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Toxicoproteomics in the Study of Aromatic Hydrocarbon Toxicity

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Abstract The aromatic hydrocarbons (AHs), which include benzene, polycyclic aromatic hydrocarbons, and dioxin, are important chemical and environmental contaminants in industry that usually cause various diseases. Over the years, numerous studies have described and evaluated the adverse health effects induced by AHs. Currently, "Omics" technologies, transcriptomics and proteomics, have been applied in AH toxicity studies. Proteomics has been used to identify molecular mechanisms and biomarkers associated with global chemical toxicity. It could enhance our ability to characterize chemical-induced toxicities and to identify noninvasive biomarkers. The proteomic approach (*e.g.* 2-dimensional electrophoresis [2-DE]), can be used to observe changes in protein expression during chemical exposure with high sensitivity and specificity. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and electrospray ionization-quadrupole (ESI-Q)-TOF MS/MS are recognized as the most important protein identification tools. This review describes proteomic technologies and their application in the proteomic analysis of AH toxicity.

Keywords: aromatic hydrocarbon, biomarkers, proteomics, toxicology

INTRODUCTION

Proteomics – a high throughput technique designed to separate, display, and identify proteins [1] – is a new technological advancement in the field of biological research. The central dogma of molecular biology is still based on the transcription of genomic DNA into mRNA and the translation of mRNA into protein. However, proteins are frequently expressed in forms that cannot be predicted on the basis of DNA or mRNA analysis alone. Posttranslational modifications of protein (*e.g.* phosphorylation and glycosylation) make it even more difficult to predict the exact structure – and, therefore, the function – of proteins based on their original genes [2]. Because proteins play an important role in cellular processes and indicate the dynamic states of cells, proteomic technologies have become important methods of study in various fields of biology.

Two-dimensional electrophoresis (2-DE), a crucial proteomics tool, has major advantages for exploring deviations from normal protein expression in metabolic disorders and for identifying specific proteins responding to a certain effect in uncharacterized crude samples [3]. Most of the proteins found in biological samples can be detected and identified. Thus, it is possible to find a protein or group of proteins that are associated with a specific disease or toxicity and use them as biomarkers. Recently, proteome expression in normal and abnormal states was compared in biological samples and was found to be applicable to clinical and biomedical research [4].

The field of toxicology is likely to benefit the most from the application of proteomics [5-7]. 2-DE is a highly sensitive method for screening toxicity and searching for mechanisms underlying toxicity. By comparing proteins expressed following the exposure of a biological system to a chemical with proteins in untreated conditions, it is possible to identify biochemical changes by monitoring changes in sets of proteins that may be associated with the toxicity. Once a large library of proteomic signatures has been compiled for compounds with known mechanisms of toxicity, it will be possible to use it to assess the toxicity of compounds whose mechanisms of toxicity is not known. A significant advantage of proteomics is its utility in analyzing proteins by using high throughput, automated techniques that can be applied to the analysis of tissue samples, cell cultures, and body fluids (*e.g.* plasma, serum, urine, cerebrospinal fluid, and synovial fluid) [5]. It has been suggested that proteomics has great potential for screening new markers of early changes in human tissue resulting from continuous exposure to toxic agents and gain an understanding the mechanisms of toxicity. To date, few large-scale studies using proteomics have entered the public domain; therefore, its full potential in toxicology has yet to be realized. We will review the new field of "Omics" (transcriptomics and proteomics), as it is applied in toxicology. We will also review a number of proteomic techniques that are currently in use and proteomic studies of the toxicity of aromatic hydrocar-

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bons (AHs).

Application of "Omics" Technologies in Toxicology

The primary goal of toxicologists is to detect the adverse effects of a toxic compound on living organisms based on observed symptoms or biomarkers of such effects. A wide variety of mechanisms underlie toxicity at the cellular level. To distinguish among these mechanisms, the current selection of study techniques and biomarkers used in toxicology must be expanded.

"Omics" is the general term used to describe several rapidly growing scientific fields. The suffix "-omics" generally indicates the study of a complete set of biological molecules. The field of "genomics", for example, utilizes a broad spectrum of technologies to explore the molecules that carry information about every aspect of cellular structure and function (*i.e.* DNA or RNA). The central dogma of the field of molecular biology is that the genes encoded in DNA are transcribed into messenger RNA (mRNA), which is then translated into proteins that become part of the cell's structure and carry out cellular functions. Currently, there are 2 main approaches to the analysis of molecular expression patterns: the generation of mRNA expression maps (transcriptomics) and examination of the proteome, *i.e.* the expression profile of proteins (proteomics). Thus, toxicological studies that utilize transcriptomics and proteomics comprise an area of research known as "toxicogenomics" [8].

In the new research discipline of toxicogenomics, genomic and proteomic technologies are integrated with classical methods of study to explore toxicity at the molecular level in relation to pathophysiological changes in the organism. In toxicogenomics, there are 2 major goals: to identify useful biomarkers of toxin exposure, and to elucidate molecular mechanisms of toxicity.

Application of Transcriptomics in Toxicology

Transcriptomics is a research discipline that focuses on measuring the full complement of activated genes, mRNAs, or transcripts in a particular tissue at a particular time, typically using microarray technology [9]. Microarrays use immobilized cDNA or oligonucleotide probes to simultaneously monitor the expression of thousands of genes to obtain a global view of gene expression. Because of its potential to facilitate the rapid discovery of specific biomarkers and the mechanisms of action for toxic compounds, transcriptomics is being used with increasing frequency in toxicology [10].

Transcriptomics has been shown to be a powerful tool in the assessment of mechanisms of toxic responses [11,12]. Gene expression profiles identified with the use of microarrays can be used to distinguish among tissue samples exposed to different classes of toxic compounds, to predict the toxicity of unknown compounds, and to study cellular mechanisms that lead to or result from toxicity. Microarray data will also provide a means of distinguishing among array data patterns indicative of the adverse effects of different toxic compounds. If array data can be combined with conventional methods of toxicity analysis, such as histopathology and clinical chemistry, it will be possible to find evidence of a toxic exposure before evidence of its clinical or pathologic effects are manifested [13]. This approach could lead to our ability to identify early biomarkers of toxic exposures.

Application of Proteomics in Toxicology

Proteomics uses a number of techniques to measure the structural and functional properties of proteins – especially 2-DE or liquid chromatography – then identify those proteins using various forms of mass spectrometry (MS).

The application of proteomics in toxicology can be divided into 3 categories: screening toxicology, predictive toxicology, and mechanistic toxicology [5]. *Screening toxicology* is used to establish a relationship between toxic effects and protein molecular markers (*i.e.* to identify toxicological biomarkers). To be clinically useful, a toxicity-related biomarker must be measurable in an accessible body fluid, such as serum or urine [6]. Because these fluids are rich in protein, proteomics may offer the best chance of discovering early changes in secreted proteins. *Predictive toxicology* is based on the assumption that a specific group or class of toxic compounds will induce a specific pattern of change in protein expression. It may be possible to use predictive techniques to develop a database of changes in protein expression for specific organs that are induced by different toxic compounds. Once that is accomplished, the toxicity of a new compound in a specific organ and the mechanisms underlying that toxicity could be studied by comparing its effects on the proteome with the effects of known toxic compounds, which are already stored in the database. Finally, *mechanistic toxicology* may be useful in identifying new molecular targets for known toxic compounds or in providing new insights into their mechanisms of action.

Combined Approach of Transcriptomics and Proteomics in Toxicology

The combination of transcriptomics and proteomics will serve as a very powerful tool for detecting early changes in human tissue induced by toxicity; therefore, they should be considered complementary technologies. For example, when few transcripts of a particular gene are available, it may not be easy to quantify the protein product using standard 2-DE. In this case, gene expression may be quantified more easily at the mRNA level using polymerase chain reaction (PCR)-mediated target amplification instead of attempting to detect proteins. Biopsy samples and culture media typically yield both mRNA and proteins of good quality. However, the quality of mRNA isolated from body fluids is often poor, because it is degraded more quickly than proteins. For this reason, RNA samples from body fluids such as serum or urine are often not very meaningful markers for toxicity, while the proteins that had been secreted into these fluids, which are more likely to be suitable. Methods of detecting posttranslational modification events, which often help determine the function of a protein, are restricted to protein expression analysis. There is also growing evidence

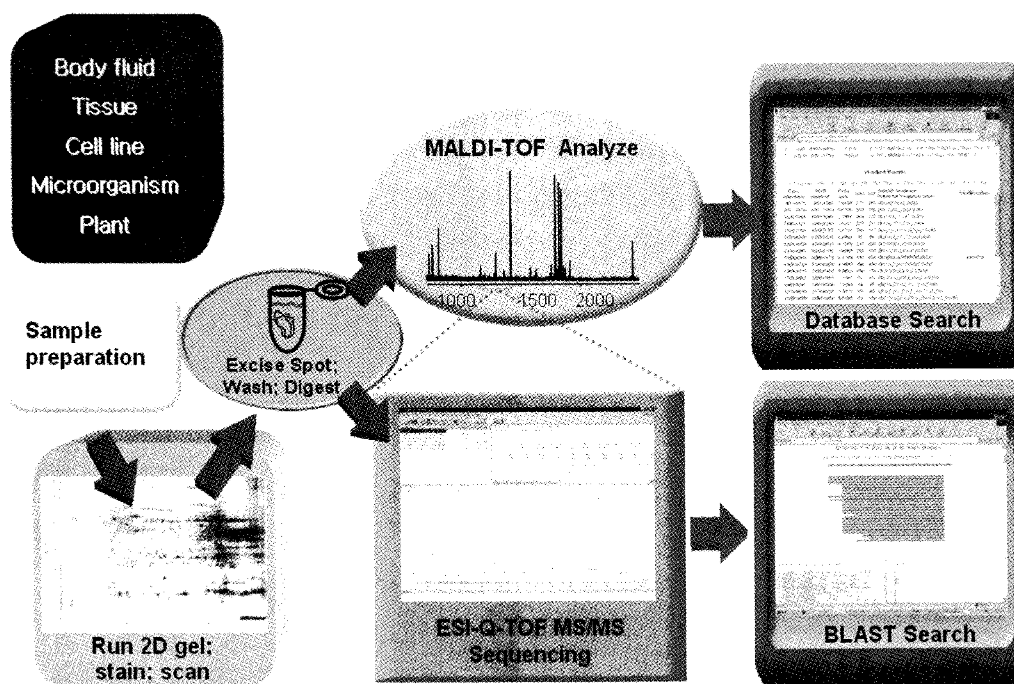

Fig. 1. The whole procedure of 2-DE based proteomics.

of a poor correlation between mRNA and protein abundance [14], which further suggests that mRNA and protein profiling are complementary and should be applied in parallel.

Technologies for Proteome Analysis

Two-Dimensional Gel Electrophoresis-Based Technologies

Two-Dimensional Gel Electrophoresis

For many years, it has been possible to determine the arrangement of proteins in complex mixtures using 2-DE, which separates proteins by pH using isoelectric focusing (IEF) in the first dimension then by molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Thus, protein separation occurs along a pH gradient that is generated by synthetic amphoteric electrolytes. It is extremely difficult to control the pattern of separation produced by this method, largely because of the non-fixed nature of pH gradients in IEF gels. This problem has been overcome by the development of a method of immobilizing the pH gradient (IPG), known as IPG IEF [15]. Immobilines (Amersham Pharmacia Biotech, Uppsala, Sweden) is a series of acrylamide derivatives that serves as a buffer at different p*K* values and is used in IPG IEF gels. The product is a rectangular pattern of protein spots that are typically revealed by Coomassie blue, silver, or fluorescent stainings.

Mass Spectrometry

Currently, techniques based on MS are driving the progress in proteomics. MS is indispensable for the identification and quantification of proteins from complex mixtures such as plasma and other tissues [16]. The introduction of matrix-assisted laser desorption/ionization (MALDI) in particular greatly expanded the range of proteins that could be analyzed using MS. Peptide mass fingerprinting (PMF), which is a fast and efficient way to identify proteins analyzed by MS, was subsequently introduced. This method involves the selective cutting of a protein with an enzyme, usually trypsin, and comparing the fragments with theoretical peptides, the digestion of which is simulated by computer using existing databases. MALDI-time of flight (TOF) is usually used for PMF [17]. In the MALDI-TOF system, enzymatically digested peptides are mixed with an energy-absorbing matrix (EAM), such as sinapinic acid, to induce crystallization. The crystallized proteins are then placed in a vacuum chamber, in which the crystal is subjected to a nitrogen laser. The matrix molecules absorb energy from the laser and transfer it to the proteins, causing the proteins to undergo ionization. A detector measures the mass of each protein species based on its velocity in the TOF analyzer and derives the mass:charge ratio (m/z) of the protein from the TOF, which is used to determine the amount of protein that is present. Peaks in the intensity plot ideally correspond to individual proteins. Electrospray ionization-quadrupole (ESI-Q)-TOF MS/MS is a rapid and precise method of determining the mass of proteins and peptides and can be used to validate protein sequences. Peptide fragmentation by collision-induced dissociation produces MS/MS spectra, which can be used to identify proteins through a database search [18]. MS/MS analysis is more accurate than PMF in identifying proteins, but

needs complete genome information to do so.

Both MALDI-TOF MS and ESI-Q-TOF MS/MS can detect low levels of proteins and are suitable for automation. MALDI-TOF MS can analyze several thousand proteins isolated from gels in a week. The entire procedure involved in 2-DE-based proteomics is illustrated in Fig. 1.

Technologies Not Involving Two-Dimensional Gel Electrophoresis

Isotope-Coded Affinity Tags (ICAT)

Many methods that do not use conventional 2-DE have been developed recently. ICAT, for example, is a class of reagents with 3 functional elements: specific chemical reactivity, an isotopically coded linker, and an affinity tag [19]. The ICAT method includes the following sequential steps. First, the side chains of cysteinyl residues in a reduced protein sample are linked with isotopic tags that are similar in structure and chemical properties but different in mass, *i.e.* the side chains of proteins obtained in one cell state are "tagged" with the isotopically light form of the ICAT reagent (containing 8 hydrogen atoms) and equivalent groups in the sample that represents a second cell state are tagged with the heavy form of this reagent (containing 8 deuterium isotopic forms). Second, the 2 samples are combined then enzymatically cleaved to generate peptide fragments (some of which are tagged), the mixture is applied to a column of avidin beads to isolate the ICAT-tagged peptides by affinity chromatography, and the isolated peptides are separated and analyzed by means of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Third, both the quantity and sequence of proteins from which the tagged peptides originated are determined by automated multistage MS. The peptides are quantified by measuring (in the MS mode) the relative signal intensities for peptide pairs (one tagged with a light ICAT reagent and the other with the heavy form) with identical sequences but a different mass (indicated by the different forms of the ICAT reagent) using the mass differential encoded in the ICAT reagent. By combining the results of the MS and MS/MS analyses of the labeled peptides, the investigator can determine the relative quantities of the components of protein mixtures as well as the sequence of these components in a single automated operation.

Available literature indicates that ICAT technology can be used routinely to identify 300 to 400 proteins per sample; this is far less than what is typically achieved using 2-DE technology. Although the number of proteins identified in a typical ICAT run are far less than the number reported by 2-DE, the high throughput, quantitative nature, and reproducibility of its results makes ICAT one of the most powerful emerging proteomic technologies, and it will be used with increasing frequency for high throughput proteomic characterization of biological specimens. Thus far, ICAT technology has been used successfully to study protein expression in mammalian cells, [20] including liver cells [21]. To facilitate the comparison of the proteomes of 2 different samples simultaneously, ICAT has been used with a multidimensional protein identification technology (MudPIT).

Multidimensional Protein Identification (MudPIT)

MudPIT involves tryptic digestion of a protein mixture, which is followed by multidimensional LC-MS/MS and a database search using the SEQUEST algorithm [22]. Importantly, the use of MudPIT allows us to identify proteins that are present in concentrations far below that of promiscuous housekeeping proteins, which tend to be present in abundance and tend to be dominant in proteomic expression profiling studies using 2-DE [23]. The development of MudPIT was a major step toward the development of comprehensive high-throughput methods, because not only does it allow us to detect and identify proteins that are low in abundance, but it allows us to detect peripheral and integral membrane proteins, as well.

Surface Enhanced Laser Desorption/Ionization (SELDI)

Ciphergen Biosystems (Fremont, CA, USA) developed the SELDI technology, which has facilitated selective protein retention using distinct, factory-prepared, chromatographic surfaces. MALDI and SELDI are similar, in that both permit "soft" ionization of biological molecules, such as peptides and proteins. The SELDI method begins with the application of a biological sample to a precoated stainless steel slide. The coating helps the surface bind preferentially to a specific class of proteins based on their physicochemical properties. The chemical surfaces described thus far have hydrophobic, hydrophilic, anionic, or cationic properties, and they can bind with such biological surfaces as antibodies, antigen-binding fragments, DNA, or receptors. The chemical surfaces are used to capture a class of proteins with the corresponding chemical affinity, and the biochemical surfaces are custom-made by the user to bind with the molecule of interest. The bound proteins are washed with buffers to remove nonspecifically bound proteins and mixed with an EAM, which causes the mixture to crystallize. The crystallized proteins are then ionized and quantified using a TOF analyzer.

Application of Proteomics in the Study of Aromatic Hydrocarbon Toxicity

Benzene

Benzene, which is the simplest AH, is a colorless mobile liquid with a characteristic odor. It is also a ubiquitous environmental contaminant, being a component of cigarette smoke, gasoline, and automobile emissions [24]. Benzene is a known carcinogen that causes chromosomal aberrations *in vitro*. Available data suggest that prolonged exposure to benzene at a concentration of 64 mg/m^3 or more may induce chromosomal aberrations [25]. Earlier publications suggested that prolonged exposure to benzene causes genetic damage, such as sister chromatid exchanges, DNA cross-linking, DNA adduct formation, and impairment of DNA repair mechanisms [26]. Inhaling high concentrations of benzene causes necrosis, and concentrations of 400 to 800 ppm inhibit bone marrow

Table 1. Identification of differentially expressed spots on benzene-exposed gels by MALDI-TOF MS [33]

Spot No.	MW (kDa)	pI	Classification	Identification	Matches[a] (%)
445	59	6.4	New Spot	Zinc finger protein Helios	25
488	54	5.3	New Spot	Collagenase 3 precursor (MMP-13)	20
492	47	7.0	New Spot	Hypothetical zinc finger protein	20
499	53	5.2	New Spot	IRF-6	20
519	51	5.7	New Spot	FKBP51	20
609	36	7.5	New Spot	RAS-related protein RAB-36	20
661	40	5.3	New Spot	Guanine nucleotide-binding protein G(0), Alpha subunit 1	20
671	40	6.0	New Spot	Homer-2 protein	35
709	34	6.2	New Spot	Phosphoinositol 4-phosphate adapter protein-1	20
734	44	4.6	New Spot	TRF2-interacting telomeric RAP1 protein	20
811	30	6.1	New Spot	FKBP23 isoform	25
815	39	6.4	New Spot	A6 related protein	25
943	20	4.9	New Spot	Hypothetical protein	20
1015	21	5.9	New Spot	TCR-β 1	30
1019	12	5.7	New Spot	Immunoglobulin light chain variable region	30
464	56	4.7	Upregulated	Interleukin-4 receptor α chain	20
591	37	5.9	Upregulated	T cell surface glycoprotein CD1b precursor (CD1 b antigen)	36
605	49	4.4	Upregulated	Angio-associated migratory cell protein	20
956	22	6.5	Upregulated	Cyclin-dependent kinase inhibitor p27	20
967	24	4.8	Upregulated	Ras-related protein Rab-3D	20
1012	20	5.6	Upregulated	TCR-β chain C region	25

[a]Match (%) is based on number of peptide masses matched in NCBInr databases using MS-Fit searching program with MALDI-TOF MS data.

formation and suppress immune function [27]. In addition, long-term inhalation of benzene has been associated with hematotoxicity and hematopoietic dysfunction in humans [28,29]. There is sufficient evidence that benzene induces the development of aplastic anemia and leukemia in humans and in laboratory animals, but the mechanism by which it exerts these effects remains unknown.

Many attempts have been made to find effective biomarkers of benzene exposure. In relatively high levels of exposure, benzene oxide reacts with cysteinyl residues in hemoglobin and albumin to form protein adducts [26,30]. Exposure to benzene is also evaluated by detecting trans, trans-muconic acid (t,t-MA) [31] and *S*-phenylmercapturic acid (*S*-PMA) – which are the benzene metabolites – and detecting benzene levels in urine and blood [32]. However, the usefulness of these biomarkers can be limited by confounding factors, such as smoking, food, toluene, and lack of standardized measures. Additionally, these biomarkers only reflect recent benzene exposure. Therefore, more specific and direct biomarkers of chronic benzene exposure are needed.

Analyses using 2-DE have led to the discovery of several potential diagnostic markers for benzene exposure. A 2-DE analysis of the proteins in plasma taken from workers (n = 50) at a printing company who were exposed to benzene revealed that their protein profiles differed significantly from the profile in a group of nonexposed individuals (n = 38) [33]. Fifteen 2-DE spots expressed only in benzene-exposed workers were selected and identified using MALDI-TOF MS (Table 1). Interestingly, 5 of the 15 upregulated proteins were components of the immune system. In these 15 spots, the most prominently expressed protein was T-cell receptor (TCR)-β. The rate of upregulation in 6 spots from the benzene-exposed samples was more than 4 times that in spots taken from unexposed individuals (Table 1). Interestingly, the TCR-β C region and T-cell surface glycoprotein Cd1b precursor (CD1 b antigen) were again identified in this group. This could indicate tissue leakage. Given that proteins that normally function intracellularly can be released into the plasma as a result of cell damage [34], these include many of the most important diagnostic markers [35]. To verify the identity of new spots, expression levels of TCR-β, matrix metallo protease (MMP)-13, and FKBP51 were confirmed by Western blot and noted to be significantly increased in benzene-exposed workers. t,t-MA is a known biomarker for recent benzene exposure [31], and

the comet assay, which detects DNA damage, can be used as a biomarker for continual benzene exposure [36]. The results of the t,t-MA and comet assay of samples obtained from workers exposed to benzene were also reported [37]. The Spearman correlation analysis was applied to find a correlation between TCR-β expression and tail moments of lymphocytes, and between TCR-β expression and the urinary excretion of t,t-MA in benzene-exposed workers [38]. The investigators in this study found that TCR-β expression and the tail moments of lymphocytes were significantly correlated (r-value, 0.428). However, there was no significant correlation between TCR-β expression and the value of t,t-MA.

The 11 plasma proteins that were differentially expressed in Sprague-Dawley (SD) rats following 2 or 6 weeks of benzene inhalation exposure were identified using 2-DE and MALDI-TOF MS [39]. They included TCR-α chain protein. The level of expression for these proteins was confirmed by Western blot and found to be similar to the level of upregulation of TCR-β of benzene-exposed workers [33].

TCR is a heterodimer composed of either α and β chains or γ and δ chains. The majority of T cells (95%) express the α/β heterodimer, while roughly 2 to 5% express the γ/δ heterodimer [40]. The increase in TCR-α upregulation in benzene-exposed rats, therefore, correlates with the increase in TCR-β upregulation in humans. TCR-α and TCR-β are highly expressed in T cells when the immune system is active. Thus, the proteins identified in these studies may be useful as biomarkers of benzene exposure and in helping us understand the toxic effects of benzene.

The SELDI method was used recently to identify changes in the serum proteome of benzene-exposed workers (n = 20:10 benzene-exposed workers, 10 controls) [41]. Two downregulated proteins were identified by SELDI-TOF MS in serum of benzene-exposed workers. Two platelet-derived CXC-chemokines (platelet factor 4 and connective tissue activating peptide-III were subsequently confirmed using a ProteinChip array-based immunoassay (Cypergen Biosystems). Given that altered expression of the platelet-derived CXC-chemokines could not be explained by changes in absolute platelet counts, this study demonstrated the possibility that SELDI-TOF analysis could be used to identify biomarkers in a limited number of benzene-exposed and non-exposed individuals.

Polycyclic Aromatic Hydrocarbon (PAH)

PAHs comprise a ubiquitous class of hydrophobic organic compounds consisting of 2 or more fused benzene rings in linear, angular, or cluster arrangements. Many industrial processes result in the generation and dissemination of PAHs. Coal-processing waste products, petroleum sludge, asphalt, creosote, and other wood preserva tive waste products all contain high levels of PAHs. Dietary PAH intake is a very significant source of human exposure. Grilling and smoking both cause the release of high levels of PAHs from meat. More recently, residential heating sources, refuse burning, and vehicle exhaust have been identified as contributing to the widespread distribution of PAHs, which are now found over large geographic areas and in numerous population groups [42]. PAHs persist within the ecosystem for years, owing to their low water solubility and their high capacity for adsorption to solid particles. PAH compounds are known or suspected to be toxic, mutagenic, or carcinogenic [43] and elicit many adverse biological effects, including immunosuppression, teratogenicity, tumorogenesis, and hormonal effects. Among these, the main effect of PAHs in humans is the induction of tumors, primarily in the lungs, bladder, or skin.

In the past decade, several biomarkers have been used to assess human exposure to PAHs. Among these, urinary 1-hydroxypyrene (1-OHP) and 2-naphtol have served as good markers of PAH exposure, mostly in occupational workers [44,45]. Other possible markers include the exchange of sister chromatids, chromosomal aberrations, and mutagenicity of urine and feces. Unfortunately, these markers have poor specificity for PAH exposure [32]. Therefore, it is necessary to delineate more specific and direct biomarkers for chronic PAH exposure.

A 2-DE analysis of plasma proteins was performed to investigate the differential expression of these proteins during a PAH exposure [46]. The plasma was exposed to PAH obtained from gas pollution waste measurers (n = 48) working at an automobile emission inspection center and compared with plasma taken from member of an unexposed control group (n = 33) (Fig. 2). 1-OHP (a urinary PAH metabolite that is used as a biomarker for PAH exposure) was present in a concentration of 0.28 μmol/mol creatinine in PAH exposure groups and 0.078 μmol/mol creatinine in unexposed control groups. In a previous study, 1-OHP levels in automobile emission inspectors (n = 24) and waste incineration workers (n = 28) were 0.27 and 0.57 μmol/mol creatinine, respectively [47]. The average working period for these PAH-exposed workers was 9.4 years, and their 1-OHP levels were much lower than the average biological exposure index (2.3 μmol/mol creatinine) [48], in spite of their long exposure to PAHs. Therefore, it was suggested that the workers in this study were exposed to only low levels of PAHs, albeit over a long period of time.

The proteins that were expressed in significant quantities in the plasma of PAH-exposed workers were identified using MALDI-TOF MS and confirmed by ESI-Q-TOF MS/MS and Western blotting. In this study, 5 proteins were upregulated during PAH exposure and one new protein was expressed (Table 2). The upregulated proteins in PAH-exposed workers were an albumin precursor, a hemopexin precursor, a fibrinogen γ-A chain precursor, and the TCR-β C region, all except the TCR-β C region have been identified as antioxidants [49-51]. Given that PAHs (like many other toxic materials) induce high oxidative stress, the increased expression of these proteins in PAH-exposed individuals could be the result of a mechanism that protects the cell from oxidative damage. In addition, a fragment of transient receptor potential (TRP)-6, the putative capacitative calcium entry (CCE) channel, was identified as a new protein that is expressed during PAH exposure. It was known that PAH-

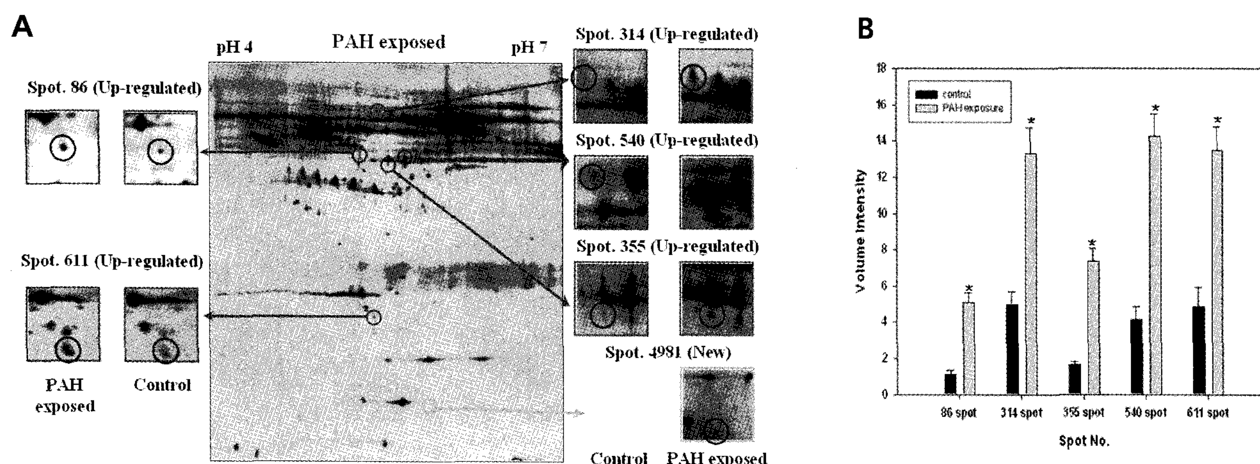

Fig. 2. The differentially expressed proteins in PAH-exposed plasma [46]. (A) Blow-up images of spots were compared between PAH-exposed and non-exposed control gels. 2-DE was performed with 40 μg of plasma proteins using 24 cm pH 4-7 IPG strip and 11 to 16% SDS-PAGE. The gels visualized by silver staining and 2-DE gel images were analyzed using Image Master Software. The 2-DE gel images of 5 upregulated spots and one newly expressed spot (Spot No. 4981) were magnified in the left and right lane. (B) Relative volume intensity of upregulated spots in PAH-exposed workers. The volume of the spot was calculated by single-spot normalization, and the quantity of each spot was expressed as a relative intensity. Each bar represents the mean ± SEM. Significant differences from control plasma samples were assessed using a 2-tailed Student's t-test (*$P<0.001$).

Table 2. Identification of differentially expressed spots on PAH exposed gels by MALDI-TOF MS and/or ESI-Q-TOF MS/MS [46]

Spot No.	MW (kDa)	pI	Classification	Identification	Confirmed by	Match[b] (%) & Score[c]
86	55	5.3	Upregulated Spot	Fibrinogen γ-A chain precursor	WB[a], MS/MS	68
314	83	5.4	Upregulated Spot	Hemopexin precursor	MS/MS	119
355	52	5.5	Upregulated Spot	Fibrinogen γ -A chain precursor	WB, MS/MS	83
540	59	5.7	Upregulated Spot	Albumin precursor	MS/MS	102
611	22	5.4	Upregulated Spot	T-cell receptor β chain C region	WB	15 (%)
4981	18	6.0	New Spot	Putative Capacitative Calcium entry Channel	WB	22 (%)

[a]WB, Western blotting
[b]Match (%) is based on number of peptide masses matched in NCBInr databases using MS-Fit searching program with MALDI-TOF MS data.
[c]Score is −10 × Log(P), where P is the probability that the observed match is a random event, it is based on NCBInr database using MASCOT searching program as MALDI-TOF MS or ESI-Q-TOF MS/MS data.

induced elevation in calcium levels occurs in T cells, B cells, and monocytes, as well as human mammary epithelial cells, among others [52]. PAHs were also believed to target immune cells, thereby altering calcium homeostasis by several mechanisms [52] and constituting a major cause of apoptosis [53]. Therefore, the CCE channel, a plasma membrane protein, and the TCR-β C region could be released from the membrane to enter the plasma as a result of PAH exposure, since that exposure triggered the alteration in calcium homeostasis which, in turn, induced apoptosis through a sense of signaling pathway.

In summary, 1 protein was newly expressed and 5 proteins were upregulated in PAH-exposed workers. These proteins could be used as biomarkers to diagnose low levels of PAH exposure.

Dioxin

Dioxins comprise a class of persistent polyhalogenated AHs that induce a wide spectrum of toxic responses in animals. The majority of environmental dioxins are gen-

erated during specific industrial processes or incineration [54]. Because of their environmental stability and lipophilicity, many enter the food chain and some can persist in biologic tissues for long periods of time. Therefore, by far the greatest source of human exposure to dioxins is food [55]. While there are 210 possible distinct chlorinated dioxins and furans, only 17 are considered important for health risk assessments. These 17 share the common structural feature of chlorine in the 2, 3, 7, and 8 positions. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent of the dioxins [56]. TCDD is formed as an unwanted byproduct of the manufacture of chlorinated hydrocarbons [57]. Many individuals have been exposed to TCDD, primarily through diet and occupational or accidental exposures.

An epidemiological study of populations exposed to high levels of TCDD or dioxin-like compounds, whether through work or by accident, provided invaluable information about the determination of relationships between human exposure to these compounds and adverse health outcomes. The structure of TCDD is related to that of a compound that initially binds to a cytosolic protein known as the aromatic hydrocarbon receptor (AHR). The AHR affected the activation of several genes, including the cytochrome p450 genes CYP1A1 and CYP1B1 [58]. Expression of other enzymes was also affected by exposure to dioxin-like compounds. These proteins may be important for understanding the physiological and pathological mechanisms of TCDD and dioxin-like compounds, and for developing bioanalytical methods of detecting and measuring dioxin-like activity [59]. However, these proteins are not only induced by dioxins, but can also be activated in other situations. For instance, CYP1A1 was induced by stressful conditions such as hyperoxia and hydrodynamic shearing and during the differentiation of monocytes and keratinocytes [60]. Therefore, studies conducted to identify biomarkers of TCDD exposure in combination with diverse studies to clarify the physiological mechanisms of TCDD toxicity are becoming important.

Alterations in rat plasma protein alterations caused by TCDD exposure were examined using 2-DE [61]. In this study, 2 groups of SD rats were exposed to TCDD, one group receiving short-term exposure in the form of a single dose of 1, 10, 20, or 50 μg/kg body weight and the other receiving long-term exposure to a daily low dose of 0.01, 0.1, 1.0, or 2.5 μg/kg body weight for 1 month. One new protein and 3 upregulated proteins were identified and characterized by MALDI-TOF MS and ESI-Q-TOF MS/MS. Plasma glutathione peroxidase precursor (GSHPx-P) was newly expressed in both groups. It is well known that GSHPx-P protects cells and enzymes from oxidative damage [62] and that TCDD is a pro-oxidant that causes lipid peroxidation and protein oxidation [63]. Therefore, the expression of GSHPx-P in TCDD-exposed rats may be a protective mechanism to safeguard the cell from oxidative damage. Cytokeratin 8 (CK8) was upregulated in the single high-dose TCDD exposure group. CK8 has recently been used as a circulating tumor marker, because CK is widely overexpressed in lung, colon, and breast cancer cells. It has also been reported that CK8 expression correlates with the invasiveness of tumors, both *in vitro* and *in vivo* [64]. The immunoglobulin lambda (Ig λ) 1 and 2 chain C region was upregulated in rats exposed to long-term low doses of TCDD. This supports the theory that TCDD is very powerful carcinogen at high concentrations [65], but stimulates immune activity at low doses [66]. In TCDD-exposed rats, the liver weight also significantly increased [61]. In studies of organ weight in experimental animals exposed to TCDD, investigators have reported that hepatomegaly is the main reason for increased liver weight, even at low doses. The enlarged liver was caused by hyperplasia and hypertrophy of parenchymal cells and, more specifically, by proliferation of the smooth endoplasmic reticulum. This morphological change was accompanied by the induction of microsomal monooxygenase activity and the coordinated activity of other expressed enzymes [67].

In succession, the 2-DE analysis was employed to screen a number of rat liver proteins that are differentially expressed after exposure to TCDD [68]. In this study, 13 upregulated protein spots and 2 newly expressed spots were detected. Apolipoprotein-AIV (Apo-AIV) was upregulated in the single high-dose TCDD exposure group. It was reported that Apo-AIV regulated the activity of key enzymes involved in lipoprotein mechanism [69]. Workers exposed to TCDD had elevated lipid levels and a mild liver lesion, and one of them died of atherosclerosis [70]. Given that Apo-AIV prevented modification of apolipoprotein structure and the physicochemical properties that are associated with lipid peroxidation (thereby conferring protection against atherosclerosis) [71], the elevated expression level of Apo-AIV in the TCDD-exposed rat liver might exert protection against TCDD-induced lipid peroxidation and atherosclerosis. It is noticeable that the transthyretin (TTR) precursor and the phosphoglycerate mutase (PGAM) type B subunit were upregulated at the 1 μg/kg TCDD dosage in the daily low-dose exposure group. TTR, which is synthesized in and secreted from the liver, is a circulatory protein that plays an important role in the transport of thyroid hormones to target tissue [72]. PGAM is a glycolytic enzyme that catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. In previous studies, PGAM activity and expression in the liver were increased by hyperthyroidism [73] and the morphology and function of thyroid were altered by TCDD [74]. Taken together, these phenomena suggest that it is conceivable that the expression of PGAM can be increased by hyperthyroidism induced by TCDD exposure.

After *in vivo* study in rats, plasma protein profiles from waste incineration workers (determined by 2-DE) were created to characterize the toxic effects of TCDD in humans [75]. During a comparison of the results in TCDD-exposed waste incineration workers (n = 31) versus healthy controls (n = 33), 7 upregulated spots and one newly expressed spot were identified in the TCDD group (Fig. 3). Adrenomedullin-binding protein was uniquely expressed in the TCDD samples, and α-fetoprotein (AFP),

Table 3. Identification of differentially expressed spots on TCDD exposed gels by MALDI-TOF MS and/or ESI-Q-TOF MS/MS [75]

Spot No.	MW (kDa)	pI	Classification	Identification	Match[a] (%) & Score[b]
150	57	6.0	Upregulated Spot	alpha-feto protein	33 (%)
540	59	5.7	Upregulated Spot	Albumin precursor	102
553	52	5.5	Upregulated Spot	Fibrinogen λ-A chain precursor	119
713	33	5.8	Upregulated Spot	XAP-5	18 (%)
1755	51	6.1	Upregulated Spot	human rab GDI	13 (%)
2525	38	5.5	Upregulated Spot	follistatin	14 (%)
2970	42	6.4	Upregulated Spot	fibronectin	96.5
5199	40	6.0	New Spot	AMBP	9 (%)

[a]Match (%) is based on number of peptide masses matched in NCBInr databases using MS-Fit searching program with MALDI-TOF/MS data.

[b]Score is −10 × Log(P), where P is the probability that the observed match is a random event, it is based on NCBInr database using MASCOT searching program as MALDI-TOF/MS or ESI-Q-TOF MS/MS data.

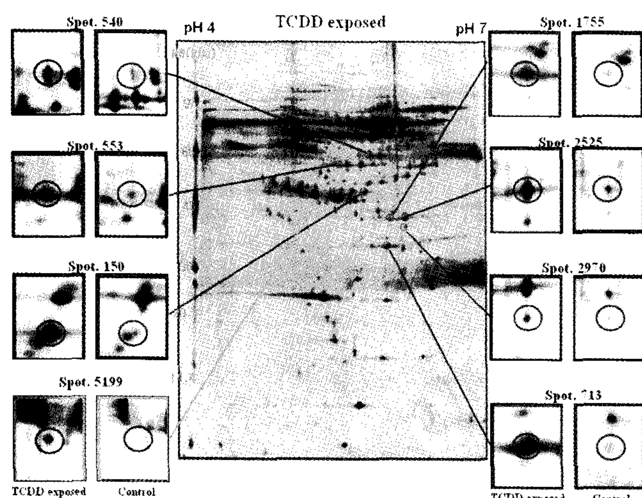

Fig. 3. The differentially expressed proteins in TCDD-exposed plasma [75]. (A) Blow-up images of spots were compared between TCDD-exposed and non-exposed control gels. 2-DE was performed with 40 μg of plasma proteins using 24 cm pH 4~7 IPG strip and 11 to 16% SDS-PAGE. The gels visualized by silver staining and 2-DE gel images were analyzed using Image Master Software. The 2-DE gel images of 7 upregulated spots and one newly expressed spot (Spot No. 5199) were magnified in the left and right lanes.

fibronectin, pre-albumin, fibrinogen γ-A chain precursor, XAP-5, human rab GDI, and follistatin were upregulated (Table 3). It has been reported that fibronectin and its integrin receptors have important functions in several stages of tumor development [76] and are detected in the hearts of TCDD-exposed animals [77]. Thus, fibronectin has been proposed for use as a biomarker of TCDD exposure. AFP expression also increased as the result of TCDD exposure. In adults, AFP is expressed abundantly during both liver regeneration and hepatocarcinogenesis, and can be used as a biomarker for hepatocellular carcinoma [78]. Also, albumin expression was significantly reduced in 15 of the 31 TCDD-exposed human plasma samples; this was verified in an *in vitro* study in HepG2 cells. Exposure of HepG2 cells to TCDD resulted in an increase in the mRNA level and protein expression of AFP, but reduced expression of albumin. Reduced albumin levels in the blood denote chronic liver or kidney disease, systemic lupus erythematosus, rheumatoid arthritis, or cancer [79]. Therefore, these results strongly suggested that TCDD had hepatotoxic and carcinogenic effects. Taking the previous results into consideration [61,68], it seems that TCDD exposure can induce liver-associated disease or cancer.

Concluding Remarks and Future Perspectives

AHs are ubiquitous environmental pollutants of air, water, and soil that are produced as a consequence of the incomplete combustion of organic materials. They can induce various diseases in humans. The proteomic approach to revealing AH toxicity has only recently been introduced, but available papers on comparative proteome research reveal the potential of proteomics to characterize molecular changes associated with AH toxicity. 2-DE, in combination with MS, is a very powerful approach to proteome research and allows researchers to find biomarkers and carry out protein expression analysis in human plasma that has been exposed to AH. There is a great possibility that identified protein biomarkers will be used to improve the evaluation of AH exposure. A proteome analysis of several AHs using proteomics tools is in progress. This will provide tremendous opportunities to develop biomarkers and identify molecular mechanism of AH toxicity.

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