Identification of Proteins Cleaved Downstream of Caspase Activation in Monocytes Undergoing Macrophage Differentiation*

Received for publication, January 18, 2006, and in revised form, April 19, 2006 Published, JBC Papers in Press, April 24, 2006, DOI 10.1074/jbc.M600537200

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We have shown previously that caspases were specifically involved in the differentiation of peripheral blood monocytes into macrophages while not required for monocyte differentiation into dendritic cells. To identify caspase targets in monocytes undergoing macrophagic differentiation, we used the human monocytic leukemic cell line U937, whose macrophagic differentiation induced by exposure to 12-O-tetradecanoylphorbol 13-acetate (TPA) can be prevented by expression of the baculovirus caspase-inhibitory protein p35. A comparative two-dimensional gel proteomic analysis of empty vector- and p35-transfected cells after 12 h of exposure to 20 nM TPA, followed by mass spectrometry analysis, identified 38 differentially expressed proteins. Those overexpressed in p35-expressing cells (n = 16) were all full-length, whereas half of those overexpressed in control cells (n = 22) were N- or C-terminal cleavage fragments. The cleavage or degradation of seven of these proteins was confirmed in peripheral blood monocytes undergoing macrophage colony-stimulating factor-induced macrophagic differentiation. In U937 cells exposed to TPA, these proteolytic events can be inhibited by expression of a caspase-8 dominant negative mutant or the cowpox virus CrmA caspase inhibitor. These cleavages provide new insights to analyze the role of caspases in this specific differentiation program.

When crossing the endothelium, peripheral blood monocytes can differentiate into scavenging macrophages or antigen-presenting myeloid dendritic cells. Cytokine conditions that regulate the balance between these two options have been identified. For example, in vitro, monocytes differentiate into macrophages in response to macrophage colony-stimulating factor (M-CSF) 1 (1) and into dendritic cells in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) 8 (2–4). Transcriptional events controlling the commitment of monocytes into macrophages versus dendritic cells are less known. It was recently proposed that dendritic cell differentiation could be instructed by high levels of the myeloid and lymphoid-specific Ets family transcription factor PU.1 together with the down-regulation of the monoocyte/macrophage-specific bZip factor MafB (5, 6).

We have shown previously that in vitro differentiation of monocytes into macrophages was associated with an activation of cellular proteases known as caspases, which was not observed in monocytes undergoing dendritic cell differentiation. We have shown also that caspase activation was required for macrophagic differentiation of U937 human leukemic cells under phorbol ester exposure (7). These results suggested that caspase activation could be one of the events that specifically promote macrophagic differentiation of peripheral blood monocytes. Accordingly, in mice, conditional deletion of the caspase-8 gene in the myelomonocytic precursors blocked the formation of macrophages without affecting the number of dendritic cells and granulocytes (8).

Caspases are aspartate-specific cysteine proteases that were identified as key players in cell death by apoptosis (9). These enzymes also contribute to processes that do not culminate in cell demise, such as cytokine maturation, T-cell activation, cell motility and migration, and cell differentiation (10). The involvement of caspases in cell differentiation was initially suspected in those whose maturation was associated with enucleation such as keratinocytes (11), lens epithelial cells (12), and erythroblasts (13). Caspase activity is also required for the formation of proplatelets from megakaryocytes (14); the differentiation of specific nucleated cells, such as skeletal myoblasts (15), osteoblasts (16), and osteoclasts (17); and, in Drosophila melanogaster, sperm differentiation and possibly oogenesis (18, 19).

Approximately 280 cellular substrates for mammalian caspases have been identified (20). It was proposed that the caspase-3-induced cleavage and activation of the serine/threonine kinase MST1 (mammalian sterile twenty-like kinase) was required for the differentiation of myoblasts into myotubes (15). In other cell types in which differentiation is associated with caspase activation, the cellular targets of these enzymes and how their cleavage contributes to the differentiation process remain poorly known. This caspase-mediated cleavage of cellular proteins must be selective to avoid the cell dismantling (e.g. the transcription factor GATA-1 is cleaved by caspases in erythroblasts undergoing apoptosis under erythropoietin deprivation, whereas this protein remains uncleaved in these cells when caspases are activated along the differentiation process) (13). To better understand the role of caspases in the
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differentiation of specific cell types, identification of their cellular tar-
ggets in each cell type is an absolute requirement.

One of the approaches used to identify natural macromolecular sub-
strates of cellular proteases and their products combines two-dimen-
sional gel electrophoresis of cell lysates with matrix-assisted laser de-
sorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry
(21–23). In the present study, this approach was used to identify caspase
targets in monocytes undergoing macrophagic differentiation by using
the baculoviral, broad spectrum caspase inhibitor, p35 protein (24, 25).
p35 is cleaved by activated caspases, which generates a fragment that
binds the caspase active site to form an irreversible inhibitor complex
(26). We compared the proteome of U937 cells stably transfected with
an empty vector and a vector expressing the p35 gene after a 12-h
exposure to phorbol esters. We identified several potential targets for
caspases activated in cells undergoing macrophagic differentiation, the
clavage of seven of them being shown to be specifically associated with
macrophagic differentiation of monocytes.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—We used mouse monoclonal antibodies
that recognize human HSC70 (Santa Cruz Biotechnology, Inc., Santa
Cruz, CA), β-actin (clone AC-15), α-tubulin (clone B-5-1-2), vinculin
(clone VIN-11-5) and heterogeneous nuclear ribonucleoprotein
(hnRNP) C1/C2 (clone 4F4) (Sigma), moesin (BD Biosciences), plas-
minogen activator inhibitor-2 (PAI-2) (American Diagnostica Inc.,
Stamford, CT), CD11b (fluorescein isothiocyanate-conjugated; Immu-
notech, Marseille, France), and CD1a and CD71 (fluorescein isothio-
cyanate-conjugated; BD Biosciences). We also used rabbit polyclonal anti-
odies that include anti-human HSP90-β (Affinity BioReagents,
Golden, CO), NPM (nucleophosmin) (Cell Signaling, Beverly, MA),
hnRNP H (Bethyl Laboratories, Inc., Montgomery, TX), and FLAG and
α-PAX (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). M-CSF, GM-
CSF, and IL-4 were obtained from R&D systems; 12-O-tetradecanoyl-
phorbol 13-acetate (TPA) and Etoposide (VP16) were from Sigma.

Cell Culture and Differentiation—The human leukemic U937 cell
line, obtained from the American Tissue Culture Collection (Manassas,
VA), was stably transfected with pTarget or pTarget containing p35
cDNA in combination with a FLAG sequence as described previously
(7) and pcDNA vector 3.1 or a caspase-8 dominant negative form or the
cowpox virus caspase-1 and -8 inhibitor CrmA (pcDNA, C8DN, and
CrmA; kindly provided by S. Grant, Medical College of Virginia, Rich-
mond, VA). These cells were grown in suspension in RPMI 1640
medium containing fetal calf serum (10%, v/v) in an atmosphere of 95%
O2 and 5% CO2 at 37 °C, and transfected cell populations were selected
by fluorescence-activated cell sorting (FACS) (24, 25).

Transfection was monitored by RT-PCR. Briefly, RNA extraction
was performed with the Nucleospin RNA II kit (Macherey-Nagel, Hoerd,
France), and the OneStep RT-PCR kit (Qiagen, Courtaboeuf, France)
was used according to the manufacturer’s instructions and the following
specific primers for the p35 gene (forward, ATGGATTAAAAAGAT-
GATGATGATAAAATGTTAATTTT; reverse, TTTATTATGTG-
TGTATTATACATTATGTTTGTGATGC) with the β2-micro-
globulin gene as a control (forward, ACCCCCATGTTAAGAGATG;
reverse, ATCTCTCAGCAACTCCATGATG).

The p35 protein was detected by immunoblotting of cell lysates using
an anti-FLAG antibody. To promote their differentiation, cells were
suspected at a density of 0.5 × 106 cells/ml in fresh medium for 24 h to
ensure their exponential growth and then treated with 20 nM TPA for up
to 48 h. Differentiation was monitored by following cell adhesion and
plasma membrane expression of the glycophorin CD11b by flow
cytometry analysis. To promote their apoptosis, cells were treated with
50 μM VP16 for up to 4 h. Apoptosis was monitored by following the
percentage of cells with chromatin condensation.

Human peripheral blood monocytes were obtained from healthy
donors with informed consent and purified using a monocyte isolation
kit with a light-scattering column according to the manufacturer’s
instructions (Miltenyi Biotec, Paris, France) and then incubated (2.5 ×
107/ml) for up to 6 days in RPMI medium, 10% fetal calf serum, in the
presence of either 100 ng/ml M-CSF to trigger their differentiation into
macrophages or a combination of GM-CSF (100 ng/ml) and IL-4 (10
ng/ml) and B-mercaptoethanol (50 μM) for inducing their dendritic
differentiation. The differentiated phenotype was identified by flow cyto-
metry analysis of CD71 and CD1a at the cell surface. FAM-VAD-fmk, the
fluochrome inhibitor of caspases, a carboxyfluorescein analog of benzyl-
loxycarbonyl-tetrapeptide-fluoromethylketone that becomes fluorescent
upon cleavage by caspases, was used to measure caspase activity by flow
cytometry (Serotech, France).

siRNA Transfection—Human primary monocytes were transduced
using the Human Monocyte Nucleofector Kit (Amaxa, Köln,
Germany) according to the manufacturer’s instructions. Briefly, 5 × 106
monocytes were resuspended into 100 μl of nucleofector solution with
2 μg of either caspase-8 siRNA (forward, AGGGAAUUCAGACAC-
CAGtt; reverse, CUGUGUCUGAGUUCCCUttt) (Ambion, Austin,
TX) or luciferase siRNA (Qiagen) (forward, CUUACGCGAGUC-
UCGAttt; reverse, UCGAAUGCGUCGGUAAGt) before nucleo-
fection with nucleofector 1. Cells were then immediately removed and
incubated overnight with 1 ml of prewarmed monocyte nucleofector
medium containing 2 mM glutamine and 10% of fetal bovine serum.
Cells were then resuspended into complete RPMI medium and treated
with appropriate cytokines to induce their differentiation into macro-
phages or dendritic cells.

High Resolution Two-dimensional Gel Electrophoresis—Cells (5 ×
106) were treated with 20 nM TPA for 12 h and then suspended in 25 μl
of isotonic sucrose buffer (250 mM sucrose, 10 mM Tris, pH 7.5) before being
stored at −80 °C. They were lysed in lysis buffer (0.3% (w/v) SDS, 50 mM
Tris, pH 7.5, 1 mM NaF, 2 mM EGTA, 1 mM sodium pyrophosphate, 40 mM
dithiothreitol) supplemented with protease inhibitors, 5 μg/ml DNase,
and 1 μg/ml RNase for 30 min at 4 °C. Cell debris and the remaining intact cells
were removed by centrifugation for 15 min at 20,000 × g and 4 °C. Total proteins
were precipitated with ice-cold acetone and in a solution containing
8 M urea, 2.5 M thiourea, 4% (v/v) CHAPS, 50 mM dithiothreitol, 0.5% (v/v)
ampholines at pH 4–7 (Amersham Biosciences) before being cleared
on 0.2-μm filters. This solution (800 μg of proteins in 450 μl) was loaded
onto a dehydrated immobilized pH gradient strip (24 cm, pH 4–7 linear;
Amersham Biosciences), hydrated for 12 h at 30 °C under mineral oil before
isoelectric focusing of the first dimension by progressive increase of voltage
up to 66,000 V-h at 20 °C in an IPGPhor (Amersham Biosciences). Then strips
were equilibrated with 60 μl dithiothreitol in equilibration buffer (6
M urea, 30% (v/v) glycerol, 50 mM Tris-HCl, pH 8.4, 4% (w/v) SDS)
for 15 min to reduce proteins that were subsequently carbamidomethylated for 15
min with 135 mM iodoacetamide in equilibration buffer. Then proteins
were separated according to their molecular mass using 11% SDS-poly-
acrylamide gels at 35 °C in an Ettan Dalt II system (Amersham Biosciences).
Then the gels were washed three times in deionized water for 5 min; fixed
in 10% (v/v) formalin, 20% (v/v) ethanol for 1 h, washed again; soaked in 0.05%
(w/v) 2,7-naphthalenedisulfonic acid solution overnight; washed six times
for 20 min; and incubated in ammoniacal silver nitrate solution for 1 h.
After three washes, gels were incubated in 0.01% (w/v) citric acid and 0.1%
(w/v) formaldehyde for 3–5 min before adding a stop solution containing 2%
(w/v) acetic acid and 0.5% (v/v) ethanolamine. Three analytical gels were run
for each sample. These gels were scanned (ImageScanner; Amersham Biosciences), and spots were characterized using the Melanie 4.03 software (GeneBio, Geneva, Switzerland) that permits measurement of the relative volume (percentage of volume) of each spot. Experimental variations were neutralized by reporting the volume of each spot relative to the volume of all spots in the gel, and gel-matching algorithms were used for comparison. Heuristic clustering and factorial analysis were used to assess the reproducibility of the method. A more than 2-fold change in a spot volume between control- and p35-transfected U937 cells was considered for further analysis.

Mass Spectrometry Analysis—High resolution two-dimensional gels performed with initial loading of 1.6 mg of proteins were used for mass spectrometry analysis. Staining was performed either with silver nitrate as above, except for fixation in 30% ethanol and 5% acetic acid as described (27). Alternatively, gels were fixed in a solution containing 30% ethanol and 2% phosphoric acid before three washes in 2% phosphoric acid for 20 min and stained with a colloidal Coomassie solution (10% (v/v) phosphoric acid, 10% (w/v) ammonium sulfate, 0.12% (w/v) Brilliant Blue G, and 20% (v/v) methanol) as described (28). Selected spots were excised and incubated either in 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 15 min (silver nitrate staining) or in 25 mM ammonium bicarbonate at pH 8 for 10 min (Coomassie staining). After three washes in 50% acetonitrile, 25 mM ammonium bicarbonate, the gel pieces were soaked in 100% acetonitrile for 5 min and dried. The proteins were reduced in 100 mM dithiothreitol, 25 mM ammonium bicarbonate for 1 h at 56 °C and then alkylated in 55 mM iodoacetamide, 25 mM ammonium bicarbonate for 45 min in darkness, washed, and dried again. The proteins were rehydrated with trypsin (Roche Applied Science) (12.5 μg/ml in 25 mM ammonium bicarbonate, pH 8) and incubated overnight at 37 °C. Peptides were extracted twice with 25 mM ammonium bicarbonate and twice with 50% acetonitrile, 1% trifluoroacetic acid at room temperature and dried. For mass spectrometric analysis, peptides were solubilized by sonication in 10 μl of formic acid (2%), concentrated on a C18 Zip-Tip column (Millipore Corp., Billerica, MA), and eluted with 5 μl of 50% acetonitrile, 0.1% trifluoroacetic acid. One μl of each peptide sample was dried on a Teflon-coated MALDI target plate (Applied Biosciences) before adding 1 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.01% trifluoroacetic acid). Peptide mass fingerprinting was performed on a reflectron MALDI-TOF mass spectrometer (Applied Biosciences) with internal calibration using trypsin autolysis peaks, and protein identification was performed by using the MS-FIT software and SWISS-PROT or NCBI protein databases (available on the World Wide Web at prospector.ucsf.edu/).

Immunoblot Analysis—Whole-cell lysates were prepared by lysing the cells in boiling buffer (1% SDS, 1 mM sodium vanadate, 10 mM Tris, pH 7.4) in the presence of protease inhibitors. Protein concentration was measured using the Bio-Rad DC protein assay kit (Ivy sur Seine, France). Fifty micrograms of proteins were incubated in loading buffer (125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromphenol blue), separated by SDS-PAGE, and electroblotted to nitrocellulose membrane (Bio-Rad). After blocking nonspecific binding sites overnight by 5% nonfat milk in TBS-Tween (0.05 μl Tris base, 0.9% NaCl, pH 7.6, Tween 20 (0.1%), the membrane was incubated for 2 h at room temperature with the primary antibody. After two washes in TBS-Tween, membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature and then washed twice in TBS-Tween. The immunoblot was revealed using an enhanced chemiluminescence detection kit (Amersham Biosciences) and autoradiography.

RESULTS

p35-mediated Caspase Inhibition Prevents TPA-induced Differentiation—We transfected U937 cells with either the empty pTarget vector (U937/Co) or the pTarget vector including the sequence encoding the baculovirus p35 protein tagged with an N-terminal FLAG (U937/p35). A stably transfected population was selected by culture for
4 weeks in the presence of G418 (1 mg/ml). The expression of the p35 gene in the transfected cells was analyzed by RT-PCR, and the protein was detected by immunoblotting using an anti-FLAG antibody to demonstrate the efficacy of the transfection (Fig. 1A). As described previously (29), exposure of U937/Co cells to 20 nM TPA induced their progressive differentiation into macrophages, as assessed by cell adhesion to the plastic of culture flasks (Fig. 1B) and an increase in the expression of CD11b at the cell surface (Fig. 1C) (7). Using FAM-VAD-fmk as a permeant substrate, the TPA-induced differentiation of U937/Co cells was observed to be associated with a transient activation of caspases.
The expression of p35 in U937 cells prevented TPA-induced cell adhesion (Fig. 1C), CD11b expression increase (Fig. 1C), and caspase activation (Fig. 1, D and E), indicating, in accordance with previous studies, that caspase inhibition could prevent macrophage differentiation (7, 8). 

Table 2: Proteins overexpressed in U937/Co after 12 h of TPA exposure

Spot numbers indicate the locations of proteins on two-dimensional gels. Those whose mean volume was at least 2-fold higher in U937/Co compared with U937/p35 were annotated with T. The protein name was identified using the MS-FIT software (the accession numbers from the SWISS-PROT and NCBI protein data bases are shown). Predicted (from the amino acid sequence) and observed (from two-dimensional gel analyzed using Melanie software) molecular weights (Mr) and isoelectric points (pI) are indicated. The volume ratio was obtained by dividing the mean volume of a spot in the four U937/Co gels by its mean volume in the three U937/p35 gels. T indicates a spot whose maximal activity was observed 12 h after exposure to 100 nM TPA in U937/Co cells. Peptide coverage in mass spectrometry (MS) analysis corresponded either to the full-length protein (F) or to its carboxyl-terminal (C) or amino-terminal (N) fragment. *, proteins selected for immunoblot analyses (see “Results”).

Table 3: Selection of proteins potentially cleaved by caspases in monocytes undergoing differentiation into macrophages

A selection of proteins was made in Table 1, based on data from the literature indicating potential cleavage by caspases. The reference and the model in which the cleavage was provided, are as well as the cleavage site and the fragment sizes when described. N and C, N-terminal and C-terminal fragments, respectively. N-ter and C-ter, the location of the epitope recognized by the antibody used for Western blot analyses.

whose maximal activity was observed 12 h after the beginning of TPA exposure (Fig. 1D). Caspase activation was further confirmed by the time-dependent appearance of caspase-3 and -8 cleavage fragments, whose size was similar to that observed in U937 cells undergoing apoptosis upon VP16 exposure (Fig. 1E). In this positive control, about 60% of cells demonstrated an apoptotic phenotype 4 h after exposure to 100 μM VP16 (not shown). Caspase activation occurred in the absence of any apoptotic feature (e.g. nuclear chromatin condensation and phosphatidylserine externalization were not observed (not shown), and the well characterized caspase target poly(ADP-ribose)polymerase, which was cleaved in U937 cells undergoing VP16-induced apoptosis, remained uncleaved in U937 cells undergoing differentiation (Fig. 1E).
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65% between U937/Co and U937/p35 gels. Two different methods of clustering, namely heuristic clustering (Fig. 2 B) and factorial analysis (not shown), both indicated a good separation between U937/Co and U937/p35 gels, suggesting that the differences between gel profiles were a consequence of caspase inhibition rather than variations in experimental conditions. Using a more than 2-fold change in the mean spot volume between U937/Co and U937/p35 cells as a cut-off value (e.g. see Fig. 2 C), we identified differences in 90 spots, including 54 whose volume was more than 2-fold higher in U937/Co compared with U937/p35 cell lysates and 36 spots whose volume was more than 2-fold higher in U937/p35 compared with U937/Co cell lysates.

The 90 spots that exhibited high reproducibility and more than 2-fold changes in abundance between the two studied conditions were excised from two-dimensional gels, digested with trypsin, and analyzed by mass spectrometry, which allowed identification of the corresponding proteins in 47 cases. Other spots could not be identified, either because they were insufficiently abundant or because the mass spectrometry results could not be matched to a peptide sequence in the data base. Since some spots were generated by distinct forms of the same protein, we eventually identified 39 distinct proteins that were differentially expressed in U937/Co and U937/p35 cells after 12 h of TPA treatment. Interestingly, the proteins overexpressed in p35-expressing cells (n = 16) were all full-length (i.e. their apparent molecular weight was similar to the predicted molecular weight, and the peptide coverage in mass spectrometry indicated isolation of the complete molecule), whereas about half of those overexpressed in control cells (11 of 23) were N- or C-terminal fragments (i.e. their apparent molecular weight was lower than predicted molecular weight, and peptide coverage in mass spectrometry was limited to one molecule extremity) (Table 1 and 2). Peptides identified by mass fingerprint never overlapped a characterized cleavage site by caspases (data not shown), and the size of the fragments identified by analyzing the two-dimensional gel electrophoreses was similar or complementary to that reported for fragments generated by caspase-mediated cleavage (Table 3).

Identification of Proteins Whose Cleavage or Degradation Is Prevented by p35 in TPA-treated U937 Cells—The higher expression of some proteins in U937/p35 compared with U937/Co cell lysates suggested caspase-mediated cleavage along TPA-induced differentiation. In order to check this hypothesis, we selected four of these proteins (indicated by an asterisk in Table 1) (PAK-2, β-actin, nucleophosmin, and heterogeneous nuclear ribonucleoprotein (hnRNP C1/C2)), whose cleavage had been previously associated with apoptosis and, in most cases, demonstrated to be mediated by caspases (Tables 1 and 3). We used Student’s t test to confirm that differences measured in mean spot volumes calculated from the four U937/Co gels and the three U937/p35 gels were statistically significant (Fig. 3). Then we performed immunoblot analyses of cell lysates obtained in the same conditions as those used for two-dimensional gel analysis. These experiments identified PAK-2 and β-actin cleavage fragments and demonstrated the degradation of NPM and the two identified isoforms of hnRNP C in TPA-treated U937 cells. The size of PAK-2- and β-actin-identified fragments was in accordance with that described for caspase-mediated cleavage (Table 3). These experiments also confirmed that these events were prevented by the expression of p35.

The protein fragments identified in U937/Co but not in U937/p35 cell lysates also suggested caspase-mediated cleavage along the differentiation (Table 2). We selected six proteins (indicated by an asterisk in Table 2) that had been previously described as caspase targets in cells undergoing apoptosis to check this hypothesis. Again, we used Student’s t test to confirm that differences measured in mean spot volumes calculated from the four U937/Co gels and the three U937/p35 gels were statistically significant (Fig. 4). Then immunoblot experiments demonstrated the differentiation-associated cleavage of α-tubulin, PAI-2, HSP90-β, and moesin into fragments whose size was in accordance with a cleavage by caspases (Table 3), and the degradation of hnRNP H without a detectable cleavage fragment. In accordance with two-dimensional gel analyses, these experiments also showed that TPA-induced U937/Co cell differentiation was associated with an increase in the expression of full-length PAI-2 and one of the two full-length isoforms of vinculin, two events that were not observed in TPA-treated U937/p35 cells. Last, these immunoblot experiments failed to detect any cleavage fragment of vinculin in TPA-treated U937/Co cells (Fig. 4).
The Cleavage or Degradation of Some of These Proteins Is Specifically Associated with Macrophagic Differentiation of Peripheral Blood Monocytes—We then explored whether the differentiation-associated changes in these selected proteins identified in TPA-treated U937 cells were also observed in peripheral blood monocytes undergoing M-CSF-induced macrophagic differentiation. As a control, we tested the expression of these proteins in monocytes exposed to GM-CSF and IL-4 to induce their dendritic cell differentiation. Analysis of CD71 and CD1a expression at the cell surface was used to monitor the macrophagic and dendritic cell differentiation processes, respectively (Fig. 5A) (7). Using FAM-VAD-fmk as a permeant substrate, we detected caspase activation after 3 days of culture in the presence of M-CSF, whereas no caspase activity was detected in monocytes cultured in the presence of GM-CSF and IL-4 (Fig. 5A). The specific activation of caspase-3 and caspase-8 along the macrophage differentiation pathway was confirmed by immunoblot analysis showing the time-dependent cleavage of the proenzymes into active fragments, detected 3 days after the beginning of exposure to M-CSF (Fig. 5B). Caspase activation in cells exposed to M-CSF was not associated with apoptosis, as demonstrated by the lack of nuclear chromatin condensation (not shown) and the lack of cleavage of lamin B, a well known target of caspases in cells undergoing apoptosis (Fig. 5B).

These immunoblot analyses identified a cleavage of PAK-2, α-tubulin, and PAI-2 in monocytes undergoing macrophagic differentiation (Fig. 5C). This cleavage was similar to those observed in TPA-treated U937 cells (Figs. 3 and 4). These experiments confirmed the degradation of hnRNP H observed in TPA-treated U937 cells (Fig. 5B) and detected cleavage fragments of NPM, hnRNP C, and vinculin that had not been identified by immunoblot analyses of TPA-treated U937 cells (Fig. 5D). The size of the cleavage fragments of the studied proteins was always compatible with caspase-mediated cleavage of these proteins (Table 3). All of these events were not observed in monocytes undergoing dendritic cell differentiation. On the other hand, these experiments did not detect any significant cleavage or degradation of moesin, β-actin, and HSP90-β in monocytes undergoing differentiation, either into macrophages or dendritic cells (Fig. 5E), which may be related to the specificity of the differentiation process in TPA-treated U937 cells as compared with M-CSF-treated primary monocytes.

Caspase-8 Inhibition Prevents Proteolytic Events Associated with Macrophage Differentiation—Using U937 cells that stably express either a dominant negative mutant of caspase-8 or the cowpox virus caspase inhibitor CrmA (8), we show that caspase-8 inhibition prevents TPA-induced U937 cell differentiation into macrophages (Fig. 6A) and caspase activation (Fig. 6, B and C), suggesting that caspase-8 is the upstream enzyme in the proteolytic cascade associated with macrophagic differentiation. Using these stably transfected cells, we further demonstrate that caspase inhibition prevents the TPA-associated cleavage of PAK2, PAI-2, and NPM (Fig. 6A) and caspase-8 activation (Fig. 6, B and C), suggesting that caspase-8 is the upstream enzyme in the proteolytic cascade associated with macrophagic differentiation. Using these stably transfected cells, we further demonstrate that caspase inhibition prevents the TPA-associated cleavage of PAK2, PAI-2, and NPM (Fig. 6A), further indicating that these proteolytic events may be a direct or indirect consequence of caspase activation. We also down-regulated caspase-8 in primary monocytes by the use of specific siRNAs, which decreased the generation of active caspase-8 fragments and prevented the cleavage of PAK2, PAI-2, and

FIGURE 4. Immunoblot analysis of potential caspase substrates in TPA-treated U937 cells. Six proteins were selected among those whose fragments were identified by two-dimensional gel analysis in TPA-treated U937/Co cells (Co), not in TPA-treated U937/p35 (P35) cells: α-tubulin, PAI-2, HSP90-β, moesin, hnRNP H, and vinculin. Upper panels, enlarged areas of silver-stained two-dimensional gels comparing U937/Co and U937/p35 cells treated with 20 nm TPA for 12 h. Spots corresponding to the studied protein are indicated by an arrow. Middle panels, mean ± S.D. of the spot volumes measured in the four U937/Co gels and the three U937/p35 gels (**, p < 0.01; *, p < 0.05; Student’s t test analysis). Lower panels, immunoblot analyses of cell lysates obtained in the same conditions than those used for two-dimensional gel analysis. Molecular masses are shown in kDa. *, a cleavage fragment. One representative of at least two independent gels is shown.
NPM specifically associated with M-CSF-induced macrophage differentiation (Fig. 6D). Interestingly, the cleavage fragments generated along the macrophage differentiation pathway were similar in size to those generated by VP16-induced apoptosis in U937 cells (Fig. 6C and supplemental figure).

DISCUSSION

The present study enforces the previous demonstration that caspases were required for the differentiation of monocytes into macrophages (7, 8) and identifies proteins that are cleaved or degraded along with the differentiation process. Western blot analyses using specific antibodies confirmed several results from two-dimensional analysis and verified that the same cleavages were occurring in normal monocytes exposed to M-CSF. The ability of the broad spectrum caspase inhibitor p35, a dominant negative caspase-8 mutant, or the cowpox virus caspase inhibitor CrmA to prevent these events in TPA-treated U937 cells, the size of the cleavage fragments when compared with those detected in U937 cells undergoing VP16-induced apoptosis, the correlation of these events with caspase activation, their dependence on caspase-8 activation, and their absence in monocytes undergoing in vitro dendritic cell differentiation upon GM-CSF and IL-4 exposure all argue for a caspase-mediated proteolysis of most of these proteins in monocytes undergoing differentiation into macrophages. However, at least for some of them, we cannot rule out a role for other proteases that would be activated downstream of caspases along the differentiation pathway.

FIGURE 5. Immunoblot analysis of monocytes undergoing differentiation upon appropriate cytokine stimulation. Peripheral blood monocytes were purified and exposed for indicated times to M-CSF to induce their macrophagic differentiation (M-CSF) or to GM-CSF and IL-4 to induce their dendritic cell differentiation (GM-IL4). A, time-dependent increase in CD71 and CD1a expression at the surface of monocytes exposed to M-CSF or GM-CSF plus IL-4 for the indicated times in days, respectively (untreated monocytes (white curves) and cytokine-treated monocytes (gray curves)). FAM-VAD-fmk cleavage was determined by flow cytometry in untreated monocytes (gray curves) and in M-CSF- and GM-IL4-treated monocytes (white curves). B, Western blot analysis of caspase-3 (C3), caspase-8 (C8), and lamin B (L8) in monocytes exposed for the indicated times to cytokines. HSC70 was used as a loading control. C, Western blot analysis of proteins whose cleavage or degradation is similar in M-CSF-treated monocytes and TPA-treated U937 cells. D, Western blot analysis of proteins whose cleavage or degradation is observed in M-CSF-treated monocytes, not in TPA-treated U937 cells. E, Western blot analysis of proteins whose cleavage or degradation is observed in TPA-treated U937 cells, not in M-CSF-treated monocytes. Molecular masses are indicated in kDa. *, cleavage fragments. One representative of at least three experiments is shown in each panel.

FIGURE 6. Effects of caspase-8 inhibition on protein cleavage along macrophagic differentiation. A–C, we used U937 cells stably transfected with the empty pcDNA3 (pcDNA) vector or pcDNA3 encoding a caspase-8 dominant negative mutant (C8DN) or the cowpox virus caspase inhibitor CrmA. A, these cells were left untreated (Co) or exposed to 20 nM TPA for 48 h (TPA) before analyzing CD11b expression by flow cytometry. B, FAM-VAD-fmk cleavage was determined in untreated (white areas) and TPA-treated (20 nM, 12 h; gray areas) cells. C, Western blot analysis of the indicated proteins in cells exposed for the indicated times to 20 nM TPA. Molecular masses are in kDa. D, monocytes were transfected with either luciferase-targeting (Control siRNA) or caspase-8-targeting siRNA (Casp-8 siRNA). Expression of caspase-8 (C8), lamin B (L8), and other indicated proteins was studied by immunoblotting at the indicated times (days). *, cleavage fragments. All panels, one representative of at least three experiments is shown. MAC, macrophages; DC, dendritic cells.
Some of the protein cleavages identified in TPA-treated U937 cells and prevented by caspase inhibition were not subsequently observed in primary monocytes exposed to M-CSF. These discrepancies may be related to the caveats of leukemic cell lines when used as models for studying hematopoietic differentiation. Nevertheless, several proteins were observed to be cleaved similarly in both TPA-treated U937 cells and M-CSF-treated monocytes, suggesting that their cleavage was a characteristic event along macrophage differentiation.

This differentiation process is associated with changes in the cytoskeleton, cell adhesion and caspase inhibition in U937 cells prevent differentiation-associated cell adhesion. Several of the proteins whose cleavage or degradation was identified along differentiation into macrophages are involved in cytoskeletal regulation and cell adhesion. One of these is PAK-2, which belongs to the PAK family of proteins. Members of this family can be activated by the monomeric G proteins Cdc42 and Rac. In their GTP-bound state, Cdc42 and Rac bind to a conserved region within the N-terminal domain of PAKs to release inhibition of the catalytic site by an overlapping autoinhibitory domain. The ubiquitous PAK2 is unique among PAKs, since it can be activated also by caspase-mediated cleavage that removes most of the autoinhibitory domain and generates an active C-terminal fragment that contains the entire catalytic domain (30, 31). PAK2 is primarily inactive in dividing cells and is transiently activated under moderate stress conditions (e.g. throughout phosphorylation by the phosphatidylinositol-3-kinase (PI-3K)/Akt signaling pathway). Activated full-length PAK-2 protects from cell death, in part by phosphorylation and inhibition of the pro-apoptotic, Bel-2 family member Bad (32), whereas caspase-mediated cleavage and activation of PAK-2 was originally associated with cell death induction (33). The present study demonstrates that this proteolytic event is possibly involved also in cell differentiation, independently of apoptosis. Interestingly, PAK2 demonstrates similarities with MST1, a target of caspases, when activated in skeletal myoblasts undergoing differentiation (15).

Another caspase target involved in cell adhesion is the serine protease inhibitor (serpin) PAI-2. This multifunctional protein is partly secreted to the entire catalytic domain of PAKs (36), and TPA causes a strong increase in expression of the PAI-2 fragment identified in monocytes undergoing M-CSF-induced differentiation (15).

Caspase Targets in Macrophagic Differentiation

Calpain-mediated cleavage of vinculin could facilitate the redistribution of the protein to the cytoskeleton along with aggregation of platelets (54). Whether one of the two identified fragments generated by active caspases in monocytes undergoing macrophagic differentiation demonstrate a biological activity by themselves remains to be determined.

Several other cytoskeletal proteins are cleaved in various differentiation processes associated with caspase activation, including spectrin (lens fibers), lamin B (erythropoiesis), gelsolin (megakaryopoiesis), and fodrin (skeletal myoblast), suggesting a role for caspases in differentiation-associated cytoskeletal changes (13, 14, 55, 56).

Another caspase target identified in cells undergoing macrophagic differentiation is NPM. This 38-kDa phosphoprotein regulates various cellular functions, including centrosome duplication, gene transcription, and cell proliferation (57, 58). Its overexpression prevents retinoic acid-induced granulocytic differentiation of HL-60 leukemia cells (59), whereas the protein is cleaved in K562 leukemia cells undergoing TPA-induced megakaryocytic differentiation (60). The npm gene is involved in various chromosome translocations that characterize malignant hematopoietic diseases (61, 62). npm mutations that cause aberrant cytoplasmic localization of the protein are found with a high frequency in acute myelogenous leukemias (63), and npm+/−/− mice demonstrate features of human myelodysplastic syndromes (64). NPM is cleaved in cells undergoing apoptosis, and caspase-3 generates a 20-kDa N-terminal fragment of the protein in vitro (65). The decrease in NPM protein expression along with macrophagic differentiation may be related to caspase-mediated cleavage, generating an amino-terminal fragment of 20 kDa, as detected in M-CSF-treated monocytes.

We also identified a cleavage or degradation of several hnRNPs. These proteins contribute to pre-mRNA processing to mature RNAs before export from the nucleus, several of them are also involved in the control of pre-mRNA splicing and translation (66–68), and some of them are targets for caspases (69) or other proteases (70).

About 3,000 individual proteins were resolved on each gel, and this technology was efficient for the discovery of several potential substrates of caspases in monocytes undergoing differentiation into macrophages. This method also identified proteins whose expression decreases along with the differentiation process without identified cleavage, which could be related either to the use of antibodies that do not recognize the exposed epitopes or rapid degradation of cleavage fragments or decreased expression in the absence of cleavage (e.g. through transcriptional regulation). Since the approach used does not allow identification or transmembrane proteins, several other unidentified targets could be cleaved by caspases or other proteases, downstream of caspase activation, along the differentiation process. We are now exploring the functional role of the identified cleavages in macrophagic differentiation.

Another important question will be to determine how some proteins such as PARP1 and lamin B are protected from caspase-mediated proteolysis in cells undergoing differentiation, whereas they are cleaved in those undergoing apoptosis.

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