Alexafluor 488) anti-mouse IgG antibody (1:500). Evaluation of fluorescence was performed with a confocal laser scanning microscope (LSM 410; Carl Zeiss, Jena, Germany).

2.6. Preparation of protein from UVB irradiated and non-irradiated skin

The UV B 200 mJ/cm^2 irradiated and non-irradiated mouse skin samples were cut into pieces of 2 mm and immersed in 8 M urea, 2 M thiourea, 3% CHAPS solution for 2 days at 4 °C. The sample was homogenized in the above solution and the homogenate was centrifuged at 10,000 × *g* at 4 °C for 30 min. The supernatant was analyzed by two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE).

2.7. Two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE)

Isoelectric focusing in the first dimension was performed in 11 cm ReadyStrip™ IPG Strips (pH 3-10) (BioRad, Hercules, California, U.S.A.). The electrophoresis in the second dimension was performed in a slab gel containing 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide.

2.8. Detection of D-β–Asp containing proteins and AGE-modified proteins by immunoblotting

After the 2-D-PAGE, the separated proteins were transferred to Immobilon™ Transfer PVDF Membrane (0.45 μm) (Millipore, Billerica, Massachusetts, U.S.A.) under semi-dry conditions with the use of Transblot (Millipore). The membranes were blocked by incubation with 5% skimmed milk in PBS at 4 °C overnight. Subsequently, the membranes were washed three times with PBS containing 0.05% Tween 20 for 15 min each, and incubated with the diluted (1:300) polyclonal anti-peptide 3R or anti-CML antibodies at room temperature for 1 h. After being washed, the membranes were incubated with horseradish peroxidase (HRP)-conjugated (1:5000) anti-rabbit IgG antibody for 1 h at room temperature. The western blots were visualized using a chemiluminescence method

using an ECL Western blotting detection kit (Amersham, Buckinghamshire, England) on LAS 4000 mini (Fuji Film, Tokyo, Japan).

2.9. In-gel digestion

The 2-D-PAGE gels were stained with coomassie brilliant blue (CBB). The 2-D gel spots that were judged to include D-β-Asp-containing protein and CML-modified protein from the immunoblotting were excised and cut into pieces. The CBB stain was removed by 50% acetonitrile, 50 mM NH_4HCO_3. The gel pieces were then saturated in 100% acetonitrile and dried in an evaporator. For trypsin digestion (using modified, sequencing grade enzyme from Promega, Fichburg, Wisconsin, U.S.A), the dried gel pieces were re-swollen in trypsin reagent (30% acetonitrile, 50 mM NH_4HCO_3 containing modified trypsin) and incubated overnight at 37 °C. After digestion, the supernatant was desalted using ZipTipc18 tips (Millipore).

2.10. MALDI-TOF/MS/MS

The desalted supernatant (5 μl) was mixed with an equal volume (5 μl) of matrix solution. As matrix solution, α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg) was dissolved in 1 ml of solution containing a 1–1 ratio of aqueous 0.1% trifluroacetic acid and acetonitrile. The mixed solution was spotted onto a 384-spot MALDI target and then dried. All spectra were obtained using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (AXIMA TOF2, Schimadzu, Kyoto, Japan). The MALDI-TOF equipment was operated with a nitrogen laser with a wavelength of 337 nm and ion acceleration voltage of 20 kV. The data were collected in reflection mode as signals of positive ions.

3. Results

3.1. Immunohistochemical staining

As shown in Fig. 1a and c, the upper to mid dermis of the sun-exposed skin (glabellar skin) was strongly stained with the anti-peptide 3R antibody and anti-CML antibody but this was not the case for the sun-protected skin (inguinal region) taken from a 70 year-old individual donor (Fig. 1b and d). Since the sun-exposed regions which were stained both with the anti-peptide 3R antibody and with the anti-CML antibody seemed to be the same area, we performed double immunofluorescence labeling of the sun-exposed skin using both antibodies. The red and green colors show the immunoreactive area recognized by the anti-peptide 3R antibody (Fig. 2a) and the anti-CML antibody (Fig. 2b), respectively. The co-localization is demonstrated by the yellow-color in the same section (Fig. 2c). These results suggest that the formation of D-β-Asp residues in proteins from sun-damaged skin occurs concomitantly with CML modification. This result clearly indicates that the D-β-Asp formation and AGEs modification in the proteins are simultaneously accelerated by sun-light exposure. Although several samples were used in this study, there were no significant differences in the results from the immunohistochemistry analysis over the 70–80 year range.

To further confirm the effect of sun-light irradiation on the skin, we performed immunohistochemistry on several specimens obtained from UV B-irradiated (100 mJ/cm^2 and 200 mJ/cm^2) and non-irradiated mouse skin using the anti-peptide 3R antibody and anti-CML antibody. As shown in Fig. 3b and c, both the epidermis and the upper to mid-dermis strongly reacted with anti peptide 3R antibody in a UV B dose dependent manner. The anti-CML antibody also recognized the same area of UV B-irradiated skin as that strongly recognized by anti-peptide 3R antibody (Fig. 3e and f). However, both antibodies failed to react to non-irradiated skin (Fig. 3a and d). These results also clearly show that D-β-Asp formation and CML are co-localized in the same area of UV B irradiated skin.

3.2. Detection of D-β-Asp-containing proteins and CML-modified proteins in UV B irradiated mouse skin

In order to identify the proteins which reacted with both anti-peptide 3R antibody (Fig. 3c) and anti-CML antibody (Fig. 3f), the proteins were extracted from the UV B 200 mJ/cm^2 irradiated mouse skin with 8 M urea, 2 M thiourea, 3% CHAPS solution, then analyzed by 2-D-PAGE followed by western blotting using anti-peptide 3R and anti-CML antibodies. Fig. 4a shows the CBB stained 2-D-PAGE of the UV B 200 mJ/cm^2 irradiated mouse skin. Fig. 4b and c shows the results of western blotting of the same sample using the anti-peptide 3R and anti-CML antibodies, respectively. Eleven proteins indicated by arrows in Fig. 4a, clearly showed common reactivity with the anti-peptide 3R antibody and anti-CML antibody.

3.3. Identification of D-β-Asp-containing and CML-modified proteins by MALDI-TOF–MS analysis

In order to identify the proteins detected by immuno-staining with both anti-peptide 3R and anti-CML antibodies on the 2-D gel, we analyzed the selected proteins which were stained with CBB by mass spectrometry. We selected 11 spots (indicated by arrows in Fig. 4a) from the 2-D gel, which were tentatively identified as D-β-Asp-containing and CML-modified proteins, for in-gel trypsinization and analysis by MALDI-TOF–MS. The mass data was fitted by MASCOT using the Swiss-Prot database search analysis. From the database search, we identified 6 proteins from 11 spots in Fig. 4a with high scores. Spot number 4 was identified as keratin-1 with a high MOWSE Score. Total coverage represented 32% of the predicted protein sequence. Spots 1–3 were also identified as keratin-1 but the MOWSE Scores were low compared with spot number 4. This may be a result of the post-translational modifications of keratin-1. Spot number 10 in Fig. 4a was identified as keratin 10 with a high MOWSE Score (Fig. 5). Total coverage of spot number 10 represented 22% of the predicted protein sequence. Spot number 11 was identified as keratin 14 with a high MOWSE Score. Total coverage of spot number 11 represented 20% of the predicted protein sequence. Spot numbers 7–9 were also identified as keratin 6B with high MOWSE Scores. Several spots with *pI* values different from that of keratin 6B may result from the modification of the protein. The results are summarized in Table 1.

4. Discussion

The racemization of Asp residues and CML modification in proteins has been studied independently. Both modifications of proteins have been observed in age-related diseases under oxidative stress. The present study uses immunohistochemistry with anti peptide 3R antibody and anti-CML antibody to demonstrate for the first time that these two different kinds of non-enzymatic modifications are generated in the same tissues. The D-β-Asp and CML modifications occur in a common region of sun-exposed skin (Fig. 1a and c) but not in sun-protected skin (Fig. 1b and d). Furthermore, the D-β-Asp and CML modified proteins were observed to be co-localized to the same section of the epidermis and dermis of the sun-exposed skin by a double-labeling method with anti-peptide 3R and anti-CML antibody (yellow color in Fig. 2c). This result suggests that both modifications are related to the effects of UV-B irradiation. Therefore, we irradiated normal mouse back skin with a dose of UV B of 100–200 mJ/cm^2. UVB irradiation induced the

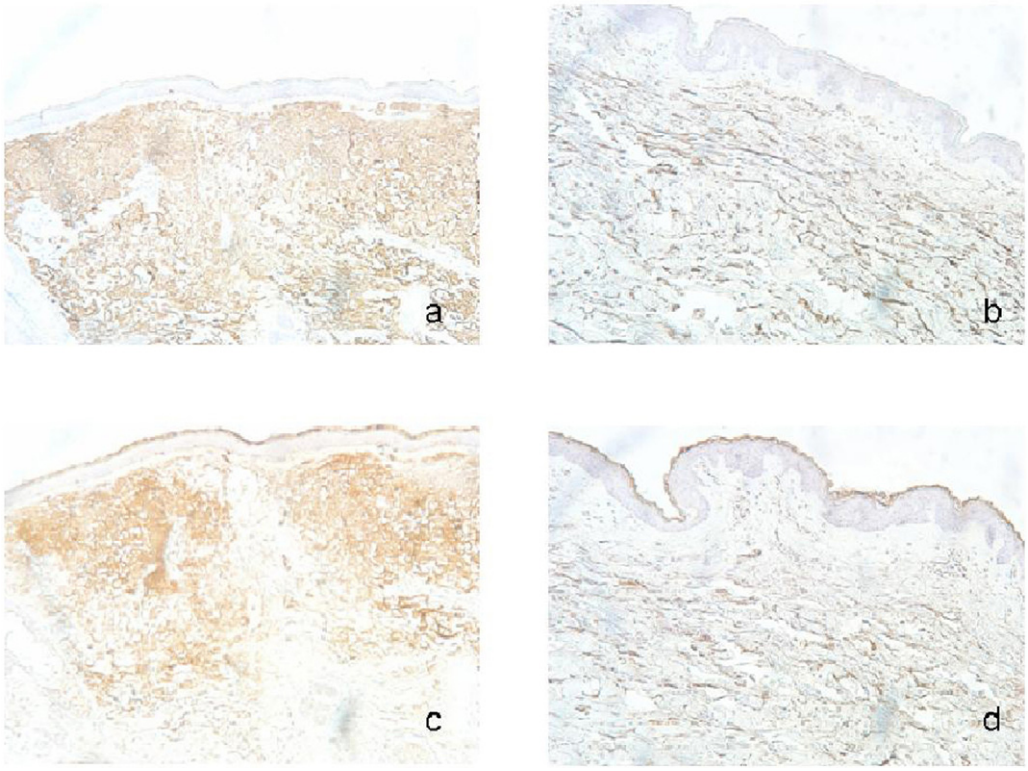

Fig. 1. Immunohistochemical localization of D-β-Asp-containing protein and AGE-modified protein in the skin from an 80 or 70-year-old donor. (a) Immunohistochemistry of sun-exposed skin (glabellar skin) using anti-peptide 3R antibody. (b) Sun-protected skin (inguinal region) using anti-peptide 3R antibody. (c) Immunohistochemistry of sun-exposed skin (glabellar skin) using anti-CML antibody. (d) Immunohistochemistry of sun-protected skin (inguinal region) using anti-CML antibody. Skin specimens were embedded in paraffin then immunoreacted with the antibodies for peptide 3R and CML. The sections were incubated with rabbit anti-Ig antibody at 1:50 dilution for 2 h. Antigen–antibody complex was visualized by avidin–biotin complex.

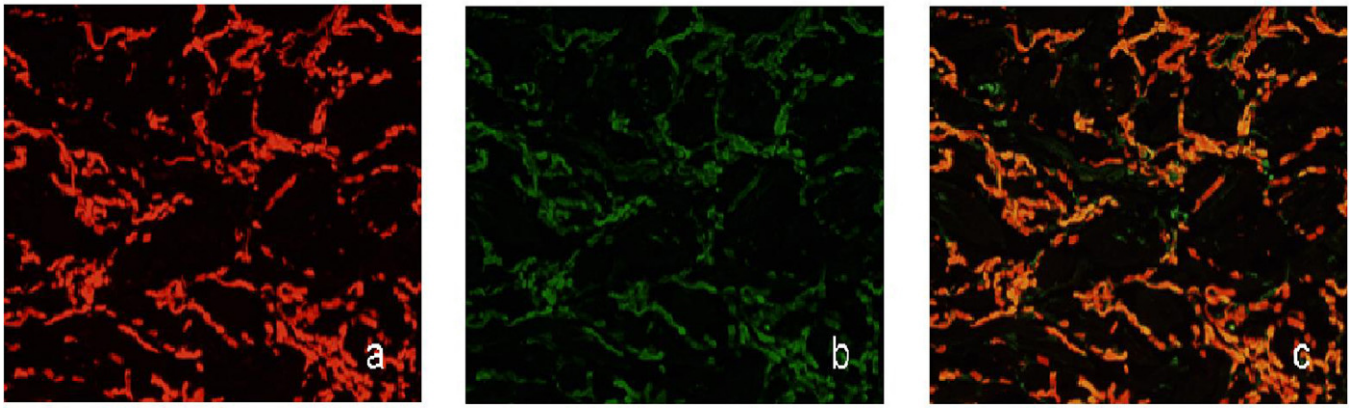

Fig. 2. Double immunofluorescence labeling of sun-damaged skin (80 or 70-year-old glabellar skin) using the antibodies for D-β-Asp containing peptide (peptide 3R) and CML. Cryostat sections were incubated with polyclonal antibody for peptide 3R (a) or monoclonal antibody for CML (b) or both (c). Bound antibodies were visualized with rhodamine-conjugated anti-rabbit Ig antibody (1:40) or fluorescein-conjugated anti-mouse Ig antibody.

Table 1
List of D-beta Asp and AGE modified proteins of mouse skin after UVB 200 mJ/cm^2 irradiation.

Spot no. in Fig. 4a	Identified protein	Accession no.	MW (Da)	*pI*	Sequence homology (%)	Score
4	Keratin-1	NP_032499	66079	8.39	32	81
7	Keratin-6B	AAI22885	60533	8.50	31	66
8	Keratin-6B	AAI19556	60578	8.33	25	57
9	Keratin-6B	AAI19556	60578	8.33	27	67
10	Keratin-10	NP_034790	57178	5.00	22	132
11	Keratin-14	NP_058654	53176	5.10	20	64

Type of search: peptide mass fingerprint.
Enzyme: trypsin.
Fixed modifications: carbamidomethyl.
Variable modifications: oxidation (methionine), phospholyration (serine, threonine, tyrosine).
Mass tolerance: ±0.5 Da.

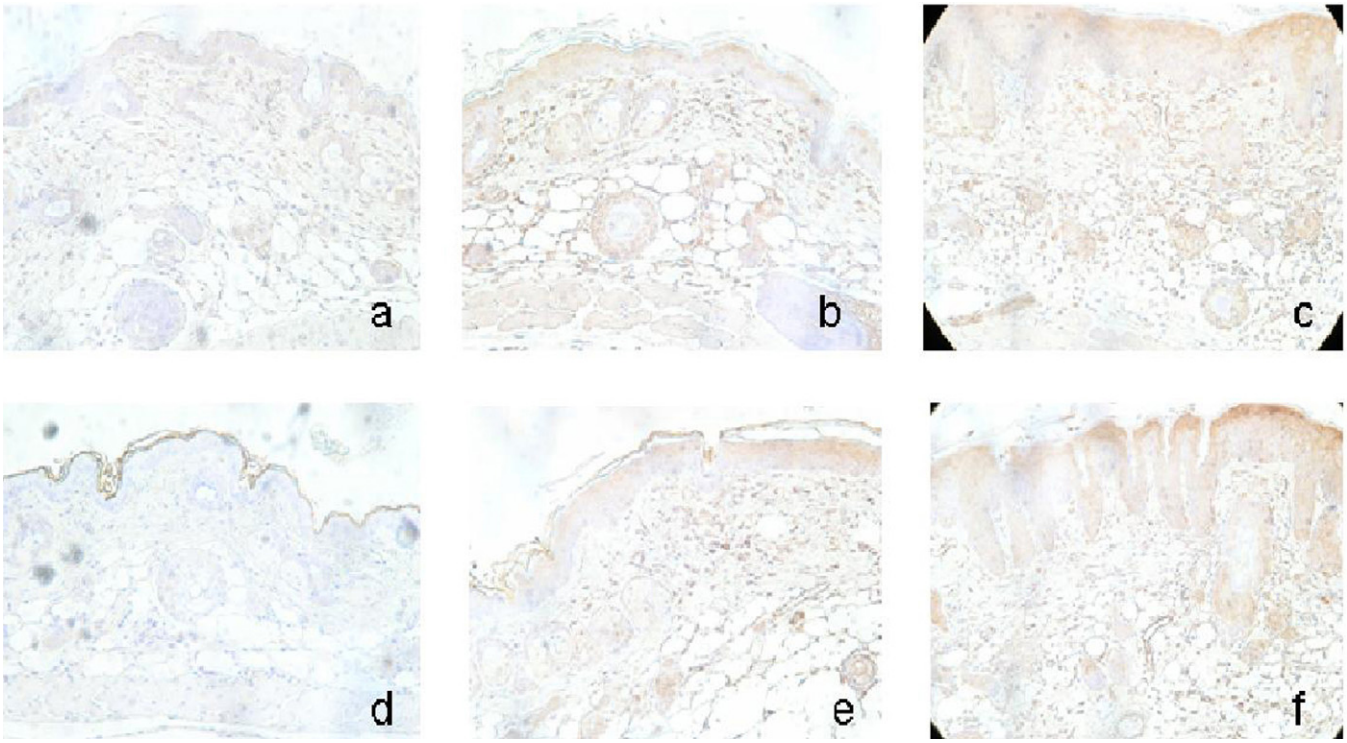

Fig. 3. Immunohistochemical localization of D-Asp-containing protein and AGE-modified protein in UV B irradiated mouse skin. The proteins were extracted from mouse skin 3 days after irradiation. (a) Immunohistochemistry of non-irradiated mouse skin using anti-peptide 3R antibody. (b) Immunohistochemistry of the 100 mJ/cm^2 UV B irradiated mouse skin using anti-peptide 3R antibody. (c) Immunohistochemistry of the 200 mJ/cm^2 UV B irradiated mouse skin using anti-peptide 3R antibody. (d) Immunohistochemistry of non-irradiated mouse skin using anti-CML antibody. (e) Immunohistochemistry of the 100 mJ/cm^2 UV B irradiated mouse skin using anti-CML antibody. (f) Immunohistochemistry of the 200 mJ/cm^2 UV B irradiated mouse skin using anti-CML antibody.

production of these modified proteins in the epidermis and dermis of the mouse (Fig. 3).

Our previous study showed that D-β-Asp containing proteins exist in the elastic fibers of dermis from sun-exposed skin of elderly people [5]. This abnormal proteins were suggested to be elastin by western blotting with an anti-elastin antibody [5]. We undertook to extract the abnormal proteins of the dermis and identify by mass spectroscopy the peptide fragments of the proteins but we did not succeed in the analysis because of the insolubility of the protein. Since elastin in human skin has three Asp residues, we synthesized three Asp-containing model peptides corresponding to sections of elastin and analyzed the kinetics of Asp racemization in these peptides. The results suggest that the racemization of Asp residues in these peptides occurred rapidly and the time required for the racemization of Asp residues in elastin is such that it can occur in a human lifetime [22].

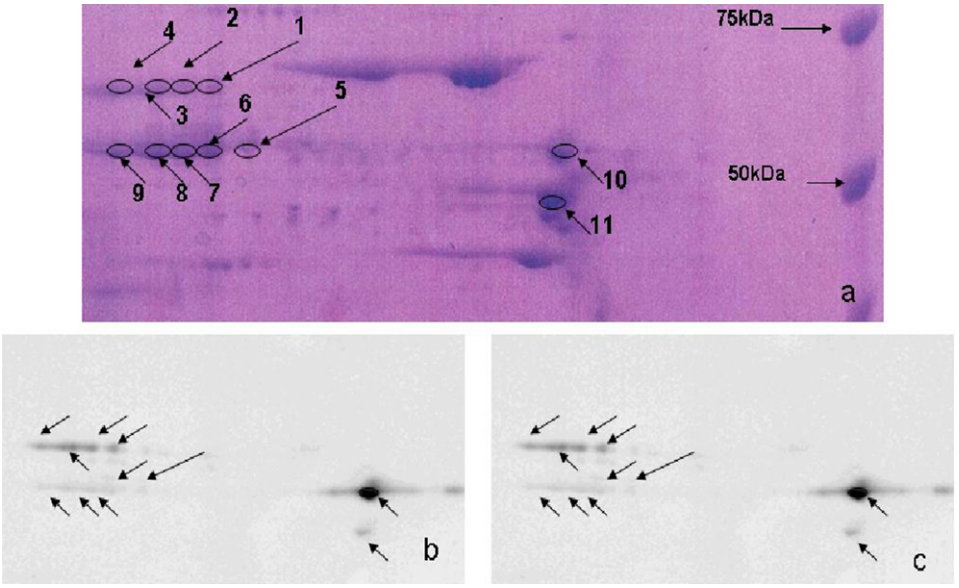

Fig. 4. 2-D PAGE and western blot analysis of the proteins extracted from mouse skin 3 days after 200 mJ/cm^2 UV B irradiation. Detection was performed using (a) Coomassie blue-staining, (b) anti-peptide 3R antibody and (c) anti-CML antibody.

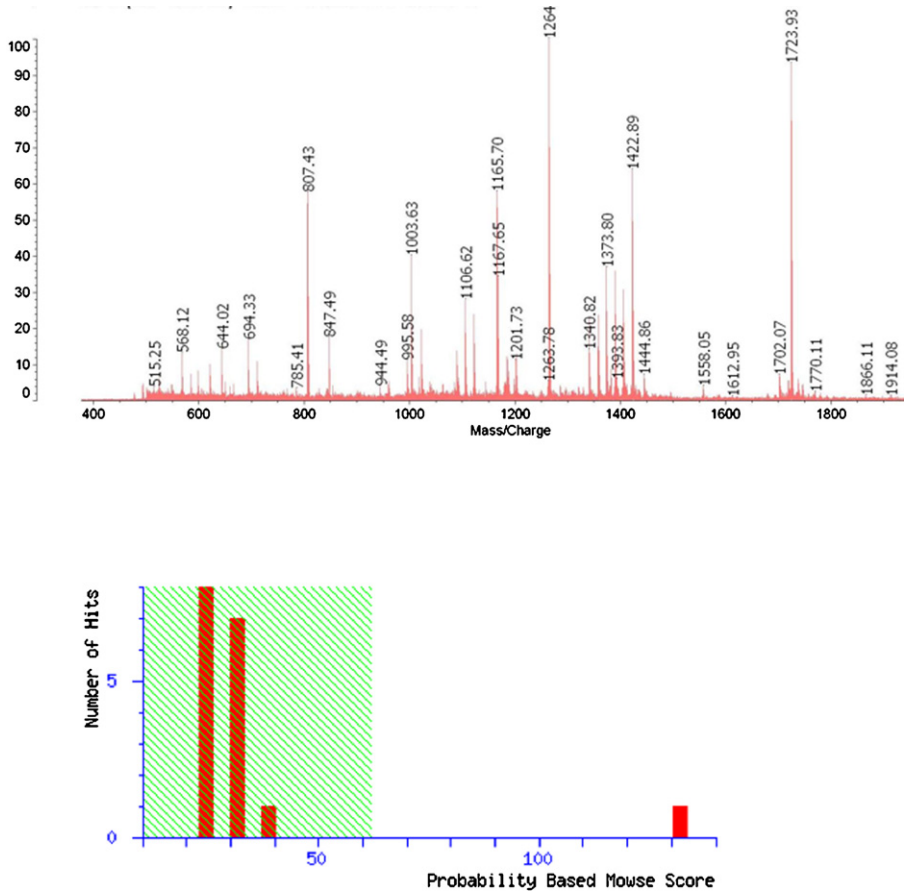

Fig. 5. (a) MALDI-TOF–MS spectrum of spot 10 in Fig. 4, and (b) the result of MASCOT analysis.

In this study, we succeeded in identifying the D-β-Asp containing proteins in the epidermis from UV B-200 mJ/cm^2 irradiated mouse skin. The extraction of proteins from the epidermis is easier than that from dermis. Fig. 4a shows the extracted proteins from UV B-200 mJ/cm^2 irradiated mouse skin. Eleven of the visible spots clearly reacted with both anti-peptide 3R and anti-CML antibodies. These proteins were treated with trypsin and the resulting peptides were analyzed by mass spectrometry and identified using the MASCOT online database searching algorithm. These abnormal proteins were identified as keratin family proteins such as keratin-1, 6B, 10 and 14.

Aspartic acid is the most easily racemizable amino acid in proteins. Usually, this reaction proceeds with difficulty in mild conditions such as those found in the living body. However, Asp residues in protein are susceptible to racemization because D-Asp formation in proteins occurs via a succinimide intermediate as follows: (i) When the carbonyl group of the side chain of the L-α-aspartyl residue is attacked by the nitrogen of the amino acid residue following the Asp residue, L-succinimide is formed by intramolecular cyclization; (ii) L-succinimide may be converted to D-succinimide through an intermediate that has the prochiral α-carbon in the plane of the ring; (iii) the D- and L-succinimide are hydrolyzed at either side of their two carbonyl groups, yielding both β- and α-Asp residues, respectively. The rate of succinimide formation is expected to depend on the neighboring residue of the Asp. When the neighboring amino acid of the Asp residue has a small side chain, such as glycine, alanine or serine, the formation of succinimide occurs easily because there is no steric hindrance. Racemization and isomerization of amino acids in protein can cause major changes in structure, since different side chain orientations can induce an abnormal peptide backbone. Therefore, these posttranslational modifications can induce the partial unfolding of protein leading to a disease state. In fact, αA-crystallin from eye lens from elderly donors, contains relatively high amounts of D-β-Asp which results in abnormal aggregation to form huge and heterogeneous aggregates and a loss of its chaperone activity [23]. The relationship between generation of D-β-Asp and UV irradiation is not well understood. However, we have demonstrated that the amount of D-β-Asp increased in proteins from lens [24] and skin [5] upon exposure to UV irradiation. Succinimide-related reactions to form D-β-Asp are known to occur more readily in unfolded proteins than in native proteins [25]. Hott et al. [26] reported that the unfolding of gamma-II crystallin was induced by UV irradiation while Belcher et al. [27] indicated that UV irradiation causes a large unfolding of the protein with an electron-transfer mechanism that is capable of triggering structural changes in the protein. Therefore, UV B irradiation may induce localized unfolded structure surrounding specific Asp residues in keratin proteins thus accelerating succinimide formation which ultimately generates D-β-Asp residues.

AGEs are another well known non-enzymatic modification of proteins. This study indicated that keratin-1, 6B, 10 and 14 simultaneously reacted with both the anti-peptide 3R antibody and anti-CML antibody. The glycation reaction occurs between the carbonyl group of sugars and the free amino group of proteins. AGE modified proteins were reported in diabetes [11], atherosclerosis [12], Alzheimer's disease [13], cataract [14,15] and UV B-irradiated skin [10]. The AGEs are generally pigmented or fluorescent adducts of proteins and participate in the formation of protein cross-links [17]. Formation of CML may alter the surface charge of the protein, leading to conformational change, which in turn may effect protein–protein and

protein–water interactions. The present result suggests that the racemization of Asp residues and glycation proceed in keratin-1, 6B, 10 and 14 proteins at the same time upon UVB irradiation.

Keratin filaments are abundant in keratinocytes in the cornified layer of the epidermis (these are cells which have undergone keratinization). During the process of epithelial differentiation, cells become cornified as keratin protein is incorporated into longer keratin intermediate filaments. Eventually the nucleus and cytoplasmic organelles disappear, metabolism ceases and cells undergo a programmed death as they become fully keratinized. The keratin family is composed of approximately 20 different keratins. These keratins form "keratin pairs" between acidic and basic keratin. Keratin-1 forms a keratin pair with keratin 10 and is localized in the stratum spinosum and granular layer. Keratin 6B exists in epithelial tissue and forms a keratin pair with keratin 17. They are also generated upon inflammation. Keratin 14 exists in basal cells of epithelial tissue and forms a keratin pair with keratin 5 or keratin 15 [28].

The racemized and CML modified keratins may have abnormal properties and may lead to abnormal fibril formation in the epidermis. When skin is irradiated with UV B, defense mechanisms against UV B irradiation are activated and the activity of the squamous epidermal cells increases and subsequently the epidermal layer develops hyperplasia. In this situation, there are a lot of reactive oxygen species (ROS). CML formation is known to proceed via ROS including H_2O_2, superoxide and hydroxyl radicals. Nagai et al. [29] indicated that catalase and dismutase and hydroxylradical scavengers inhibited CML formation. The formation of CML may change the charge of the protein, leading to alteration of the tertiary structure of the protein and this may promote the racemization of Asp residues in the protein; our previous studies showed that the racemization of Asp depends on the tertiary structure of the protein. Furthermore, Ahmed et al. [18] showed the pH dependence of CML formation, *i.e.* the rate of CML formation increased at pH higher than 7.4. The tendencies are similar to the susceptibility of the racemization of amino acids.The chemical relationship between production of CML and racemization is not yet known but our findings suggest that it is important to identify and determine the levels of proteins modified by oxidative stress.

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