Identification of apoptotic tyrosine-phosphorylated proteins after etoposide or retinoic acid treatment of HL-60 cells

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A main shortcoming of using HL-60 cells as a model of granulocyte-macrophage differentiation is that some cells in the differentiating population undergo apoptosis. To address this issue, we have identified which tyrosine-phosphorylated proteins are involved in apoptosis and differentiation, respectively. HL-60 cells were induced specifically to undergo apoptosis with 68 mM etoposide, and to undergo granulocytic differentiation with 1 mM retinoic acid (RA). The corresponding two-dimensional electrophoretic maps of tyrosine-phosphorylated proteins from treated cells were compared. In the 8 h etoposide-treated HL-60 cell population, 83% of the cells were apoptotic. In the 120 h RA-treated cells, 50% of the cells were apoptotic. Eighteen cytosolic and nuclear tyrosine-phosphorylated proteins were found in both the 8 h etoposide- and the 120 h RA-treated cells, but not in the proliferating HL-60 cell population. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry analyses suggested that some of the proteins may be involved in signal transduction pathways (NFκB, GTP-binding protein, protein disulfide isomerase, Cyclophilin A), others in cell transcriptional and translational control (hnRNP H, hnRNP L, Hsp60, Hp1, Hcc-1, 26S proteasome beta-subunit, ATP synthase beta-chain), and a third group in cell cytoskeleton organization and receptor cycling (profilin, caveolin-1). An understanding of signal transduction in apoptosis initiation by screening for tyrosine-phosphorylated proteins associated with apoptosis may provide new targets for the treatment of leukemia.

Keywords: Apoptosis / Differentiation / Etoposide / Tyrosine phosphorylation

1 Introduction

The human promyelocytic leukemia HL-60 cell line has been used repeatedly to study the control mechanisms of cell proliferation and differentiation [1–6]. Various compounds induce differentiation of HL-60 cells into granulocyte- or monocyte-like cells and cause inhibition of cell growth. Retinoids represent the most potent inducers of promyelocyte differentiation to granulocytes [6]. Due to this property they have been proposed as future radical theraeutics for treatment of acute promyelocytic leukemia [7, 8]. Under certain conditions proliferating or retinoic acid (RA)-induced differentiating cells also undergo apoptosis [4, 9, 10], which may cause some adverse side effects. Thus, in vitro studies of the relationship between granulocyte differentiation and apoptosis should be useful for the elucidation of the control mechanisms of normal hematopoiesis and for the strategy of differentiation therapy.

RA has been suggested to cause cytotoxic effects via apoptosis, but the mechanisms are not fully understood. The cytotoxic effect of RA on cell growth and induction of apoptosis is mediated specifically via RA receptors [11]. Several genes have been described to be involved in the process of RA-induced apoptosis. The Bax protein and the nuclear transcription factor TR3 have been shown to be translocated from the cytoplasm to mitochondria, and bcl-2 is down-regulated during RA-induced apoptosis.

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; RA, retinoic acid

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Protein phosphorylation and dephosphorylation play pivotal roles in intracellular signalling. In cancer, as well as in other proliferative diseases, unregulated cell proliferation, differentiation and survival frequently result from abnormal protein phosphorylation. The receptor tyrosine kinase signalling network plays a central role in regulating cellular differentiation and proliferation. Kim and Feldman [19] have, for instance, shown that introduction of activated protein tyrosine kinase Fes into U937 monocytic precursors induced differentiation into functional macrophages. Moreover, Wang et al. [20] observed that receptor tyrosine kinase EphB4 enforces preferential megakaryocytic and erythroid differentiation and may be a molecular bridge between angiogenesis and hematopoiesis. The receptor protein-tyrosine phosphatase DEP-1 has been implicated in the regulation of cell growth, differentiation and transformation, and has been identified as a potential tumour suppressor [21]. It is known that RA induces sustained activation of various mitogen-activated protein kinase signalling molecules, including extracellular signal-regulated kinase 2, src-like kinases, and adapter molecules. The regulation and function of Raf kinases that interact with adaptors, cofactors (14-3-3), chaperones, activators (Ras, kinases), substrates (MEK-1/2), inhibitors (Raf kinase inhibitor protein), and regulators of subcellular location (Ras; Bcl2) have been reviewed by Dhillon and Kolch [22]. Recently, Sedlik et al. [23] have shown that Syk protein tyrosine kinase is dispensable for dentritic cell differentiation in vitro and in vivo.

A balance between cell proliferation, differentiation and apoptosis is crucial for normal development. A number of observations indeed suggest that signalling between differentiation and cell death machinery occurs. Apoptosis is characterized by a rapid entry of cells into a programmed cell death cascade involving cell shrinkage, chromatin condensation around the nuclear periphery, plasma membrane blebbing, controlled DNA degradation and fragmentation of cells into membrane bound apoptotic bodies [24]. Regulation of apoptosis requires various protein kinases and phosphatases. The role of PTK, for instance, has been assessed with specific inhibitors of tyrosine phosphorylation, i.e. herbamycin A [25, 26] and genistein [27]. The activity of Burton's tyrosine kinase was critical for the apoptotic response to ionizing radiation in chicken DT40 lymphomic cells [28], and the Src-family tyrosine kinases are activated during Fas-mediated apoptosis [29]. Phosphatases have also been implicated in control of the apoptotic pathway. The tyrosine phosphatase FAP-1 has been reported to inhibit Fas-induced apoptosis [30]. However, besides being positive regulators of apoptosis, protein tyrosine kinases can also be anti-apoptotic. Thus, Bcr/Abl kinase delays the onset of apoptosis in hematopoietic cells [31–33]. In the present study, we examined tyrosine phosphorylation of cytosolic and nuclear proteins during RA-induced differentiation and etoposide-mediated apoptosis. We detected several tyrosine-phosphorylated proteins, which may be related to the induction or execution of apoptosis.

2 Materials and methods

2.1 Cell culture

The human promyelocytic leukemia cell line HL-60 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin in a 5% CO2 humidified incubator at 37°C. Cells were seeded at a density of 4 × 10⁵ cells/mL and allowed to attain a maximum density of 1.5 × 10⁶ cells/mL before being transferred to fresh medium. Granulocytic differentiation was induced by treating 5 × 10⁵ cells/mL with 1 μM RA. Stock solutions of 2 mM RA in 95% ethanol were stored at −20°C. The extent of differentiation was assayed by the ability of the cells to reduce nitroblue tetrazolium (NBT) to insoluble blue-black formazan on stimulation by phorbol myristate acetate (PMA) [34]. One hundred μL of cell suspension from the cultures was mixed with an equal volume of 0.2% NBT dissolved in PBS containing 40 ng/mL PMA and incubated further at 37°C for 30 min. Blue and granulated NBT-positive cells were counted using a haemocytometer. At least 200 cells were scored for each determination and the number of NBT-positive cells was expressed as a percentage of the total number of viable cells as determined by the exclusion of 0.2% trypsin blue. Differentiating populations containing no less than 50–60% differentiated cells were used. Apoptosis was induced by treating logarithmically growing HL-60 cells with 68 μM etoposide. The
stock solution was 68 μM etoposide in dimethyl sulfoxide. After 8 h, 80–85% of cells were scored as apoptosis using acridine orange and ethidium bromide [35]. Apoptotic cells were assessed by the appearance of green and/or orange apoptotic bodies in live and/or dead apoptotic cells, respectively, using a fluorescent microscope.

2.2 Isolation and fractionation of apoptotic DNA fragments

After harvesting, cell samples were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 s with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5; 10 μL per 10^6 cells, minimum 50 μL). After centrifugation for 5 min at 1600 g, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 h with RNase A (Sigma, St. Louis, MO, USA; final concentration

2.4 Isolation of cytosolic and nuclear proteins

Nuclei were isolated as described by Antalis and Godbolt [38] with some modifications. Cells were collected, washed twice in 0.1 M Na_2HPO_4, 0.1 M Na_2HPO_4, 0.1 M NaCl, pH 7.5 (PBS) containing 10 mM NaF and 1 mM Na_3VO_4, resuspended to 3 × 10^7 cells/mL in solution A (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl_2, 0.05% NP-40, 1 mM PMSF, 1 μg/mL aprotinin, leupeptin and pepstatin, 10 mM NaF, 1 mM sodium orthovanadate) and incubated for 15 min at 0°C to induce swelling. The cell suspension was then shaken vigorously by hand and immediately mixed 1:1 v/v with solution A containing 0.6 M sucrose (solution B). The cell homogenates were then centrifuged at 1500 g for 5 min. The supernatant, corresponding to the cytosol fraction, was clarified by centrifugation at 20 000 g for 30 min and frozen at −76°C.

2.5 Gel electrophoresis

Cytosolic and nuclear proteins were resolved by 2-DE (IEF/SDS). In some experiments two parallel gels were used if needed. An Immobiline DryStrip Kit, (Amersham Biosciences, Uppsala, Sweden) pH range 3–10, and Exel Gel SDS (gradient 8–18%) were used for 2-DE. We used 11 cm long Immobiline DryStrips, (110 × 3 × 0.5 mm after rehydration). In order to characterize specific proteins in a total cytoplasmic or nuclear protein mixtures the pH range 3–10 was chosen. Proteins were reduced and alkylated to eliminate disulfide bridges prior to 2-DE. For this we used DTT as the reductant and iodoacetamide as the alkylating reagent. The second-dimension separation was performed on the Multiphor II flatbed system (Amersham Biosciences) using buffer strips. For the anode SDS buffer strip 0.45 mol/L Tris-Acetate, pH 6.6, 4 g/L SDS and 0.05 g/L Orange G were used. For the cathode SDS buffer strip 0.08 mol/L Tris, 0.80 mol/L Tricine and 6 g/L SDS, pH 7.1 were used. 2-DE was carried out according to the manufacturer’s instructions (Immobiline DryStrip Kit for 2-DE with Immobiline DryStrip and ExelGel SDS; Amersham Biosciences). For analysis of total cytosolic and nuclear proteins, 2-DE gels were stained using the Pharmacia Silver Staining Kit (Amersham Biosciences, Uppsala, Sweden). For protein identifi-
using the Brilliant Blue R Stain Kit (Sigma) according to the manufacturer’s instructions. For scanning of 2-D gels and Western blotting the Fluor-S Multilager (Bio-Rad, Hercules, CA, USA) and PDQUEST software were used.

2.6 Immunoblotting analysis

After 2-DE proteins were transferred to an Immobilon™ PVDF transfer membrane (Millipore, Bedford, MA, USA) and then blocked with 5% BSA dissolved in PBS containing 0.1% Tween-20 by incubation overnight at 4°C. After washing in PBS/Tween-20, the filters were probed with anti-phosphotyrosine antibody. Specifically, for tyrosine-phosphorylated protein analysis, the membranes were incubated for 1 h at room temperature with 1:4000 dilution of anti-phosphotyrosine antibodies (IgG2b) in PBS/Tween-20. They were then washed four times in 30 min with PBS/Tween-20 and incubated further with 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in PBS/Tween-20 for 1 h at room temperature. The filters were then washed with PBS/Tween-20 as described above and immunoreactive bands were detected by enhanced chemiluminescence following the manufacturer’s instructions (Amersham Biosciences).

2.7 In-gel digestion and MALDI-TOF MS

Regions of interest were isolated from the gel and subjected to overnight in-gel tryptic digestion [39, 40]. Briefly, the gel slices were dehydrated with 50% ACN and then dried completely using a centrifugal evaporator (SpeedVac, SC100 Savant, Albertville, USA). The protein spot was rehydrated in 30 μL of 25 mM ammonium bicarbonate (pH 8.3) containing 25 μg/mL modified trypsin (Promega, Madison, WI, USA), and the samples were incubated overnight at 37°C. The tryptic peptides were subsequently extracted from the gel slices as follows. Any extraneous solution remaining after digestion was removed and placed in a fresh tube. The gel slices were washed two times with 5% TFA in 50% ACN, with occasional shaking. The digestion and extract solutions were then combined and evaporated. For MALDI-TOF analysis, the peptides were redissolved in 3 μL of 30% ACN and 0.01% TFA and then prepared with a matrix (z-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed on a Voyager MALDI-TOF MS (Perspective Biosystems, Framingham, MA, USA) and externally calibrated using synthetic peptides with known masses. The spectra were obtained in the positive ionization mode at 25 kV. The mass information generated from the composite spectrum was submitted to a search performed with the PeptideIt (www.expasy.org/tools/peptident.html), EMBL (www.ebi.ac.uk/embl/), and MS-Tag (www.prospector.ucsf.edu) databases.

3 Results

3.1 Time-dependent extent of apoptosis and DNA degradation in RA- and etoposide-treated HL-60 cell cultures

During RA-induced HL-60 cell differentiation to granulocytes some cells undergo apoptosis [10]. Table 1 displays the time-dependent proportion of apoptotic and differentiated cells in the 1 μM RA-induced differentiating and 68 μM etoposide-treated population of HL-60 cells, as assessed by morphology. With etoposide treatment apop-

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Table 1. Time-dependent extent of differentiation, apoptosis and DNA degradation in RA- and etoposide-treated HL-60 cells

<table>
<thead>
<tr>
<th>Duration (h) of treatment of HL-60 cells with 68 μM etoposide</th>
<th>Extent (%) of apoptotic cells in cell culture</th>
<th>Duration (h) of treatment of HL-60 cells with 1 μM retinoic acid</th>
<th>Extent (%) of apoptotic cells in cell culture</th>
<th>Extent (%) of differentiated cells in cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>5</td>
<td>0 (control)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>24</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>48</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>72</td>
<td>10</td>
<td>40</td>
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<td>3</td>
<td>60</td>
<td>96</td>
<td>20</td>
<td>60</td>
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<tr>
<td>8</td>
<td>85</td>
<td>120</td>
<td>50</td>
<td>65</td>
</tr>
<tr>
<td>18</td>
<td>96</td>
<td></td>
<td></td>
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</table>
Figure 1. Flow cytometry analysis of cell cycle distribution in proliferating, differentiated and etoposide-treated HL-60 cells. Representative example of flow cytometric analysis of control HL-60 cells, treated with 68 μM etoposide for various lengths of time or with 1 μM RA for 120 h. An hypodiploid peak (subG1) represents the amount of apoptotic cells (AP). The percentages of apoptotic nuclei after 0.5, 1, 2, 3, 8, 18 h of etoposide treatment were 5 ± 0.7%, 7 ± 1.2%, 35 ± 2.3%, 60 ± 2.5%, 83 ± 3.2%, and 96 ± 2.3%, respectively. After 120 h of RA induction 50 ± 2.5% of apoptotic nuclei were detected. Results represent the means of three separate experiments.
totic cells appeared first at 2–3 h. The proportion of apoptotic cells during differentiation started to increase during the commitment stage, i.e. after 48 h, and reached 50–55% after 120 h. In parallel, the number of differentiated cells increased from 24% at 48 h to 65% at 120 h after induction of differentiation. Thus, comparison of proteins from cells treated with etoposide for 3 h or 8 h (yielding 60 and 85% apoptotic cells, respectively) with those treated with RA for 120 h (50% apoptotic cells and 65% differentiated cells) should allow proteins of apoptotic origin to be distinguished. This was accomplished by analyses of 2-D electrophoretic spot patterns, which appeared characteristic both for the 8 h etoposide- and 120 h RA-treated HL-60 cells.

Representative examples of flow cytometric analysis of control cells, cells treated with 68 μM etoposide for 0.5–18 h or differentiated for 120 h with 1 μM of RA are shown in Fig. 1. A hypodiploid peak (sub G1) represents the amount of apoptotic cells (AP). The percentages of apoptotic cells after 1, 2, 3, 8, 18 h of etoposide treatment were 7 ± 1.2, 35 ± 2.3, 60 ± 2.5, 85 ± 3.2, and 96 ± 2.3%, respectively. There were 50 ± 2.7% apoptotic cells in the differentiating population after 120 h of induction. Thus, the flow cytometric analysis suggests that after 1 h etoposide treatment the cells are still similar to control cells. However, as suggested by morphological and DNA fragmentation criteria, after 2 h with 68 μM etoposide part of the cells had started to undergo apoptosis. Figure 2 shows the DNA fragmentation patterns. These data are consistent with those obtained by morphological methods (Table 1). In RA-treated cells DNA fragmentation started at 72 h of differentiation and at 120 h the smallest DNA fragments reached the nucleosomal level (145 bp), while in etoposide-treated cells DNA was degraded at the nucleosomal level after 3 h of treatment (Fig. 2).

3.2 2-D electrophoretic patterns of total cytoplasmic and nuclear proteins in proliferating, etoposide-treated, and differentiated HL-60 cells

Cytosolic and nuclear proteins from proliferating, etoposide- or RA-treated HL-60 cells were resolved by 2-DE. Up to 700 polypeptides were detected by silver-staining. As can be seen in Fig. 3, the number of cytoplasmic polypeptides was much higher in HL-60 cells treated for 8 h with etoposide (85% apoptotic cells) or for 120 h with RA (50% apoptotic cells), than in control cells or cells treated for 1 h with etoposide (both populations contained 5–7% apoptotic cells). Incidentally, we have recently shown that 96 h RA-treatment of HL-60 cells results in insignificant changes in cytoplasmic protein patterns [41]. Furthermore, no qualitative changes in nuclear protein patterns were detected [42]. However, we did detect some qualitative differences between cytoplasmic protein patterns of control cells, cells treated with etoposide for 3 h (data not shown) or 8 h, and cells differentiated for 120 h with RA. As these changes mostly encompass small molecular mass polypeptides it is likely that they are degradation products of high molecular mass proteins of apoptotic origin.

3.3 Tyrosine phosphorylation of cytoplasmic and nuclear proteins from control, etoposide- and RA-treated HL-60 cells

To identify tyrosine-phosphorylated proteins in silver-stained 2-D gels, twin gels were Western blotted with anti-phosphotyrosine antibodies and compared. Figure 4 shows that tyrosine phosphorylation of cytoplasmic and nuclear proteins of cells treated with either etoposide or RA was different from control proliferating cells. This was observed in populations containing both apoptotic and differentiating cells. Already, after 1 h etoposide treat-
Figure 3. 2-DE maps of cytosolic and nuclear proteins from proliferating, differentiated and etoposide-treated HL-60 cells. Cytoplasmic and nuclear proteins from proliferating HL-60 cells (Control), cells treated for 1 or 8 h with 68 μM etoposide, and cells differentiated with RA for 120 h were fractionated by 2-DE and the gels were silver-stained. Arrows indicate the position of tyrosine-phosphorylated proteins of apoptotic cell origin, appearing in cells treated with etoposide and at a mature stage of differentiation.
Figure 4. Tyrosine phosphorylation of cytosolic and nuclear proteins in proliferating, differentiated and etoposide-treated HL-60 cells. Cytoplasmic and nuclear proteins from proliferating HL-60 cells (Control), cells treated for 1 or 8 h with 68 μM etoposide and cells differentiated with RA for 120 h were fractionated by 2-DE. Proteins were transferred to PVDF membrane and immunoblotted with anti-phosphotyrosine antibodies. Arrows mark new proteins modified by tyrosine phosphorylation in cells induced for apoptosis and a late stage of differentiation.
ment, when 5–7% of the cell population was apoptotic, new proteins had undergone tyrosine phosphorylation in the cytoplasm (Figs. 3 and 4): 17 kDa, pI 8.3 (no. 18), 20 kDa, pI 7.0–7.5 (no. 16, 17), 52 kDa, pI 5.5–5.6 (no. 3, 4), 55 kDa, pI 4.4 (no. 15), 59 kDa, pI 5.6 (no. 7), 65 kDa, pI 6.2–6.7 (no. 11–13) and 73 kDa, pI 4.75 (no. 14). Some tyrosine-phosphorylated proteins, i.e. proteins 3, 4, 7, 11–13, were also detected in the nucleus of HL-60 cells (in addition to 20 kDa, pI 4.8–5.0 (no. 8–10), and 62 kDa, pI 4.9 (no. 2)). The intensity of newly tyrosine-phosphorylated proteins increased markedly after 8 h of etoposide treatment, but only the proteins labeled no. 5, 6 (27–29 kDa and pI 5.8) and no. 1 (52 kDa and pI 4.8) in Figs. 3 and 4 appeared newly tyrosine-phosphorylated after the treatment. To summarize, we detected 17 newly tyrosine-phosphorylated proteins in the nucleus (Fig. 4), and 11 in the cytosol of HL-60 cells. It should be noted that tyrosine-phosphorylated protein 3 is characteristic for apoptosis induction, and proteins 1, 5, and 6 for the execution stage of apoptosis, i.e. after 8 h of etoposide treatment (85% apoptotic cells). Tyrosine-phosphorylated proteins 16–18, which were detected in the cytosol after 1 h etoposide treatment, translocated into the nucleus after 8 h, suggesting that they also take part in apoptosis execution.

As can be seen in Fig. 4, after 120 h of RA treatment intensive tyrosine-phosphorylated cytosolic proteins appeared mainly with acidic pIs (4.5–6.0) and molecular masses around 48–89 kDa. Tyrosine phosphorylation of nuclear proteins occurred mainly on proteins in the acidic region. In this population, we detected 50% apoptotic cells and a large number of differentiated cells (65%) (Table 1). As can be seen in Fig. 4 (120 h RA), the tyrosine-phosphorylated proteins marked by arrows were characteristic both of apoptosis initiation and execution. Other newly tyrosine-phosphorylated cytoplasmic and nuclear proteins in the RA-treated cell population could be related to granulocytic differentiation of the cells.

Proteins 11–13 (Figs. 3 and 4), which were tyrosine-phosphorylated both in the cytosol and the nucleus of proliferating HL-60 cells, underwent dephosphorylation during the apoptosis process in the cell nucleus. However, this modification also increased in the cytosol of cells treated either with etoposide or RA.

### 3.4 MALDI-TOF MS analysis of tyrosine-phosphorylated proteins in apoptotic cells

A proteomic approach was employed to identify tyrosine-phosphorylated cytosolic and nuclear proteins related to apoptosis in HL-60 cells. Separation of cytosolic and nuclear proteins from proliferating, etoposide- and 120 h RA-treated HL-60 cells by 2-DE was performed using a wide pH range IPG strip (pH 3–10) (Figs. 3 and 4). This allowed protein identification in minor spots. Novel tyrosine-phosphorylated cytosolic and nuclear proteins from apoptotic HL-60 cells were isolated and prepared for MALDI-TOF MS. All newly tyrosine-phosphorylated or dephosphorylated proteins in apoptotic HL-60 cells were subjected to proteomic analysis (Figs. 3 and 4, marked by arrows). High quality mass spectra were obtained. Three software packages (MS-Fit, PeptIdent (Swiss-Prot) and PeptideSearch (EMBL)) were used to identify protein spots. The summarized search results are presented in Table 2. Seven cytosolic spots could not be identified.

### Table 2. The summarized search results of tyrosine-phosphorylated proteins of apoptotic HL-60 cell origin

<table>
<thead>
<tr>
<th>No.</th>
<th>Mr</th>
<th>pI</th>
<th>AC</th>
<th>Description of matching, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>52</td>
<td>4.8</td>
<td>P06576</td>
<td>Chain 1: ATP syntase beta chain, 28%</td>
</tr>
<tr>
<td>2.</td>
<td>62</td>
<td>4.9</td>
<td>P10809</td>
<td>Chain 1: 60 kDa heat shock protein, 28%</td>
</tr>
<tr>
<td>3.</td>
<td>52</td>
<td>5.5</td>
<td>P31943</td>
<td>Heterogeneous nuclear ribonucleoprotein H (hnRNP H), 24.1%</td>
</tr>
<tr>
<td>4.</td>
<td>52</td>
<td>5.6</td>
<td>P31943</td>
<td>Heterogeneous nuclear ribonucleoprotein H (hnRNP H), 31.2%</td>
</tr>
<tr>
<td>5.</td>
<td>29</td>
<td>5.8</td>
<td>P82979</td>
<td>Nuclear protein Hcc-1 (HSPC316), 30%</td>
</tr>
<tr>
<td>6.</td>
<td>27</td>
<td>5.8</td>
<td>P49720</td>
<td>Proteasome subunit beta type, 18%</td>
</tr>
<tr>
<td>7.</td>
<td>59</td>
<td>5.6</td>
<td>Q04206</td>
<td>Transcription factor p65 (Nuclear factor NF-κB p65 subunit), 27.6%</td>
</tr>
<tr>
<td>8.</td>
<td>20</td>
<td>5.0</td>
<td>Q13185</td>
<td>Chromobox protein homolog 3 (HP1), 23.6%</td>
</tr>
<tr>
<td>9.</td>
<td>19.9</td>
<td>4.9</td>
<td>Q03135</td>
<td>Chain 1: Caveolin-1 alpha, 33.5%</td>
</tr>
<tr>
<td>10.</td>
<td>20</td>
<td>4.8</td>
<td>P21181</td>
<td>G25K GTP-binding protein (GP), 22.5%</td>
</tr>
<tr>
<td>11.</td>
<td>65</td>
<td>6.2</td>
<td>P14866</td>
<td>Heterogeneous ribonucleoprotein L (hnRNP L), 35.8%</td>
</tr>
<tr>
<td>12.</td>
<td>65</td>
<td>6.6</td>
<td>P14866</td>
<td>Stress induced-phosphoprotein 1 (ST11), 13%</td>
</tr>
</tbody>
</table>
possibly due to low protein concentration even though their tyrosine phosphorylation signals were very strong. Table 2 lists tyrosine-phosphorylated cytosolic and nuclear proteins related to apoptosis. For proteins 10 to 12, protein masses from one protein spot matched several different proteins. Proteins 1 to 10 and 14 underwent tyrosine-phosphorylation during the apoptosis process. They included 60 kDa heat shock protein, heterogeneous nuclear ribonucleoprotein H (hnRNP H), nuclear protein Hcc 1, chromobox protein homolog 3, GTP-binding protein, and 78 kDa Glucose regulated protein. Proteins 16 to 18 were identified as protein disulfide isomerase, peptidyl-prolyl cis-trans isomerase and profilin. They underwent tyrosine phosphorylation only in cells after treatment with etoposide but not RA. Proteins 11 to 13, which correspond to heterogeneous ribonucleoprotein L and stress-induced phosphoprotein 1, were tyrosine-dephosphorylated in the cell nucleus during apoptosis.

4 Discussion

The aim of this study was to identify tyrosine-phosphorylated cytosolic and nuclear proteins of apoptotic origin. A typical hematopoietic cell model, the human promyelocytic cell line HL-60, was employed to study protein tyrosine phosphorylation related to chemically induced apoptosis, and granulocytic differentiation leading to apoptosis. It has recently been reported that HL-60 cell differentiation in vitro towards granulocytes is associated with induction of apoptosis [4, 41–44]. Furthermore, like their normal counterparts in blood, the cells die via apoptosis [4, 41–44]. We have also shown that after RA-mediated granulocytic differentiation of HL-60 cells many cytoplasmic proteins undergo tyrosine phosphorylation [41]. Other studies using leukemia cell lines, such as HL-60 and MO7e suggest a role for tyrosine phosphorylation in the regulation of programmed cell death [45]. To date, an understanding of the basic mechanisms of apoptosis initiation and the proteins which contribute to this mechanism is limited. There is, thus, a great interest in the signal transduction of apoptosis initiation and in screening for molecules associated with apoptosis. The identification of tyrosine-phosphorylated proteins related to apoptosis of hematopoetic cells could help in the development of new leukemia therapies.

In the present study, we analyzed the time-dependent extent of differentiation and apoptosis in RA- and etoposide-induced HL-60 cells. Our data demonstrate that after induction of cell differentiation the number of differentiating and apoptotic cells is proportional to the time of differentiation. In the differentiating population the proportion of apoptotic cells started to increase during the commitment stage (48 h), and formed about 50% of the population in the maturation stage (120 h). Analysis of control, differentiated and apoptosis-induced HL-60 cells by flow cytometry demonstrated that a hypodiploid peak (sub G1), representing the amount of apoptotic cells, appeared in particular after 2 h of etoposide treatment. In cells treated with RA for 120 h, the percentage of cells in the subG1 phase increased about 10-fold in comparison to control proliferating HL-60 cells. Thus, flow cytometric analysis showed that HL-60 cells started to accumulate in the G1 phase of the cell cycle after induction of differentiation and apoptosis.

We induced HL-60 cells for apoptosis with etoposide and for granulocytic differentiation with RA, to compare proteins undergoing tyrosine phosphorylation in these cell populations. 2-DE in combination with immunoblotting with antibodies against phosphotyrosine and MALDI-TOF MS enabled us to identify proteins which undergo tyrosine phosphorylation during apoptosis induction. We detected over 30 cytosolic and nuclear proteins with altered tyrosine phosphorylation during apoptosis induction. A few tyrosine kinases have been detected in the nucleus including Fes/Fer, Fyn, Lyn, Wee1, c-Abl, and Rak [46]. Functionally, tyrosine phosphorylation has been linked to the progression of the cell cycle and the regulation of transcription factor activity. For example, the anti-apopto-

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Activation of GTP-binding proteins by post-translational modifications suggest that these modifications are essential for their interaction with their respective effector proteins. Our finding that the 25 K GTP-binding protein undergoes tyrosine phosphorylation clearly supports the hypothesis that this modification also plays a role in the apoptosis of HL-60 cells. Peptidyl-prolyl isomerases are a group of cytosolic enzymes first characterized by their ability to catalyze the cis-trans isomerization of cis-peptidyl prolyl bonds [53]. Cyclophilin A (CypA), identified by us, is present in the cytoplasm of HL-60 cells and is tyrosine phosphorylated. This modification became more intense after induction of HL-60 cells to undergo apoptosis. CypA appeared in two positions on the 2-DE gel. This may be a result of modifications of the CypA precursor.

In the present study, we also identified a number of proteins involved in cell transcriptional and translational control. The functions of hnRNP proteins range from mRNA packaging and transport, to mRNA splicing and silencing [54]. Hence, hnRNP proteins play diversified roles both in the nucleus and cytoplasm, and regulate gene expression at various levels. We found that hnRNP H undergoes tyrosine phosphorylation during apoptosis, and the levels of hnRNP L decrease slightly in the nucleus. However, in the cytosol hnRNP L tyrosine phosphorylation increased during apoptosis and in the terminal stage of differentiation. This may be because of translocation of hnRNP L into the cytoplasm, where it affects mRNA translation, characteristic for the apoptotic process.

The up-regulation of several stress proteins, heat-shock proteins and glucose-regulated proteins which function as molecular chaperons, is required for tolerance against environmental stress. Huang et al. [55] observed an increase in Hsp60 levels during lipopolysaccharide-induced apoptosis in PC12 cells. Gupta and Knowlton [56] showed that reduction in cytosolic Hsp60 levels caused by its translocation to the plasma membrane is accompanied by apoptosis. We identified Hsp60 as a tyrosine-phosphorylated protein in apoptotic cells, further supporting its importance in apoptosis. In our studies 78 kDa glucose-regulated protein exhibits tyrosine phosphorylation in apoptotic cells. At the same time, protein disulfide isomerase, acting as a chaperone was found to be tyrosine-phosphorylated in the cytosol of HL-60 cells after induction of apoptosis. The 26 S proteasome is a multi-subunit complex that recognizes and degrades, in an ATP-dependent manner, various proteins, including poly-ubiquitinated, and misfolded and short-lived regulatory proteins [57]. It is found in the cytosol, endoplasmic reticulum and nucleus. In addition, recent evidence suggests a role for the ubiquitin-proteasome pathway in the process of cell death. We showed that the proteasome beta subunit is tyrosine-phosphorylated both after induction of apoptosis and at the terminal stage of granulocytic differentiation. It seems likely that the mechanisms of RA- and etoposide-induced apoptosis include proteasome-dependent degradation.

The HP1 class of chromobox genes encode an evolutionarily conserved family of proteins involved in the packaging of chromosomal domains into a repressive heterochromatic state. In our studies, HP1 was found to be tyrosine phosphorylated immediately after induction of apoptosis and at the terminal granulocytic differentiation.
stage. These results support the view that modification on tyrosine residues affects heterochromatin-induced repression, gene silencing for differentiation and apoptosis. A novel protein, Hcc-1, was identified by analysis of the hepatocellular carcinoma-M cell proteome [58]. We detected Hcc-1 in the nucleus of proliferating, apoptotic, and terminally differentiated HL-60 cells. However, Hcc-1 undergoes tyrosine phosphorylation only in the late stages of apoptosis in the cell population consisting of 50–85% apoptotic cells.

It has been demonstrated in many cell types that mitochondria play a crucial role in apoptosis. We identified mitochondrial ATP synthase beta-chain as being tyrosine-phosphorylated both in terminally differentiated HL-60 cells and after 8 h of etoposide treatment. Another category of tyrosine-phosphorylated and apoptosis-associated proteins are those involved in cell cytoskeleton restructuring. Profilin, a cytoskeleton protein regulated by phosphatidylinositol bisphosphate forms a complex with G-actin [59]. By being an important link between a receptor and the nucleus, the cytoskeleton likely plays a vital role in physiology, as well as in differentiation and apoptosis. It is interesting to note, that profilin in Phaseolus vulgaris is phosphorylated on tyrosine residues [60]. Vemuri et al. [61] described phosphorylation of profilin by different PKC isozymes, and recent studies also propose a role for profilin as a tumour suppressor [62]. We found that profilin undergoes tyrosine phosphorylation in the nucleus after 8 h of etoposide treatment when 85% of apoptotic cells appear in the population. Profilin could be an important link between signal transduction pathways and cytoskeletal dynamics during apoptosis and differentiation. Dynamic rearrangements of the actin cytoskeleton are important to the morphological changes observed both in apoptosis and granulocytic differentiation, allowing for instance cell motility and phagocytic capacity.

Another important protein we identified was caveolin-1. Sanguinetti and Mastick [63] suggested that it is phosphorylated on tyrosine by c-Abl in response to cellular stress. Caveolin is crucial for caveolae formation, receptor signalling and interaction with the cytoskeleton. We observed that caveolin-1 undergoes tyrosine phosphorylation during both etoposide-induced apoptosis and RA-mediated granulocytic differentiation in HL-60 cells. Clusters of signalling molecules, including caveolin-1, exist at sites of actin attachment to the plasma membrane and serve to modulate numerous cellular events including stress-induced signalling [64]. The data presented by us suggest a role for profilin and caveolin in apoptosis possibly linked to the regulation of the actin cytoskeleton.

5 Concluding remarks

In summary, our data suggest that tyrosine-phosphorylated cytosolic and nuclear proteins are involved in execution mechanisms of apoptosis, while early tyrosine-phosphorylated proteins may be involved in the induction of apoptosis. In the cell population treated with RA, some tyrosine-phosphorylated proteins apparently specifically related to differentiation mechanisms. Our studies display an attempt to understand the signal transduction of apoptosis by screening the molecules associated with this process. The identification of tyrosine-phosphorylated proteins related to apoptosis of hematopoetic cells could help to further understand apoptosis mechanisms, and serve as targets for the treatment of leukemia.

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6 References


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