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Current status and perspectives of proteomics in aging research

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Abstract

The accumulation of non-enzymatic modifications on both DNA and protein molecules under the attack of reactive oxygen species (ROS), is one of the most possible factors responsible for the functional deterioration in aged cells. Direct protein modifications as well as DNA damages may be detectable, in part, by proteome analysis if the gene expression is affected by the damages on DNA. The novel term "proteome", which is a compound of "protein" and "genome", means a whole set of proteins expressed in a tissue or a cell strain to be investigated. Proteomics is a methodology for analyzing proteomes. In proteomics, two-dimensional gel electrophoresis is performed primarily to separate constitutive proteins, followed by mass spectrometry to identify each protein of interest and to determine a possible post-translational modification. Proteomics has offered us an innovative tool for investigating the molecular mechanisms of cellular aging. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

The age-dependent deterioration of cell function appears in both replicative and post-mitotic cells, in living organisms. The major function of stem cells is cell division in the regeneration of cell population and tissues. Most replicative blast cells play another role: in the excretion of cytokines for inter-cellular signal transduction. Post-mitotic cells in the brain, heart, kidney and many other organs have tissue-specific functions. Although a downstream of the cascade in cellular aging may be cell-type specific, and may proceed in a given intracellular environment that is made with all constituents of the proteome,

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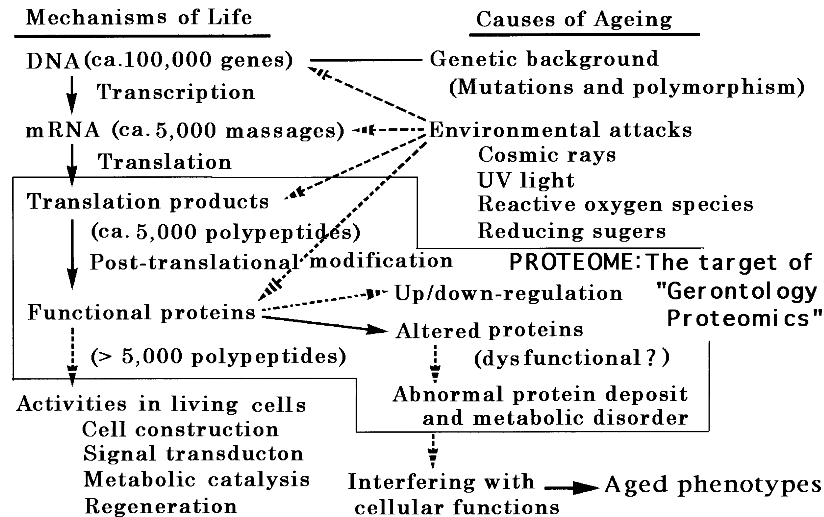

Fig. 1. Possible causes of aging in the mechanisms of life, and the target of "Gerontology Proteomics". Cascade of cellular aging may proceed in an intracellular environment that is made with cell-type-specific "proteome". Many primary causes of cellular aging are commonly speculated for all cell types including mitotic and post-mitotic cells. Genetic background is one of the most important factor that determine the quality of the defense system against environmental attacks: Cosmic rays, UV light, reactive oxygen species (ROS), and reducing sugars are significant elements of environmental attacks. Results of the battle against these environmental attacks may be revealed in their proteome profiles.

there may be common factors in the upstream of the cascade. The accumulation of molecular damages on DNAs and proteins under environmental attacks including oxidative stress, may be one of the major events that trigger the cascade of cellular aging as described in Fig. 1.

Oxidative protein modification may alter methionine residue to sulfoxide, phenylalanine to diphenyl, and lysyl residue to carbonyl (Smith et al., 1997; Wells Knecht et al., 1997; Stadtman and Berlett, 1998). In proteomics, such protein altered by post-translational modification, may be separated from the original form by high resolution two-dimensional gel electrophoresis (2-DE), and the modification may be determined by mass spectrometry (MS).

2. Materials and methods

Immobiline DryStrip and Pharmalyte were purchased from Amersham Pharmacia Biotech KK (Tokyo, Japan). Sequi-Blot PVDF membrane was obtained from the Nippon Bio-Rad Laboratories (Tokyo, Japan). Acrylamide, *N*,*N*′-methylenebisacrylamide and TEMED were from Daiichi Pure Chemicals (Tokyo, Japan). Sequencing grade endoproteinase Lys-C was from Roche Molecular Biochemicals (Indianapolis, IN, USA). Trizma base, Tricine, SDS, Triton X-100, iodoacetamide, acetonitrile, trifluoroacetic acid and alpha-cyano 4-hydroxy-cinnamic acid were from Sigma (St. Louis, MO, USA). Quick

CBB staining reagent, silver staining reagent kit "Wako", urea, and other chemicals of reagent grade were obtained from Wako Pure chemicals (Osaka, Japan).

2.1. Two-dimensional gel electrophoresis

Constitutive proteins in a cell extract were separated into isolated spots by 2-DE in an "immobilized pH gradient isoelectric focusing (IPG-IEF)/sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system" according to the standard protocol (Toda and Kimura, 1997), with minor modifications. The details of the modified protocol are shown in our web homepage: http://proteome.tmig.or.jp/2D/2DE_method.html. In brief, the first-dimensional IPG-IEF was carried out on a re-swollen Immobiline DryStrip, pH 4–8, 18-cm long (Code No. 18-1004-34) in the CoolPhoreStar IPG-IEF Type P horizontal IEF apparatus (Anatech, Tokyo, Japan). A 20-μl aliquot of the sample solution, which was absorbed in a small piece of filter paper, was applied near the cathode wick on the IPG gel. Spot proteins on a gel plate for "differential protein display" were subsequently visualized by silver staining. For the preparative use of 2-DE gel, the IPG gel was swollen in a solution containing 0.2 ml of cell extract. After 46,700-Vh electrofocusing, the IPG gel was equilibrated with the SDS-treatment solution (50 mM Tris–HCl, pH 6.8, 6 M urea, 0.5% (w/v) dithiothreitol, 2% (w/v) SDS, 0.005% (w/v) BPB, 25% (v/v) glycerol), initially for 30 min, and then followed by carbamoylmethylation in an iodoacetamide-containing buffer (50 mM Tris–HCl, pH 6.8, 6 M urea, 4.5% (w/v) iodoacetamide, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue (BPB), 25% (v/v) glycerol) for 10 min. An equilibrated gel strip was placed on top of a gel slab (7.5%T, 3%C, 20×18 cm), and firmly contacted to the top of a gel slab by pressing the gel strip with a shark-teeth comb. SDS-PAGE was run vertically in the CoolPhoreStar SDS-PAGE Tetra-200 vertical slab gel electrophoresis apparatus (Anatech, Tokyo, Japan) using a tricine buffer system.

2.2. Protein staining and image processing

Protein spots were visualized on the gel slab for a differential protein display analysis by silver staining, using the original version of the reagent kit "Wako" as it showed the widest dynamic range in optical density among all the commercially available reagents tested. The 2-DE gel images were acquired using a Sharp JX-330 scanner. Noise reduction, background subtraction, spot detection, spot quantification, gel-to-gel matching and differential protein analysis were carried out using a PDQuest software (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Endoproteinase digestion and peptide mass finger printing

After scanning, Coomassie-stained proteins in the gel slab were re-solubilized and transferred onto a PVDF membrane as follows. The stained gel was rinsed in pure water thrice, each time for 30 min, incubated in a re-solubilization buffer (0.2% (w/v) SDS, 0.3% (w/v) Tris, 0.7% (w/v) glycine) for 10 min and mounted on an electrotransfer

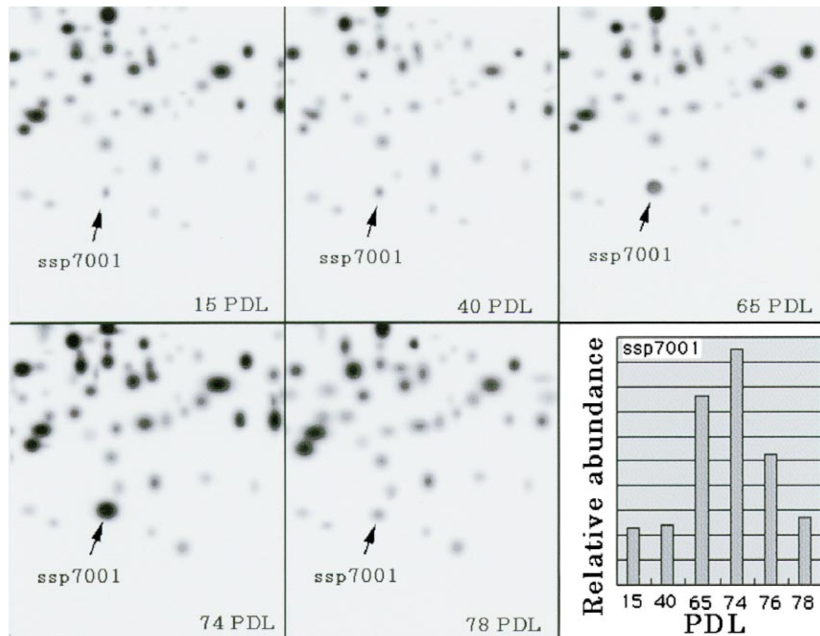

Fig. 2. Detection of age-related protein alteration by 2D gel electrophoresis and quantitation of relative intensity by image processing in proteome analysis. The relative intensity of ssp7001 shows a transitional increase around 65 PDL, which is the border between phases 2 and 3 of cellular aging of normal human diploid fibroblasts TIG-3.

blotting chamber. The electrotransfer was carried out overnight at 4 V/cm in 0.1% (w/v) SDS, 0.3% (w/v) Tris and 1.5% (w/v) glycine.

Protein spots were excised from the PVDF membrane and the Coomassie dye was removed by rinsing in 60% (v/v) methanol. For the MS analysis, the piece of membrane was incubated with 4.5% (w/v) polyvinyl pyrolidone 25 in 2.5 mM Tris–HCl, pH 7.7, for 30 min, followed by a brief rinsing in 5% (v/v) acetonitrile. The digestion was carried out overnight with 0.1 μg of sequencing grade endoproteinase Lys-C in 30 μl of 8% (v/v) acetonitrile, 2.5 mM Tris–HCl, pH 7.7, at 37°C. After digestion, the reaction mixture was supplemented with 10 μl of acetonitrile and sonicated for 5 min.

After removing the acetonitrile by blowing with nitrogen gas, peptides in the digestion mixture were trapped on C18 resin packed in a ZipTip$_{C18}$ (Millipore Corporation, MA, USA), and eluted in 5 μl of 75% (v/v) methanol, 1% (v/v) formic acid. Nano-ESI-MS for peptide-mass fingerprinting was performed using a Micromass Q-Tof system, equipped with a NanoFlow Probe Tip Type F (Micromass UK Ltd., Manchester, UK). The peptide solution was put into a bolosilicate capillary tip and subjected to electrospray ionization (ESI) at a flow rate of 10 nl/min. The MS spectrum was analyzed with the ProteinLynx software. Protein identification through a database search was carried out using the MS-FIT proteomic tool at the UCSF web server, through the internet.

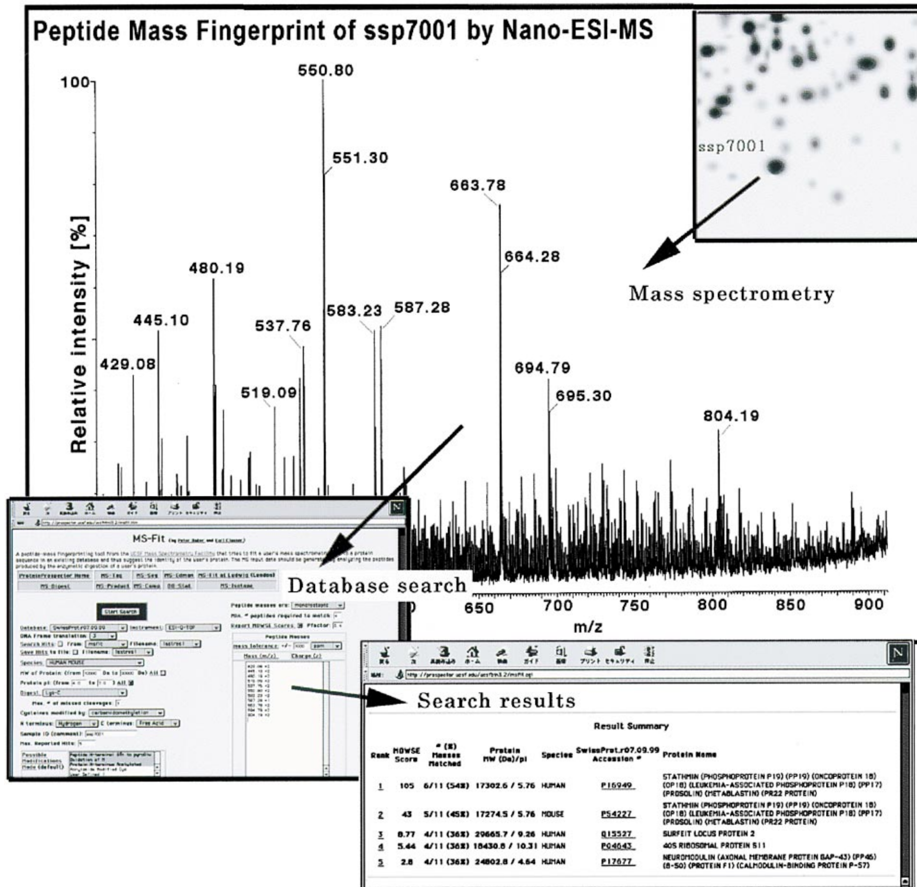

Fig. 3. Identification of spot protein by MS. The spot protein was transferred to a PVDF membrane, and digested with lysylendoproteinase Lys-C. The mass spectrum of peptide fragments was obtained using a Micromass Q-Tof MS system. The mass fingerprint database search was executed on the internet MS-FIT site. The ssp7001 was identified as phosphoprotein stathmin.

3. Results

3.1. Screening of age-related protein alteration by proteome profiling and differential protein display

Alterations in the relative intensity of protein spots appearing on proteome profiles, may be the results of various molecular alterations that occur during aging. Quantitation of the integrated optical density of each protein spot is required for the 2D gel electrophoresis of protein, to screen out the molecular events that may be responsible for the functional deterioration in senescent cells.

Coomassie Blue staining is the most reliable method for the quantitative demonstration

of proteins on a gel slab. However, the sensitivity of Coomassie staining is not high enough to detect minor components of cellular proteins. Autoradiography of [^{35}S]methionine-labeled proteins shows the highest sensitivity. However, it is not applicable to the detection of post-translational modification of proteins. This is due to the fact that, only the fresh proteins that are de novo synthesized during the period of labeling incubation are detected by autoradiography. Cypro ruby fluorescent staining shows almost the same sensitivity as silver staining, and quantitativity as Coomassie staining. Therefore, fluorescent staining will be included in the standard protocol of proteomics in the future. Silver staining is a practical method for spot protein visualization in proteome analysis at present, because it shows the highest sensitivity and practical reliability in relative quantitation for differential protein display, as shown in Fig. 2.

In this 2D gel area, the transitional increase of spot protein ssp7001 was observed around 65 PDL; at that level, the normal human diploid fibroblasts (TIG-3) transfer into phase 3 of the replicative cell aging and the doubling time was delayed.

3.2. Identification of spot protein by mass spectrometry in proteomics

In general, western blotting has been performed for the identification of proteins in spots for the last several years. However, the immunochemical technique was not applicable to the unknown protein for which a specific antibody was not available. Co-electrophoresis is another way to assign a spot to a candidate protein when its authentic protein standard protein is available. In modern proteomics, a sequence database search queried with peptide mass fingerprint data and/or partial sequence tag data obtained by MS, is generally performed to identify proteins on 2D gel patterns. Fig. 3 shows an example of identification of ssp7001 spot protein by peptide mass fingerprinting. The mass spectrum of Lys-C digests of ssp7001 was recorded using a Micromass ESI-Q-Tof-MS system (MICROMASS UK, Manchester, UK). The database search was queried to MS-FIT in the Protein Prospector Server at California University, and subsequently it was assigned to a microtubule associating phosphoprotein stathmin. When no candidate protein is hit by the database search, because it is really novel, molecular cloning should be done to clarify the meaning of the protein alteration in the aging process. In that case, Edman-degradation microsequencing has an advantage over mass spectrometry in sequencing longer peptide fragments of the protein in order to design proves and/or primers in molecular cloning of its corresponding cDNA.

3.3. Determination of protein modification by mass spectrometry in proteomics

Age-related protein alterations in heat stability and specific activity have been reported by many groups (Holliday and Tarrant, 1972; Gershon and Gershon, 1973; Chetsanga and Liskiwskyi, 1977; Pigeolet and Remacle 1991). Further, the accumulation of detergent-insoluble protein was also observed in aged cells and tissues (Yang and Wang, 1994). It was suggested that these age-related alterations reflect post-translational modifications such as oxidation by attacking of reactive oxygen species (Gordillo et al., 1989). Oxidative modification may produce carbonyl groups, ortho-tyrosine structures and methionine sulfoxide residues that increase the hydrophobicity of the protein surface (Chao et al., 1997). In the proteome analysis, structures of oxidative modification on spot proteins,

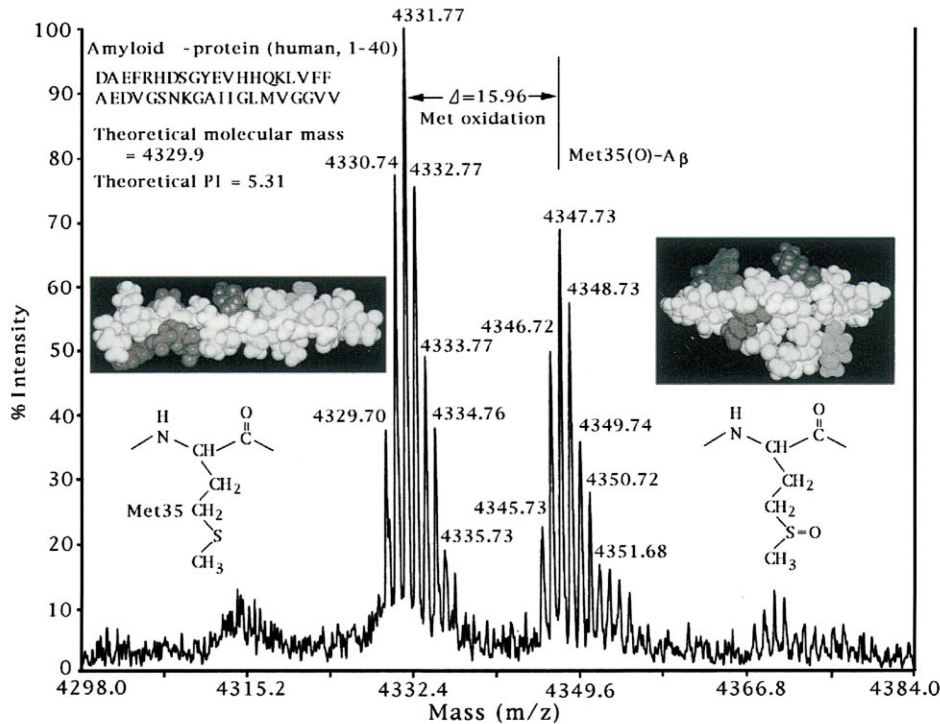

Fig. 4. Determination of methionine oxidation in human amyloid beta peptide (1–40) by MALDI-TOF-MS. The sample solution was prepared as described in Methods. One-μl aliquot was applied to the MALDI target plate. The mass spectrum was recorded with a Voyager-DE STR MALDI-TOF-MS system (PE Biosystems, Foster City, CA) in a reflection mode. The spectrum indicated that the authentic preparation of human amyloid beta peptide (1–40) contained both native and Met35-oxidized forms. Detected ions were all monovalent and the 15.96-Da mass shift was derived from the methionine oxidation.

isolated by 2D gel electrophoresis, can be determined by MS. Human amyloid beta (Aβ)1–40 peptide contains a methionine, and its oxidation induces the alteration of the 3D structure. The Met35-oxidized Aβ shows a higher molecular mass of 16 Da than native Aβ, as shown in Fig. 4. Carbonylation does not result in a sufficient shift in molecular mass (−1 Da); however, theoretically its dinitrophenylhydrazine derivatives gives an increase of 180 Da.

4. Discussion

We have obtained an excellent methodology, proteomics, which is most suitable for investigating protein factors in molecular mechanisms of cellular aging. Alterations in gene expression that are the results of DNA damages, and accumulation of altered proteins that are made by oxidative modification can be detected by methods in proteomics. The advanced method of two-dimensional gel electrophoresis, in which isoelectric focusing is

carried out on an immobilized pH gradient for the first dimensional separation, offers the highest resolution of proteins. More than thousands of spots are detected on a gel slab in an optimized condition. The proteome database of normal human diploid fibroblasts TIG-3 has been established, and is displayed on our Gerontology Informatics database: the PDL-dependent alterations of protein spots are demonstrated here. Although the resolution of 2D gel electrophoresis is still not high enough to separate all the proteins in a cell extract, it will be improved by partitioning the pH and molecular mass range using narrow pH range IPG-IFE gel strips and various concentrations of acrylamide gel slabs for SDS-PAGE. The multiplication of 2D gel electrophoresis for partitioning the range of separation may yield better results than the simple enlargement of a gel size to cover the overall range of separation. Identification of the protein by making inquiries on the proteomic database with a peptide mass fingerprint data, will be more successful after completion of the genome-sequencing projects. Most post-translational modifications that are accumulated in aged cells under attacks of reactive oxygen species (ROS), are easily detected by proteomics. The extensive application of proteomics in the investigation of altered proteins in aged cells has led to the next stage of research: the molecular mechanisms of aging.

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