Survival by Mac-1-mediated Adherence and Anoikis in Phorbol Ester-treated HL-60 Cells

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During the exposure of human myelocytic leukemia HL-60 cells to phorbol diester, nonadherent cells die by apoptosis, but adherent cells survive and growth-arrest at G1 phase of the cell cycle. Here we have shown that the adherent cells rapidly die by apoptosis after forced detachment (anoikis), indicating that phorbol diester induced apoptosis by default. Dimethylsphingosine induced apoptosis in the adherent cells, and sphingosine-1-phosphate rescued the detached cells from apoptosis. Sphingosine kinase activity in adherent cells was higher than that in nonadherent cells and was decreased by forced detachment. It is likely that the phorbol diester-induced apoptosis and the adhesion-mediated survival are modulated by sphingosine and sphingosine-1-phosphate, respectively. The adherent cells were reverted and repropagated when allowed to spontaneously detach from plastic surfaces by removal of phorbol diester. This result suggests that after removal of phorbol diester, the commitment signal of apoptosis by default is lost faster than the survival signal by adherence.

In phorbol ester-induced monocyte/macrophage-like differentiation in human leukemic HL-60 cells (1), the leukocyte integrin receptor Mac-1 is expressed on the cell surface (2), and the cells are growth-arrested at G1 of the cell cycle (3, 4). Mac-1 (CR3, aMβ2; CD11b/CD18) is a member of the β2 integrin subfamily and plays a critical role in numerous physiological functions of monocytes and macrophages that are mediated by cell-cell and cell-substrate interactions (5–8, 46). Phorbol esters activate Mac-1 receptors on monocyte such that they promote vigorous phagocytosis (9, 10). Monocytes die by apoptosis after phagocytosis of bacteria (11). Undifferentiated HL-60 and U937 cells express LFA-1 (α1β2; CD11a/CD18) (2), another member of the β2 integrin subfamily, although at low levels. CD18 subunit mRNA is thus expressed constitutively, but CD11b subunit mRNA is not expressed at detectable levels (12). Phorbol ester up-regulates the steady state levels of both CD11b mRNA (13) and CD18 mRNA (14, 15) by transcriptional induction. Firm adherence of differentiated cells to tissue culture plastic dishes is inhibited by a monoclonal antibody to CD18 (16) or CD11b (17). Transcription factor NF-κB is constitutively activated by phorbol esters (18) and is indispensable for the CD11b gene expression and cell adhesion, because both are suppressed by a dominant negative inhibitor of NF-κB expression (19) and by antisense oligonucleotides to RelA subunit (20).

Phorbol esters also induce apoptosis of HL-60 and U937 cells (21), which is accompanied by a marked decrease in bcl-2 mRNA and protein levels (22). Ectopic expression of Bcl-2 protein inhibits apoptosis but has no significant effect on differentiation (23, 24). Apoptosis of HL-60 cells induced by treatment with phorbol esters has been correlated with an increase in the steady-state level of sphingosine and elevation of the ceramide activity (25). In fact, exposure of HL-60 cells to sphingosine induced apoptosis (25, 26). Co-exposure of HL-60 cells to phorbol ester and sphingosine blocked phorbol ester-induced differentiation (27, 28). Interestingly, the cell adherence to plastic substrate protected HL-60 cells from apoptosis induced with phorbol esters (29). Recently, sphingosine-1-phosphate (SPP)1 has been shown to prevent apoptosis in HL-60 cells or U937 cells induced by tumor necrosis factor-α (TNF-α) or Fas ligand (30). The principal mediator of apoptosis in these cases is ceramide, not sphingosine, but SPP could be equally effective in the protection of adherent HL-60 cells from phorbol ester-induced apoptosis.

The several lines of experimental evidence summarized above suggested to us the following possibilities. 1) Apoptosis by sphingosine occurs by default (31) in phorbol ester-treated HL-60 cells; 2) adherence to plastic by Mac-1 elicits an anchorage-dependent survival signal, and adherent cells may undergo anoikis by forced suspension (32, 33); 3) apoptosis in nonadherent cells and anoikis, as well, may be suppressed by the addition of SPP; and 4) if the commitment signal to apoptosis is lost faster than the survival signal, phorbol ester-differentiated and adherent HL-60 cells may de-differentiate and proliferate again by removing phorbol ester without perturbing the adherent state. Here we demonstrate that our model is substantiated by a line of experimental evidence that was obtained by studying adherent and suspended cell fractions separately after isolation.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment

The human acute myeloblastic leukemia cell line HL-60 (34–36) (CCL240; ATCC, Manassas, VA) was cultured in plastic dishes at 37 °C in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA) and 100 μg/ml kanamycin. The HL-60 cells used in this work were restricted to early passages characterized by a doubling time of 47 h. Cells were passaged before cell density reached 2 × 10^6 cells/ml and seeded at 3 × 10^5 cells/ml. The cell viability determined by trypan blue exclusion was scored during the course of culture and found to be higher than 90%. For the differentiation induction we used phorbol 12,13-dibutyrate.

1 The abbreviations used are: SPP, sphingosine-1-phosphate; TNF-α, tumor necrosis factor-α; FCS, fetal calf serum; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate-13-acetate; DMS, N,N-dimethylsphingosine; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole.
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(PDB; Sigma) rather than phorbol 12-myristate-13-acetate (PMA), because PDB can be easily removed from cells by simple washings (37), provided that FCS or BSA is contained in the washing medium (38). An ethanol stock solution of PDB (0.2 mM) was diluted before use to 10 μM with the culture medium and added to a cell culture of 5 × 10⁵ cells/ml at a final concentration of 100 nM (4). In using sphingolipids as effectors, cells in logarithmic growth were first precultured for 24 h in a serum-free RPMI 1640 medium supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml) and treated with drugs in the same medium (30, 39). An ethanol stock solution (10 mM) of N,N-dimethylsphingosine (DMS; Biomol) was diluted with the serum-free medium before use. SPP (Biomol) was added as BSA-containing solutions (40) at a concentration of 125 μM in 4 mg/ml BSA (fatty acid-free). In suppression of anoikis of HL-60 cells with SPP, adherent cells prepared by pretreatment with 100 nM PDB for two days were detached by forced suspension by pipetting three times with the serum-free medium containing 20 μM SPP. PDB was removed from cells by washing three times with RPMI 1640 containing 5% FCS, and SPP was then removed by washing with serum-free medium. The cells were finally suspended in serum-free medium containing 20 μM SPP.

Separation of Adherent and Nonadherent Cell Populations

During the PDB treatment of HL-60 cells, suspended cells were separated by gentle pipetting. Culture dishes with the adherent cells were gently washed three times with 10 ml of fresh medium. A small number of cells contained in the wash fractions were discarded. The suspended cells were counted without suspending the dishes by using an inverted microscope fitted with an ocular lens with grid, because the cells formed large aggregates after suspension. A unit area of the grid with an appropriate size was chosen so that a total of 200 or more adherent cells were contained in it. The cell density/unit area was measured at 8–16 different places selected at random in a dish. The total number of adherent cells/dish was estimated by multiplying the average cell density and the total area of a dish.

Assay of Cell Proliferation

To a cell culture of 100 μl (3–10 × 10⁵ cells/ml), [3H]thymidine (55 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) was added to a final activity of 0.5 μCi/ml. After incubation for 1 h at 37 °C in a CO₂ incubator, the cells were washed three times and fixed on a slide glass. Autoradiography was done by immersing the slide glass in a photographic emulsion (type NR-M2; Konica, Tokyo, Japan) and exposing it for 4–6 days. Developed and fixed slides were counterstained with Giemsa, and more than 400 cells were counted under a light microscope. The labeling index was calculated from the number of cells with silver grains divided by the total cell counts.

Assay of Apoptosis

Nuclear Fragmentation—10⁵ cells were fixed with an equal volume of 5% paraformaldehyde, neutralized by one-tenth volume of 1 M Tris-HCl (pH 7.2) and centrifuged onto a glass slide using a cytospin apparatus. The cells were further fixed in cold methanol (−20 °C, 5 min) followed by cold acetone (−20 °C, 5 min), and the plates were allowed to dry. Dried plates were stained with 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI). Alternatively, cytospin preparations were fixed in 90% (v/v) cold methanol (−20 °C, 5 min) and dried. Dried plates were stained with 125 μg/ml acridine orange in phosphate buffer (pH 6.9). Nuclei were assessed in an Olympus OMT2 inverted fluorescence microscope equipped with the appropriate epi-fluorescence filters at a final magnification of 1500×.

DNA Fragmentation (40)—Briefly, 10⁵ cells were lysed by freeze-thawing in 0.1 M phosphate buffer (pH 7.2) containing 10 mM MgCl₂, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 20 μM ZnCl₂, 1 mM Na₃VO₄, 1 mM NaF, 10 μg/ml leupeptin and aprotanin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine. Cytoplasmic fractions were prepared by ultracentrifugation at 105,000 × g for 90 min. Sphingosine kinase activity in supernatants (50 μl) was measured by incubating with 5 μM sphingosine-BSA complex (42) and [γ-³²P]ATP (1 mM, 0.2 Ci/mmol) for 30 min at 20 °C. Labeled lipids were extracted with a mixture of chloroform/methanol/concentrated HCI (100:200:1, by volume), and the extract was partitioned into two phases by adding 2 M KCl/methanol (1:1, v/v) (43). The lower phase was washed twice with the same mixture and dried in a vacuum centrifuge at room temperature. The dried lipid was completely dissolved in 100 μl of chloroform/methanol (1:1, v/v) with occasional stirring over 30 min. Lipids were resolved on silica gel plates (Silica Gel 60; E. Merck) impregnated with 1% potassium oxalate/2 mM EDTA, using the solvent system of butanol/water/acidic acid (3:1:1) (39). The phospholipid standards were visualized with molybdenum blue spray (Sigma), and the radioactivity was measured by autoradiography in a Bioimaging analyzer (BAS2000, Fuji Film, Tokyo, Japan).

RESULTS

Plastic Adherence and Cessation of Proliferation by Exposure to PDB—PDB-treated HL-60 cells followed different fates. The number of differentiated cells that adhered to plastic surfaces began to increase after an induction period of at least 6 h (4) and reached a plateau in 24 h (Fig. 1A, closed circles). We showed by using an antisense RNA technology that the plastic adherence was mediated by Mac-1 expressed at the cell surface. The adherent cells were growth-arrested (Fig. 1B, closed circles) at the G1 phase of the cell cycle. A small number of adherent cells in the induction period were spontaneously differentiated (44). The number of undifferentiated, nonadherent cells exhibited an inverse decrease and leveled off in 24 h (Fig. 1A, open circles). The remaining nonadherent cells were apoptotic and appeared resistant to adherence. In the early stage of PDB treatment, the nonadherent cells continued to proliferate, although at a progressively reduced rate (Fig. 1B, open circles). Most of the nonadherent cells were converted to adherent cells by induction of differentiation; the remaining cells either proliferated by self renewal in the early stage or underwent apoptosis in the later stage and were gradually degraded (see below). Therefore, we plotted in Fig. 1A the number of cells for each type in a dish rather than the percentage of total.

Survival of Adherent Cells and Apoptosis of Nonadherent Cells During Exposure to PDB—Most of the nonadherent cells exhibited an apoptotic morphology of nuclear fragmentation between day 1 and day 2 of PDB treatment, but no nuclear

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fragmentation was observed in adherent cells (Fig. 2A). However, nuclear fragmentation occurred rapidly when the adherent cells were forced to suspend in medium that contained no PDB, independently of the method of suspension (Fig. 2B). This result indicates that PDB-treated adherent cells have already been committed to apoptosis (apoptosis by default). In the observed anoikis, apoptotic cell death by detachment, the apoptotic cells were later degraded and lost from the system, as indicated by the decrease in the percentage of cells with nuclear fragmentation after 12 h (Fig. 2B). The internucleosomal DNA fragmentation was consistent with the observed nuclear fragmentation. DNA fragmentation was demonstrated in non-adherent cells (Fig. 2C, lanes 4–6), whereas no fragmentation was observed in adherent cells (Fig. 2C, lanes 7–9). However, DNA fragmentation did occur in adherent cells after the forced suspension (Fig. 2C, lanes 12–13). The adherent cells did not survive long term, as they gradually detached from the plastic surface after 3 days and later died by apoptosis. Similar results were reported previously (29).

Suppression of PDB-induced Apoptosis in HL-60 Cells by SPP—SPP was investigated for its ability to block apoptosis in HL-60 cells induced by PDB, as it has been shown to block the apoptosis by TNF-α or Fas ligand (30). Apoptosis induced by PDB treatment of the nonadherent cells in either serum-free RPMI 1640 or medium supplemented with 10% FCS progressed similarly, as observed by nuclear fragmentation (Fig. 3A, open bars). SPP at 20 μM strongly inhibited the apoptosis at day 2, but the inhibitory effects were almost gone by day 3 (Fig. 3A, hatched bars). Anoikis induced by forced suspension of PDB-treated adherent cells was suppressed by SPP beyond the experimental error (Fig. 3B), although the extent of inhibition was less than in nonadherent cells (Fig. 3A). Since the anoikis progressed very rapidly after the forced suspension compared with the apoptosis of PDB-treated nonadherent cells, we tried to add 20 μM of SPP to the medium 5 h before the detachment, but it failed to protect cells from anoikis.

Induction of Apoptosis of HL-60 Cells by DMS and Its Suppression by SPP—We further examined the involvement of sphingosine in PDB-induced apoptosis of HL-60 cells and suppression of apoptosis by SPP. HL-60 cells were incubated with DMS, a strong inhibitor of sphingosine kinase, for 12 h in serum-free RPMI 1640 medium, and nuclear fragmentation was observed by staining with DAPI (Fig. 4A). The percentage of cells with fragmented nuclei increased in a dose-dependent manner. A similar result was reported previously by observa-
tion of DNA fragmentation (25, 26). The apoptosis induced by DMS was inhibited in a dose-dependent manner by the simultaneous addition of SPP (Fig. 4B). HL-60 cells remained suspended throughout these treatments. The differentiated, adherent HL-60 cells induced by the PDB treatment for 12 h also underwent apoptosis by the addition of DMS (Fig. 4C) to the same extent as did the untreated HL-60 cells (data not shown). The PDB-differentiated HL-60 cells adhered to the plastic surface by spreading pseudopodia-like structures so firmly that the cells were only suspended by vigorous pipetting. The HL-60 cells co-treated with PDB and DMS still adhered to the plastic surface, and the morphology did not change appreciably from the PDB-differentiated cells, but the cells were suspended very easily by light pipetting. Apoptosis of HL-60 cells was not induced by the treatment with 10 μM of C2-ceramide for 24 h, in agreement with previous results (25) (data not shown).

**Activation of Sphingosine Kinase by Adherence of PDB-treated HL-60 Cells**—Sphingosine kinase activity was measured for adherent and nonadherent cell fractions after the PDB treatment (100 nM) for 15 h. The percentage of adherent cells was 44 ± 3%. Fig. 5A shows that the activity was higher in both the adherent and nonadherent cells compared with the untreated cells, but the value for the adherent cells was much higher than that for the nonadherent cells. The detached cells by forced suspension also exhibited a decreased sphingosine kinase activity compared with the adherent cells (time 0) after further incubation for 4 h in the absence of PDB (Fig. 5B). The addition of PDB during the further incubation increased the activity only slightly. The sphingosine kinase activity for the detached cells at 8 h after suspension was not significantly different from the value for the adherent cells both in the presence or absence of PDB.

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Cells after Spontaneous Suspension—After the treatment of HL-60 cells with 100 nM PDB for 2 days, the cells that adhered to 10-cm plastic dishes were separated by gentle pipetting of the nonadherent cells. The medium was gently replaced by the fresh medium containing no PDB such that the cell adherence was not disturbed. During further incubation in fresh medium not containing PDB, the adherent cells spontaneously detached, as shown by the decrease in the number of adherent cells (Fig. 6A). The detached cells were collected during various time intervals after the removal of PDB, and the time course of apoptosis was examined for each fraction after suspension. Quite interestingly, those cells that adhered longer exhibited less apoptosis as assayed by the nuclear morphology (Fig. 6B). Those cells that spontaneously detached after 24 h showed essentially no apoptosis (Fig. 6B, closed circles). The HL-60 cells that spontaneously detached after 12 or 24 h did not clearly exhibit apoptosis as observed by internucleosomal DNA fragmentation (Fig. 2C, lanes 10–11). The percentage of apoptotic cells decreased with time (Fig. 6B) due to both the degradation of apoptotic cells and the repopulation of suspended cells (Fig. 6C). It is interesting to note that those detached cells that suspended earlier after the removal of PDB more rapidly entered S phase in the fresh medium, a correlation inconsistent with cell death by apoptosis. All the [3H]thymidine-labeled cells were co-labeled by immunostaining with monoclonal antibody to Mac-1, indicating that the repopulating cells were those cells that had been differentiated by the previous PDB treatment.3 These results indicate that the commitment of apoptosis by default is canceled by PDB removal before the cells lose the anchorage-dependent survival signal, resulting in de-differentiation and repopulation of the differentiated cells.

DISCUSSION

Anoikis of PDB-differentiated HL-60 Cells—In the induction of differentiation of HL-60 cells by exposure to 100 nM PDB, most cells firmly adhered to the plastic surface within 24 h (Fig. 1A), stopped proliferating, and arrested at G1 of the cell cycle (Figs. 1B, 2A, and 2C). The PDB concentration of 100 nM is thus not severely cytotoxic and can be used for the study of differentiation induction. The persistently nonadherent cells (Fig. 2A) died by apoptosis after 24 h (Fig. 2C). Similar results were reported previously (29). Differentiation as observed by Mac-1-mediated adherence was induced before apoptosis. The studies with a HL-60 cell line with overexpressed bcl-2 gene suggest that differentiation is regulated independently of apoptosis (23, 24). It is interesting to note in this regard that high passage HL-60 sublines, which have amplified sequences in a single homogeneously staining region in a chromosome, were resistant to both differentiation and apoptosis after PDB treatment.4 The HL-60 cells studied here were of low passage and have double minutes but no homogeneously staining region. Amplified sequences of genes such as c-myc are detected in both the homogeneously staining region and double minutes (45), but the genomic contents may not necessarily be identical with each other.5 Therefore, differentiation or the differentiation potential could be a prerequisite for apoptosis. Expression of β2 integrin Mac-1 on the cell surface was lost from the cells en route to apoptosis, but it was retained in the repopulating cells after removal of PDB.3

The surviving adherent cells exhibited rapid apoptosis after forced suspension and incubation in the absence of PDB (anoikis) (Fig. 2, B and C). These results clearly indicate that apoptosis is the fate by default of PDB-treated HL-60 cells. Apoptosis by default has been observed for various types of cells after growth factor deprivation (47). The important roles for the apoptosis by default have been recently emphasized in activated phagocytes for limiting tissue injury and eradicating persistent infection (48, 49).

The involvement of Sphingolipid Metabolites in the Regulation of Apoptosis or Survival in PDB-treated HL-60 Cells—It has recently been suggested that the block by PMA of apoptosis induced by TNF-α in HL-60 cells and U937 cells is mediated by activation of sphingosine-1-kinase and the resultant increase in SPP levels (30). Activation of transcription by NF-κB (50–52) could be an important upstream event. Intriguingly, PMA

3 T. Oda and H. Utiyama, H., unpublished observations.

4 H. Nakamura and H. Utiyama, unpublished observations.

5 K. Kitajima, H. Nakamura, T. Hirano, K. Hamada, M. Haque, N. Itoh, H. Shimokawa, K. Tanaka, N. Kamada, N. Shimizu, N., and H. Utiyama, submitted for publication.
increased both the SPP and sphingosine levels in the presence or absence of TNF-α (30). TNF-α increased the cellular concentration of ceramide, but TNF-α had no effect on the amount of basal sphingosine. The involvement of ceramide in TNF-α-induced apoptosis has been questioned, however, by a direct and simultaneous determination of sphingolipids using a mass spectrometric technique, that showed no generation of ceramide by TNF-α (53). In contrast to TNF-α, PMA increased sphingosine levels but did not increase basal levels of ceramide, even in the presence of TNF-α. It was reported that C2-ceramide (10 μM) induced apoptosis in HL-60 cells, but we could not detect significant apoptosis in agreement with other reports (25, 26, 54). The reason for the discrepancy is presently unknown.

The role of sphingosine and its metabolites as a second messenger has recently been given much attention (55). SPP strongly inhibited PDB-induced apoptosis in suspended HL-60 cells, but the effects were almost gone by day 3 (Fig. 3A). Anoikis was also significantly blocked by SPP, but the effect was marginal (Fig. 3B). The exact reason for the difference is presently unknown. We suggest that plastic, although never ingested, may be the target of phagocytosis by activated HL-60 cells. In this case, plastic-adherent HL-60 cells are similar to monocytes or macrophages engaged in phagocytosis. Thus, adherent cells, when detached, could be more dangerous than nonadherent cells and may be programmed to undergo vigorous apoptosis by rapid up-regulation of sphingosine. Activation of sphingosine kinase by phorbol esters (56, 57) may have little effect in detached cells. DMS, on the other hand, is a strong inhibitor of sphingosine kinase (58) and induced rapid accumulation of sphingolipids in platelets (59) or apoptosis in HL-60 cells (54). Sphingosine phosphate blocked DMS-induced apoptosis in a dose-dependent manner (Fig. 4, A and B). DMS also blocked survival of PDB-treated HL-60 cells by adherence (Fig. 4C). PDB treatment increased the sphingosine kinase activity in HL-60 cells, but the kinase level was clearly enhanced by cell adherence (Fig. 5A). The sphingosine kinase activity in PDB-treated HL-60 cells was also decreased by forced adherence of suspended cells (Fig. 5B). Collectively, these results suggest that a dynamic balance between the levels of sphingolipid metabolites (30), SPP and sphingosine, may determine whether a cell survives by adherence or undergoes anoikis by forced suspension. It remains to be investigated by mass spectroscopy quantification (53, 59) whether the cellular concentrations of sphingosine and SPP are modulated as anticipated. It will also be interesting to investigate how the extracellular-regulated kinase and c-Jun NH2-terminal kinase/stress-activated protein kinase pathways are stimulated by these metabolites in PDB-treated HL-60 cells in relation to their role in apoptosis induced by TNF-α and fas ligand (30).

Reversion of PDB-differentiated HL-60 Cells—We showed that PDB-differentiated, adherent cells reproliferate if PDB is removed from the culture medium without disturbing the adherent state (Fig. 6C). This result implies that the commitment to apoptosis by default induced by PDB is resolved faster than the loss of survival signal by adherence after the PDB removal. Those cells that adhered longer to the culture dish after the PDB removal exhibited less apoptosis after the spontaneous detachment (Fig. 6B). We have shown recently that the irreversible differentiation of HL-60 cells by exposure to dimethyl sulfoxide is induced by elimination of double minutes that are trapped in micronuclei. It is interesting to note in this regard that PDB inhibited micronucleation and elimination of double minutes. Therefore, integrity of the genomic constituents in HL-60 cells is maintained after the PDB treatment. Thus there may exist a reasonable route of de-differentiation of PDB-differentiated HL-60 cells as demonstrated in this study. Previ-
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