IDENTIFICATION OF HL60 PROTEINS AFFECTED BY 5-AMINOLEVULINIC ACID-BASED PHOTODYNAMIC THERAPY USING MASS SPECTROMETRIC APPROACH[+]

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A combination of mass spectrometric techniques was used for identification of HL60 leukemia cell proteins affected by 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT). We compared two-dimensional electrophoresis (2-DE) protein maps of ALA-treated non-irradiated and irradiated cells and found extensive changes in the proteome of HL60 cells. The silver-stained 2-DE pattern of HL60 proteins contained more than 1 350 spots. Matrix-assisted laser desorption/ionisation mass spectrometry and microcapillary liquid chromatography/tandem mass spectrometry have identified twelve proteins differing in their intensity or position following ALA-PDT. Several endoplasmic reticulum, mitochondrial, ribosomal and cytoplasmic proteins were determined showing the impact of ALA-PDT-mediated cytotoxicity on some cellular pathways.
Keywords: Mass spectrometry; Matrix-assisted laser desorption/ionisation; MALDI; Electrospray ionisation; Protein identification; Peptide mass fingerprinting; Photodynamic therapy.

Mass spectrometry has revolutionized the field of analytical biochemistry in the past decade. It is a method of choice for identification of proteins separated by two-dimensional electrophoresis[1]. 2-DE with a resolution of up to 10 000 protein spots has been used for separation of complex protein mixtures[2] and also for subtractive analysis allowing identification of disease-associated proteins[3,4]. Matrix-assisted laser desorption/ionisation[5,6] (MALDI) and electrospray ionisation[7] (ESI) are two major ionisation tech-

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niques widely used in mass spectrometric studies of proteins. The protein of interest can be identified either by peptide mass fingerprinting[8–12] using MALDI-MS or by sequencing using post-source decay[13] (PSD) MALDI-MS or ESI-MS/MS (refs[14,15]).

Peptide mass fingerprinting is one of the most commonly employed approaches in protein identification. The given protein is digested with sequence-specific protease, *e.g.* trypsin, which cleaves at the C-termini of arginine and lysine residues, producing a set of peptides which serves as a unique fingerprint. Comparison of the experimentally measured peptide masses with those predicted for each protein entry in a database retrieves a number of matching sequences. The list of the sequences is then evaluated considering the origin of the species, the protein molecular weight and p*I* value, the experimental peptide mass error, the number of matched peptide fragments and the amino acid sequence coverage of the assigned peptides.

We have investigated the effect of 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT) on the expression of HL60 proteins using 2-DE and subsequent mass spectrometric analysis of the affected proteins. Photodynamic therapy is a mode of cancer treatment in which selective tumour cell necrosis is achieved by action of visible light on photosensitive compounds, such as protoporphyrin-IX (PPIX). On irradiation, the photosensitizers induce production of cytotoxic singlet oxygen molecules (photodynamic effect) causing alterations of certain cellular components (proteins, lipids, nucleic acids) and finally resulting in cell death[16–21]. The modified and more efficient principle of PDT is based on the administration of 5-aminolevulinic acid[22–24], a natural precursor in the biosynthesis of heme *via* protoporphyrin. Due to defective heme biosynthesis in most cancer cell types, PPIX is selectively accumulated in tumour tissues. Subsequent irradiation with light of a wavelength corresponding to some of the PPIX absorption bands leads to specific eradication of cancer cells. Although the principles of ALA-based PDT are fairly understood[25], the detailed mechanism of ALA-PDT-mediated cytotoxicity still remains unknown. Moreover, no ALA-PDT intracellular targets have yet been identified.

The aim of this paper is the evaluation of global protein profiles of promyelocytic leukemia cell line HL60 and determination the ALA-PDT-affected proteins (down-regulated, up-regulated, structurally altered). Differentially expressed proteins can indicate changes in the regulatory or signal transduction processes in disease and offer possible targets for drug development. The findings can be further employed in the construction of specific probes and in the objective diagnosis of the disease. To achieve this goal, 2-DE maps of ALA-treated non-irradiated and irradiated HL60 cells

were obtained and compared by image analysis. The ALA-PDT-affected proteins were subsequently identified by peptide mass fingerprinting using MALDI-MS and some of them also by peptide sequencing using microcapillary liquid chromatography/tandem mass spectrometry (μ-LC-MS/MS).

EXPERIMENTAL

Photodynamic experiments, two-dimensional electrophoresis and 2-DE gel imaging were performed as described previously[26].

Enzymatic In-Gel Digestion

Silver-stained protein spots were excised from the gel, cut into small pieces and destained with a mixture (1 : 1, v/v) of 10 mM potassium ferricyanide and 100 mM sodium thiosulfate as described elsewhere[27]. After complete destaining, the gel was washed twice with 10 mM dithiothreitol (DDT), 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN), then with water, shrunk by dehydration in MeCN and swelled again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 0.01% 2-sulfanylethan-1-ol, 50 mM 4-ethylmorpholine acetate, 1 mM $CaCl_2$, 10% MeCN and sequencing-grade trypsin (50 ng/μl; Promega, Madison (WI), U.S.A.). The digestion was carried out overnight and the resulting peptides were extracted with 30% MeCN/0.5% trifluoroacetic acid (TFA).

MALDI Mass Spectrometry

A saturated solution of 2-cyano-3-(4-hydroxyphenyl)propenoic acid (Sigma, Germany) in 25% MeCN/25% methanol/0.2% TFA was used as a MALDI matrix. Two μl of sample and 2 μl of matrix solution were premixed in a tube, 0.5 μl of the mixture was placed on the sample target and allowed to dry at ambient temperature. Positive ion MALDI mass spectra were measured on a Bruker BIFLEX II reflectron time-of-flight mass spectrometer (Bruker–Franzen, Bremen, Germany) equipped with a SCOUT 26 sample inlet, a gridless delayed extraction ion source and a nitrogen laser (337 nm, Laser Science, Cambridge (MA), U.S.A.). Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. The spectrometer was calibrated internally using the trypsin autodigestion fragments with monoisotopic $[M + H]^+$ values at *m/z* 842.5 and 2 211.1.

μ-LC-ESI Tandem Mass Spectrometry

The tryptic peptides were loaded onto a PepMap C18 column (0.3 × 250 mm, 300 Å, 5 μm; LC Packings, Amsterdam, Netherlands) and separated using gradient elution from 5% MeCN/0.5% acetic acid to 95% MeCN/0.5% acetic acid at a flow rate of 4 μl/min for 50 min. The column was connected directly to an LCQ[DECA] ion trap mass spectrometer (ThermoQuest, San Jose (CA), U.S.A.) equipped with a nanoelectrospray ion source. The spray voltage was held at 2.2 kV and the tube lens potential was –10 V. The heated capillary was kept at 150 °C with a voltage of 32 V. Full scan spectra were recorded over the mass range 300–2 000 amu, MS/MS mass spectra were acquired using the manufacturer's software.

Protein Identification

Peptide mass maps produced by MALDI-MS were searched against a non-redundant database NCBI using the program ProFound[28]. Automated computer-assisted interpretation and database searches of tandem mass spectra obtained by μ-LC-ESI-MS/MS were performed using the algorithm SEQUEST[TM].

RESULTS AND DISCUSSION

The high-resolution HL60 2-DE map contained more than 1 350 protein spots. The silver-stained protein pattern of ALA-treated non-irradiated HL60 cells at p*I* and with molecular weight ranging from 3 to 10 and from 10 to 250 kDa, respectively (Fig. 1), was compared to that of ALA-treated cells after irradiation by visible light. The subtractive image analysis revealed extensive changes in protein expression induced by ALA-PDT (Fig. 2). Twelve protein species affected by ALA-PDT were selected for subsequent mass spectrometric analysis.

The protein spots were digested directly in gel with trypsin and first identification was done by peptide mass mapping on MALDI-MS. All recorded peptide mass maps were calibrated using trypsin autoproteolytic products as internal standards resulting in a mass accuracy better than 50 ppm allowing more evident protein identification. With this approach, nine proteins were directly identified (Fig. 3); the assignments of spots 9, 10 and 11 were ambiguous due to the relatively low number of matched peptides and poor sequence coverage values (Table I).

To confirm the preliminary results obtained by peptide mass fingerprinting, aliquots of these three peptide mixtures were also subjected to
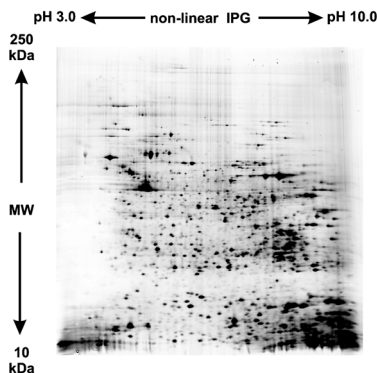

FIG. 1
2-DE protein pattern of ALA-treated non-irradiated HL60 cells

µ-LC-ESI-MS/MS, which allows a more reliable protein identification than the peptide mass fingerprinting. The acquired tandem mass spectra have provided an unambiguous sequence-specific information verifying the
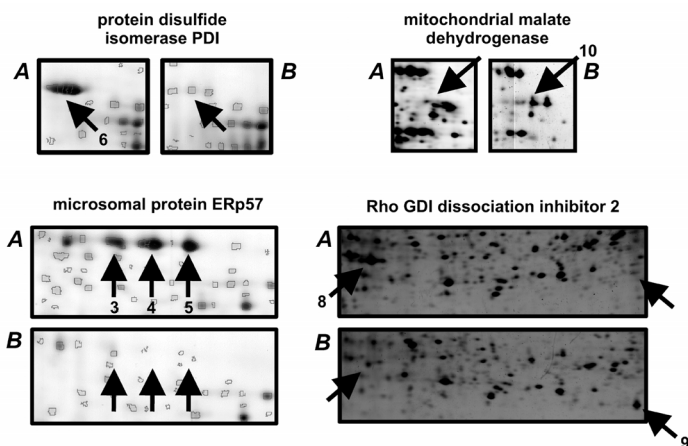

F_IG. 2

Pattern regions of ALA-treated non-irradiated (A panels) and irradiated (B panels) HL60 cells showing differences in intensity or position of several reticular and mitochondrial proteins. Protein spot numbering is in agreement with Table I
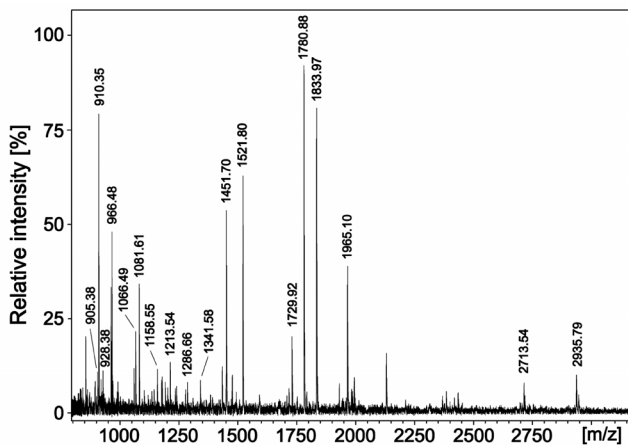

F_IG. 3

MALDI mass spectrum of the tryptic digest of human protein disulfide isomerase P55 (sequence coverage 44%). Peak labels correspond to $[M + H]^+$ ions of the individual peptide fragments

identity of the three protein spots in question (Fig. 4). The summary of all identified proteins is shown in Table I. Proteins from different cell compartments were found among them; this indicates that several cellular pathways are affected by ALA-PDT-mediated cytotoxicity. Interestingly, three of the down-regulated proteins: calreticulin, microsomal protein ERp57 and protein disulfide isomerase are all endoplasmic reticulum (ER) chaperones involved in calcium homeostasis. These findings point to the participation of endoplasmic reticulum in ALA-PDT-induced apoptosis due to oxidative stress imposed on ER proteins.

TABLE I
Identification of HL60 proteins affected by ALA-PDT therapy using mass spectrometric techniques

Spot	MW kDa	pI	No. of peptides[a]	Sequence coverage[a] %	Protein name	NCBI accession number
1	59.5	4.20	15	47	calreticulin	117501
2	57.0	4.20	17	50	calreticulin	117501
3	53.4	5.45	16	43	microsomal protein ERp57	2507461
4	53.8	5.49	21	48	microsomal protein ERp57	2507461
5	53.6	5.52	20	48	microsomal protein ERp57	2507461
6	57.8	4.61	18	44	protein disulfide isomerase	2507460
7	57.0	4.97	11	32	HSP 60	129379
8	26.5	5.03	10	59	Rho GDI dissociation inhibitor 2	1707893
9	24.9	6.19	8	57	Rho GDI dissociation inhibitor 2[b]	1707893
10	36.5	7.30	10	38	malate dehydrogenase[b]	5174541
11	17.6	5.98	6	45	ribosomal protein S12[b]	11418677
12	25.3	5.26	9	56	triosephosphate isomerase	136060

[a] Values obtained from MALDI-MS peptide mapping exclusively. [b] Protein identity was confirmed by μ-LC-MS/MS.

Moreover, as illustrated for calreticulin and microsomal protein ERp57, multiple isoforms of individual protein species were revealed by these methods (Fig. 2, Table I). The example of Rho GDI dissociation inhibitor 2 demonstrates the possibility to detect protein processing and entailing molecular weight decrease and p*I* shift induced by ALA-PDT (Fig. 2, Table I).

Peptide mass fingerprinting by MALDI and/or peptide sequencing by tandem mass spectrometry represent powerful and specific tools for protein identification working at the femtomole level. The reported approach is extremely efficient. MALDI fingerprint itself can be acquired within a few minutes, whereas an LC-MS/MS run takes about 30 min. In addition, the current public availability of protein sequence databases makes mass spectrometry a method of choice for every biochemist.

However, this approach can only identify proteins the sequence of which or the sequence of a closely homologous protein is available in the databases. It must be realized that genome sequences are still far from being complete for the majority of species. If the protein identification by peptide mass mapping fails, a partial sequence information (sequence tag) can be generated by PSD or MS/MS techniques and the identification is then based on high cross-species sequence homology. If the protein is still considered as unknown, two alternatives can be applied. The first is obtaining se-
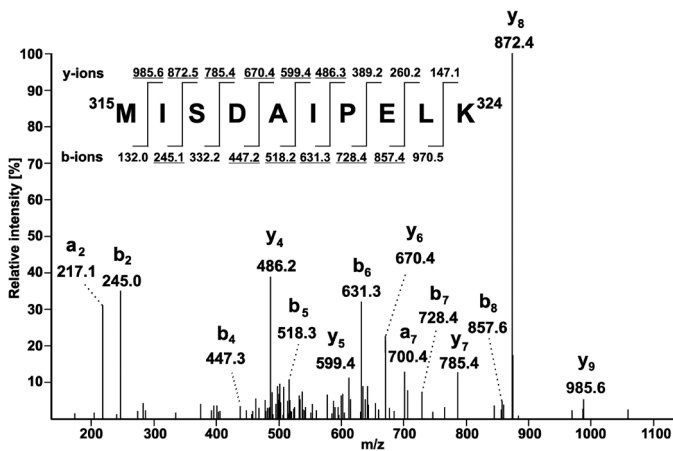

FIG. 4

μ-LC-MS/MS mass spectrum of the [M + 2 H]$^{2+}$ ion (*m/z* 559) of the peptide ^{315}M-K^{324} found in the tryptic digest of mitochondrial malate dehydrogenase. The nearly complete b- and y-ion series (for the ion nomenclature, see ref.[29]) clearly confirmed the peptide sequence shown on the top. The fragment ions observed in the spectrum are underlined

quence information suitable for construction of oligonucleotide probes and subsequent cloning/sequencing of a gene encoding the protein of interest[30,31]. In the other, if a sufficient amount of protein is isolated, the sequence can be determined *de novo* using only mass spectrometry[32].

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