Vitamin D3 Induces Autophagy of Human Myeloid Leukemia Cells*

Received for publication, March 3, 2008, and in revised form, May 13, 2008. Published, JBC Papers in Press, July 15, 2008, DOI 10.1074/jbc.M801716200

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Vitamin D3 causes potent suppression of various cancer cells; however, significant supraphysiological concentrations of this compound are required for antineoplastic effects. Current combinatorial therapies with vitamin D3 are restricted to differential effects. It remains uncertain if autophagy is involved in vitamin D3 inhibition on leukemia cells. Here we show that besides triggering differentiation and inhibiting apoptosis, which was previously known, vitamin D3 triggers autophagic death in human myeloid leukemia cells. Inhibiting differentiation does not efficiently diminish vitamin D3 suppression on leukemia cells. Vitamin D3 up-regulates Beclin1, which binds to class III phosphatidylinositol 3-kinase to trigger autophagy. Vitamin D3 phosphorylates Bad in its BH3 domain, resulting in disassociation of the apoptotic Bad-Bcl-xL complex and association of Bcl-xL with Beclin1 and ultimate suppression of apoptotic signaling. Knockdown of Beclin1 eliminates vitamin D3-induced autophagy and inhibits differentiation but activates apoptosis, suggesting that Beclin1 is required for both autophagy and differentiation, and autophagy cooperates with differentiation but excludes apoptosis, in which Beclin1 acts as an interface for these three different cascades. Moreover, additional up-regulation of autophagy, but not apoptosis, dramatically improves vitamin D3 inhibition on leukemia cells. These findings extend our understanding of the action of vitamin D3 in antineoplastic effects and the role of Beclin1 in regulating multiple cellular cascades and suggest a potentially promising strategy with a significantly better antileukemia effect.

1,25-Dihydroxyvitamin D3, the hormonally active form of vitamin D3, plays critical roles in regulating cellular and physiological responses. Treatment of vitamin D3 potently inhibits cell proliferation in a wide range of cancer cells, including myeloid leukemia and carcinomas of the breast, prostate, colon, skin, and brain and inhibits angiogenesis, tumor invasion, and metastases (1–8), suggesting that vitamin D3 has potential applications in cancer prevention and treatment. Vitamin D3 is known to inhibit cancer cell proliferation through the induction of differentiation, which is dependent on the signaling mechanisms involving down-regulation of Akt and its disassociation with Raf1 and subsequent activation of Raf/MEK/ERK2 MAPK signaling, leading to cyclin-dependent kinase inhibitor up-regulation and retinoblastoma protein dephosphorylation coupled to transcription factors E2F1 and C/EBPβ binding (9–11).

Vitamin D3 was also reported to induce apoptosis in several tumor cells. The antiapoptotic protein Bcl-2, which is overexpressed in many tumors, was down-regulated by vitamin D3 or its analogues in prostate cancer cells, MCF-7 breast cancer cells, and retinoblastoma cells undergoing apoptosis (12–14). In invasive breast cancer cells, down-regulation of Bcl-2 protein is accompanied by an increase in the proapoptotic protein Bax and a release of cytochrome c from the mitochondria followed by poly(ADP-ribose) polymerase cleavage (15). Contrary to the observation that vitamin D3 induces apoptosis in B-cell chronic lymphocytic leukemia cells (16), two studies show that vitamin D3 inhibits apoptosis of myeloid leukemia cells (17, 18), suggesting cell type-dependent mechanisms by which vitamin D3 modulates apoptosis.

Autophagy is a ubiquitous process believed to occur in all eukaryotic cells in which cytosol and organelles are sequestered within double-membrane vesicles that deliver their contents to lysosomes for degradation and/or recycling of the resulting macromolecules (19). Autophagy is frequently reduced in cancer cells compared with their normal counterparts and acts as a protective mechanism in response to nutrients depletion. However, stimulation of autophagy in cancer cells was observed in response to anticancer treatments when enhanced autophagy destroys large proportions of the cytosol and organelles, which causes irreversible cellular atrophy with a consequent collapse of vital cellular functions (20–23). Therefore, autophagy can protect cells against death or mediate cellular demise, depending on autophagy stimuli and cellular context. A vitamin D3 analog, EB1089, initially reported to induce apoptosis via a p53-independent mechanism involving p38 MAPK activation and ERK MAPK inactivation in B-cell chronic lymphocytic leukemia cells (16), triggered autophagy in breast cancer cells (23), again suggesting that the mechanisms on anticancer effects by the same compound may vary depending on cell types.

* This work was supported in part by a grant from the Unimed Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to Dr. Ray Wu for support and advice. His untimely death has saddened his many friends and collaborators in the scientific community.

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2 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LC3, light chain 3; Pi3K, class III phosphatidylinositol 3-kinase; siRNA, small interfering RNA; GFP, green fluorescent protein; mTOR, mammalian target of rapamycin; NBT, nitroblue tetrazolium.
Although vitamin D3 shows promise in counteracting tumors, significant supraphysiological concentration of this compound is required for antineoplastic effects. Such concentrations are not achievable in patients due to predictable hypercalcemia and hypercalcuria resulting from the increased intestinal absorption of calcium and the calcium-mobilizing properties of vitamin D3. To reduce the risk of vitamin D3-induced hypercalcemia and the possible immunosuppressive effects of its analogs, a combination of low doses of vitamin D3 or its analogs with administration of another agent that enhances differentiation but does not enhance the levels of circulating calcium and is not immunosuppressive, has been pursued. For example, vitamin D3 with docetaxel, dexamethasone, paclitaxel, carboplatin, and carnosic acid were tried in several types of cancers and cancer model cell lines (24–26). However, the current combinatorial therapies with vitamin D3 in the preclinical stage are restricted to differentiation effects, thus limiting the efficacy on cancer cell suppression. A better understanding of the mechanisms by which vitamin D3 exerts suppressive effects on tumor model cells is needed to develop vitamin D3 analogs and vitamin D3 combinatorial therapies targeting critical signaling pathways with a better antineoplastic effect.

In this study we show that vitamin D3 triggers autophagic death by up-regulating Beclin1 along with triggering differentiation but inhibiting apoptosis in myeloid leukemia cells. Knockdown of Beclin1 not only cripples vitamin D3-induced autophagy but also inhibits vitamin D3-induced differentiation and activates apoptotic signaling. These data suggest that autophagy cooperates with differentiation but excludes apoptosis, in which Beclin1 acts as an interface for these three different cascades. We further demonstrate that additional up-regulation of autophagy dramatically improves the antineoplastic effect of vitamin D3 on human myeloid leukemia cells, suggesting a better strategy for combating leukemia.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Materials and specific vendors were as follows: antibodies specific to C/EBPβ, Rb, Raf1, caspase 9, Bcl-2, Bel-xL, poly(ADP-ribose) polymerase (PARP), Bad, phospho-Bad, mammalian target of rapamycin (mTOR), or phospho-mTOR (Cell Signaling); antibodies specific to Beclin1, class III phosphatidylinositol 3-kinase (PI3KC3), or LC3 (Abgent); vitamin D3, Cay10443 (Cayman Chemical); rapamycin, bafilomycin A1 (Calbiochem); human Beclin1 siRNA (Santa Cruz Biotechnology), human PI3KC3 siRNA (Novus Biologicals); human Raf1 siRNA and human mTOR siRNA (Cell Signaling); DNA construct green fluorescent protein (GFP)-LC3 (Addgene); pepstatin A (Sigma); ABT-737 (Abbott Laboratories) (27).

**Cell Culture**—HL-60 human myeloid leukemia cells and its transfected were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Vitamin D3 was treated at 5 nM when cells were recultured or when transfection was performed.

**Co-immunoprecipitation**—50 μg of antibody against each bait protein was immobilized in a coupling gel, and each 50 μg of cell lysates prepared from different treatments was incubated with the antibody-immobilized coupling gel using ProFound-
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RESULTS AND DISCUSSION

Inhibition of Differentiation Does Not Efficiently Diminish Vitamin D3 Suppression on HL-60 Leukemia Cells—In addition to its induction of differentiation, shown by the induced expression of the cell surface differentiation marker CD14 determined by flow cytometric immunofluorescence and functional differentiation determined by NBT reduction assay (Fig. 1A), vitamin D3 also caused leukemia cell death at the late stage of the treatment (Fig. 1B). Previous studies found that Raf1 activation causes differentiation of human myeloid leukemia cells in response to vitamin D3 (11, 34) or retinoic acid (28). The retinoblastoma protein (Rb) up-regulation and its binding to Rb is required in monocytic differentiation cascade caused by activation of Raf1/MEK/ERK MAPK signaling in response to vitamin D3 (11). C/EBPβ is a transcription factor for monocytic differentiation, and its binding to Rb is required in monocytic differentiation of human myeloid leukemia cells in response to vitamin D3 (35). To examine if differentiation is the only mechanism by which vitamin D3 suppresses human myeloid leukemia cells, we depleted Raf1 by RNA interference in HL-60 cells. As expected, knockdown of the Raf1 crippled vitamin D3-induced Rb up-regulation and abolished C/EBPβ binding to Rb (Fig. 1C), suggesting that the vitamin D3-induced differentiation signaling was inhibited by knockdown of Raf1. Furthermore, knockdown of Raf1 effectively inhibited vitamin D3-induced expression of the differentiation marker CD14 and crippled functional differentiation (Fig. 1D), confirming successful blockage of the differentiation in response to vitamin D3. Surprisingly, although knockdown of Raf1 significantly crippled the antiproliferation by vitamin D3 due to crippled differentiation, inhibition of leukemia cell growth was not efficiently reversed, and cell viability was not improved when differentiation machinery driven by vitamin D3 was completely inhibited (Fig. 1E). These data suggest that other mechanisms may be implicated in suppressing myeloid leukemia cells under vitamin D3 induction.

Vitamin D3 Inhibits Apoptosis of HL-60 Cells—Vitamin D3 was reported to cause caspase-dependent apoptosis in several tumor cells (12–15). The vitamin D3 analog, EB1089, was also reported to induce apoptosis in leukemia cells (16), but other reports reveal that vitamin D3 causes an inhibitory effect on apoptosis of leukemia cells (17, 18). These opposing results suggest a necessity of verifying the effects of vitamin D3 on apoptosis before we could suggest any other mechanism responsible for cell death in response to vitamin D3 in HL-60 cells.

To test if caspase-dependent apoptosis is attributed to the myeloid leukemia cell suppression and cell death in response to vitamin D3, we first measured the pan-caspase activity of the HL-60 cells treated with or without vitamin D3. Flow cytometric measurements show that at 96 h of treatment, control cells showed high caspase activity possibly resulting from a high cell density and metabolic stress due to a long culture time, whereas
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Vitamin D3 analogs or other compounds have also been reported to induce caspase-independent apoptosis in human breast cancer cells (36) and human B lymphocytes (37). In support of the observation that vitamin D3 inhibits apoptotic signaling in myeloid leukemia cells, HL-60 cells treated with or without vitamin D3 were examined with a TUNEL assay, which detects apoptotic death from DNA breaks. The results show that control cells at 72 and 96 h, the late stage of culturing, reached a high TUNEL-positive percentage presumably resulting from metabolic stress due to the long culture time, whereas vitamin D3 treatment reduced the TUNEL-positive percentage (Fig. 2C), confirming that vitamin D3 inhibits apoptotic death in human myeloid leukemia cells. In addition, vitamin D3 induced the expression of Bcl-2 and Bcl-xL. Poly(ADP-ribose) polymerase did not express until 72 h after vitamin D3 induction (Fig. 2D). These data further support the role of vitamin D3 in inhibiting apoptotic signaling in myeloid leukemia cells, which was initially observed by other groups (17, 18). Because vitamin D3 also induces apoptosis in B-cell chronic lymphocytic leukemia cells (16), the regulation on apoptosis by vitamin D3 appears to be cell type-dependent.

However, the mechanism by which vitamin D3 negatively regulates apoptosis in myeloid leukemia cells has not been fully understood. Bad is a proapoptotic member of the Bcl-2 family that promotes cell death and is a member of the family of “BH3 domain only” proteins that transduces death signals from the cytoplasm to the mitochondrial membrane and induces apoptosis (38). Bad binding to Bcl-xL by the BH3 domain causes Bcl-xL to release Apaf1 or regulate other Bcl-xL activities resulting in a caspase 9-initiated cascade of proteolysis and induction of apoptosis (39). Vitamin D3 caused phosphorylation of Bad at Ser-155 in HL-60 cells (Fig. 2E). Because the Ser-155 is located within the BH3 domain of Bad that mediates its apoptotic activities via heterodimerization to the Bcl-xL at the mitochondrial membrane (38–41), phosphorylation of Bad at Ser-155 by vitamin D3 directly inhibits BH3 function and interaction with Bcl-xL, thus blocking the binding of Bad to Bcl-xL. These results are confirmed by co-immunoprecipitation detection at 96 h of vitamin D3 treatment (Fig. 2F) and are consistent with a previous report on the inactivation of Bad and suppression of apoptotic cascades (42). The above data suggest a mechanism by which vitamin D3 phosphorylates Bad Ser-155 to dissociate Bcl-xL from Bad, thus inhibiting caspase activation to prevent apoptosis in myeloid leukemia cells.

Vitamin D3 Induces Autophagy of HL-60 Cells—Our above data show that inhibition of vitamin D3-induced differentiation did not efficiently diminish vitamin D3 suppression on HL-60 cells and that vitamin D3 inhibited apoptosis. Thus, we asked if autophagy is involved in the mechanisms of vitamin D3 action on the suppression of the leukemia cells. The kinase mTOR is the major mediator of the cell survival pathway and also the principal negative regulator of autophagy (43, 44). Western
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**FIGURE 3. Vitamin D3 induces Beclin 1 and PI3KC3-dependent autophagy in leukemia cells.** A, vitamin D3 down-regulates mTOR and its kinase activity, as determined by Western blotting (WB) with antibodies against mTOR or phospho-p70S6 kinase at the designated time points. B, vitamin D3 up-regulates autophagy proteins and induces the formation of autophagy kinase complex Beclin1-PI3KC3 determined by Western blotting and co-immunoprecipitation with the designated antibodies. C, vitamin D3 causes the processing of the autophagic marker LC3 determined with autophagic flux assay by Western analysis at the designated time points of the treatment. The vitamin D3-treated cells were treated with pepstatin A (PA, 10 μg/ml) for 12 h at 60 h of vitamin D3 treatment. D, detection of autophagy by fluorescence confocal microscopy. The GFP-LC3-transfected HL-60 cells were treated with/without vitamin D3. The vitamin D3-treated cells were either simultaneously treated with/without mTOR or phospho-p70S6 kinase at the designated time points of the treatment. H and I, knockdown of Beclin1 or PI3KC3 by siRNA cripples the formation of the autophagy complex Beclin1-PI3KC3 and cripples the processing of LC3 in response to vitamin D3 treatment determined by Western blotting (left panel and right panels) and co-immunoprecipitation (middle panel). The cells were transfected with siRNA targeting Beclin 1 or PI3KC3, respectively, and treated with or without vitamin D3 for 72 h simultaneously. Knockdown cells were treated with pepstatin A for 12 h at 60 h of vitamin D3 treatment for autophagy flux assay. β-Actin is the loading control for the above Western blotting in panels A, B, C, H, and I. J, confocal fluorescence microscopic detection of autophagy in Beclin 1 or PI3KC3 knockdown cells. The GFP-LC3 stable transfectant HL-60 cells were transfected with siRNA targeting either Beclin 1 or PI3KC3 individually and simultaneously treated with/without vitamin D3 for 72 h. The cell samples were examined with a confocal fluorescence microscope. The Western blotting results show that vitamin D3 down-regulated mTOR. Down-regulation of mTOR coincided with decreased phospho-p70S6 kinase, the downstream effector of mTOR (Fig. 3A), suggesting that vitamin D3 may induce autophagy.

Autophagy is hallmarkd by accumulation of vacuoles that sequester and target cytoplasmic components for lysosomal degradation in a process dependent on autophagy proteins including Beclin-1 (31, 45). To test whether autophagy is involved in the action of vitamin D3 on leukemia cells, expression of the mammalian autophagy essential proteins Beclin1 and the PI3KC3 under vitamin D3 treatment was examined by Western analysis. These two proteins are key members of the PI3KC3 kinase complex participating in autophagosome formation, and physical interaction between the Beclin1 and PI3KC3 is a critical step in completing the autophagic cascade in mammalian cells (30, 31). The results show that the HL-60 control cells maintained a constitutive Beclin1 level. Vitamin D3 treatment enhanced Beclin1 expression at 48 and 72 h and induced the expression of PI3KC3 at 72 h (Fig. 3B). Co-immunoprecipitation and Western blotting further show that at 72 h vitamin D3 induced Beclin1 binding to PI3KC3 (Fig. 3B), suggesting that vitamin D3 triggers an autophagic response. Furthermore, the induction of the formation of the autophagy kinase complex, Beclin1-PI3KC3, is a slow event involving vitamin D3 action in myeloid leukemia cells, which is supported by the results that vitamin D3 did not cause cell death until 72 h, the late stage of the treatment (Fig. 1B).

Another established molecular marker to detect autophagy is the autophagy microtubule-associated protein 1 light chain 3 (LC3), the mammalian homologue of yeast Atg8, which specifically associates with autophagosome membranes after processing from LC3-I (18 kDa) into LC3-II (16 kDa) in the late stage of the autophagic cascade.
LC3-I is localized in the cytosol, and LC3-II is localized in autophagosomes. LC3-II was, thus, used to estimate the abundance of autophagosomes before they are degraded through fusion with lysosomes (32). Because LC3-II itself is also degraded by autophagy, the traditional detection of the LC3 conversion may lead to misinterpretation. Recently, an autophagic flux assay has been proposed to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the absence and presence of lysosomal protease inhibitors (33). The results of the detection of the autophagic flux show that control cells only exhibited an 18-kDa band representing LC3-I, vitamin D3-treated HL-60 cells revealed the presence of the processed 16-kDa LC3-II in addition to the 18-kDa LC3-I, and cells treated with vitamin D3 in combination with peptatin A showed an increase in LC3-II, indicating the accumulation of LC3-II due to the blockade of LC3-II degradation in lysosomes (Fig. 3C), further suggesting an autophagic response caused by vitamin D3.

In addition, a DNA construct expressing a fusion protein, GFP-LC3, was transfected to HL-60 cells to visually track autophagic responses under a fluorescence microscope, in particular, the processing and localization of LC3 within autophagic cells in response to vitamin D3 because GFP-LC3 fusion protein redistributes from a diffuse to a vacuolar pattern when autophagosomes are formed (32, 46). The results from fluorescence confocal microscopy show that treatment of GFP-LC3 transfectant cells with vitamin D3 caused GFP punctate pattern, indicating autophagy events (Fig. 3D). Autophagy did not occur until 72 h of vitamin D3 treatment, and longer treatment increased autophagic activity, shown by the percentage of the cells with GFP-LC3 dots in the transfected cells and the number of GFP-LC3 dots per cell in response to vitamin D3 (Fig. 3E).

Autophagy can either be protective or destructive to the cells, depending on the nature and intensity of the autophagy stimuli and cell context (47, 48). We, thus, asked if autophagy is responsible for cell death caused by a long exposure time to vitamin D3. Bafilomycin A1 is an autophagy inhibitor that is known to inhibit vacuolar H+ -ATases and block maturation of autophagic vacuoles and acidification of lysosomes by disrupting the fusion between autophagosomes and lysosomes (49). Treatment of the GFP-LC3-transfected cells with vitamin D3 in combination with 0.2 μM bafilomycin A1 for 72 h completely inhibited GFP punctate pattern induced by vitamin D3 (Fig. 3D), which counters a previous observation that a regime with a lower concentration and shorter exposure to bafilomycin A1 (0.1 μM, 90 min) did not cripple the GFP punctate pattern, whereas it inhibited autophagy (32). We, thus, treated the vitamin D3-induced cells with bafilomycin A1 with lower concentrations for shorter exposure times. The results show that short exposure and low working concentrations of bafilomycin A1 (0.05 μM, 2h) resulted in an enhanced GFP punctate pattern (Fig. 3D). These data suggest that low concentration and short exposure of bafilomycin A1 to leukemia cells results in specific inhibition on lysosomal degradation and, thus, accumulates LC3-II, whereas high concentration and long exposure of bafilomycin A1 may cause inhibition on LC3 processing, which occurs earlier than lysosomal degradation. By contrast, bafilomycin A1 treatment did not affect vitamin D3-induced expression of the cell surface differentiation marker CD14 or vitamin D3-induced functional differentiation determined by NBT reduction assay (Fig. 3F), confirming specific inhibition on autophagy but not on differentiation. Inhibition of autophagy by bafilomycin A1 significantly attenuated vitamin D3 suppression on leukemia cells and recovered the cell viability (Fig. 3G).

We, thus, propose that vitamin D3-triggered autophagy in leukemia cells is a death mechanism, not a protective one.

**Knockdown of Beclin1 or PI3KC3 Cripples Vitamin D3-triggered Autophagy of HL-60 Cells**—Beclin1 and PI3KC3 are two essential proteins for the mammalian autophagy cascade, and although they form a kinase complex participating in autophagosomal formation (30, 31), autophagy in mammalian species can be independent of Beclin1 (50). To confirm that vitamin D3-induced autophagic signaling in human leukemia cells is dependent on Beclin1 and PI3KC3, the expression of Beclin1 or PI3KC3 was knocked down by the siRNA targeting Beclin1 (Fig. 3H, left panel) or PI3KC3 (Fig. 3I, left panel) individually. The cell lysates were co-immunoprecipitated with either anti-Beclin1 or anti-PI3KC3 and subjected to Western blotting probed with either anti-PI3KC3 or anti-Beclin1. The results show that knockdown of Beclin1 or PI3KC3 by RNA interference caused a disappearance of the Beclin1-PI3KC3 complex induced by vitamin D3 (Fig. 3, H and I, middle panels) and inhibited the D3-induced LC3 processing. Treatment with peptatin A did not accumulate LC3-II in knockdown cells (Fig. 3, H and I, right panels). These data indicate that autophagy induced by vitamin D3 was crippled when Beclin1 or PI3KC3 was depleted. The fluorescence microscopy results demonstrate that a diffuse localization of GFP-LC3 in the control cells and treatment of the cells with vitamin D3 produced a punctuate pattern for GFP-LC3 fluorescence, indicating recruitment of LC3-II to autophagosomes during vitamin D3-induced autophagy. In contrast, knockdown of Beclin1 or PI3KC3 prevented the punctuate pattern for GFP-LC3 fluorescence in response to vitamin D3 (Fig. 3I). These data indicate that the formation of autophagosomes was crippled by eliminating Beclin1 or PI3KC3 and confirm that vitamin D3 induces autophagy dependent on both Beclin1 and PI3KC3. Thus, formation of the autophagy kinase complex Beclin1-PI3KC3 is a key step in executing the autophagy cascade in HL-60 human myeloid leukemia cells.

The dependence of autophagy on Beclin1 presence has been documented in numerous previous observations. Previous studies also suggested that basal autophagy is constitutive and needed for normal cell mechanisms to clean up damaged organelles and long-life proteins. However, one unresolved question is why constitutive Beclin1 levels are incapable of triggering autophagy. Our data show that an autophagic response did not occur until Beclin1 was significantly elevated, PI3KC3 was induced, and the kinase complex Beclin1-PI3KC3 was formed (Fig. 3, B and C). We, thus, suggest that induction of autophagy requires an elevated level of Beclin1 which must reach a certain threshold to trigger autophagic signaling. The constitutive Beclin1 independent of vitamin D3 stimulus is not an indicator for basal autophagy and may be involved in another cascade. Autophagy may not necessarily be a housekeeping process in certain types of cells, in particular, in some
cancer cells in which activation of autophagy may trigger a destructive mechanism for cell survival.

**Knockdown of Beclin1 Activates Apoptosis of HL-60 Cells**—Apart from the blockage of the autophagy cascade (Fig. 3), knockdown of Beclin1 protein significantly inhibited cell proliferation, reduced cell viability (Fig. 4A), and enhanced cell cycle arrest, as determined by a flow cytometric analysis of the percentage of cells in the G1 phase (Fig. 4B). Knockdown of Beclin1 excludes the autophagic death. We, thus, asked if knockdown of Beclin1 activates apoptotic signaling in myeloid leukemia cells. The Beclin1 knockdown cells and control cells were examined with pan-caspase activity at 72 h and TUNEL assay at 48 h of Beclin1 siRNA transfection and vitamin D3 treatment. The results show that knockdown of Beclin1 increased pan-caspase activity representing apoptotic signaling (Fig. 4C) and caused a significantly enhanced TUNEL-positive level representing apoptotic cell death that could be partially inhibited by vitamin D3 (Fig. 4D). These data suggest that Beclin1 may play a role in antiapoptotic signaling, which appears to be in no accordance with previous studies showing that Beclin1 positively regulates apoptosis in breast cancer cells (23) and promotes apoptosis possibly through the formation of a Beclin1-Bcl2/Bcl-xL complex (51).

To find the answer to why depletion of Beclin1 caused apoptosis in myeloid leukemia cells, we examined the expression and interaction of Bad and Bcl-xL in the Beclin1 knockdown HL-60 cells. The results show that knockdown of Beclin1 increased Bad protein expression, inhibited vitamin D3-induced Bcl-xL expression, and increased formation of the apoptotic complex Bad-Bcl-xL (Fig. 4E). These data further suggest that depleting Beclin1 activates apoptotic signaling. Because vitamin D3 phosphorylated Bad at Ser-155 to dissociate the apoptotic complex Bad-Bcl-xL (Fig. 2, D and E), we asked if Beclin1 competes with Bad in binding to Bcl-xL. Co-immunoprecipitation and Western blotting show that at 24 h, the early stage of cell culturing, there was no Bad binding to Bcl-xL (Fig. 4F). At 96 h, the late stage of cell culturing, the control cells had high levels of the apoptotic complex Bad-Bcl-xL (Fig. 4F), consistent with earlier TUNEL assay results (Fig. 2C). Vitamin D3 treatment disassociated this Bad-Bcl-xL complex (Fig. 4F). In contrast, Beclin1 bound to Bcl-xL with a basal level in the control cells, and this binding was enhanced in the late stage of vitamin D3 treatment (Fig. 4F). Because the reduced Bad-Bcl-xL complex coincided with the enhanced Beclin1-Bcl-xL complex, the decrease in Bcl-xL binding to Bad may be caused by its increased binding to Beclin1 in response to vitamin D3 treatment. These data suggest that Beclin1 may negatively regulate apoptosis by competitively binding to Bcl-xL to disrupt the apoptotic complex Bad-Bcl-xL. Because basal Beclin1 does not trigger autophagy and diminishing basal Beclin1 activates apoptotic signaling, we propose that basal Beclin1 expression is required for its suppression of apoptotic signaling.

Because vitamin D3 up-regulates Beclin1 and induces the formation of the autophagic complex Beclin1-P13KC3 and the anti-apoptotic complex Beclin1-Bcl-xL, one would ask if the Beclin1 that binds P13KC3 is the same that associates with Bcl-xL. To answer this question, the cell samples treated with or without vitamin D3 were co-immunoprecipitated with anti-body against P13KC3 and probed with antibody against Beclin1 or Bcl-xL. The results show that Bcl-xL is not in the Beclin1-P13KC3 complex, suggesting that upon vitamin D3 up-regulation of Beclin1, part of the up-regulated Beclin1 is used to form the autophagic complex Beclin1-P13KC3, and part of it is used to form the complex Beclin1-Bcl-xL. These two complexes are physically independent from each other (Fig. 4F).

The Beclin1-Bcl-xL complex has recently been suggested to inhibit autophagy because release of Beclin1 from the complex Beclin1-Bcl-xL causes autophagy (52). However, because vitamin D3 significantly up-regulates Beclin1 (Fig. 3B), it appears to have no need to recruit Beclin1 from the Beclin1-Bcl-xL complex to trigger autophagy in the context of vitamin D3 treatment. To further identify the role of the Beclin1-Bcl-xL complex in response to vitamin D3 in myeloid leukemia cells, HL-60 cells were treated with vitamin D3 in combination with ABT-737, a BH3 mimic that targets selective BCL-2 proteins (27). ABT-737 disrupted the vitamin D3-induced interaction between Beclin1 and Bcl-xL (Fig. 4G, upper panel). This combinatorial treatment compromised the vitamin D3 inhibition on apoptosis shown by TUNEL assay results (Fig. 4G, lower panel), whereas autophagic activity was not changed, shown by the detection of autophagic flux (Fig. 4H), further confirming that the Beclin1 binding to Bcl-xL is antiapoptotic and not responsible for induction or inhibition of autophagy in the leukemia cells. Because the Beclin1-Bcl-xL complex was proposed to be apoptotic (51) and anti-autophagic (52), which conflicts with our present observations, the role of the Beclin1-Bcl-xL complex may be cell-type or cell context-dependent. Similar to the antiapoptotic protein Bcl-2, which negatively regulates autophagy by binding to Beclin1 (53), Beclin1, by binding to Bcl-xL, is therefore implicated in the negative regulation of apoptotic signaling in addition to its essential regulatory role in autophagy.

**Knockdown of Beclin1 Impairs Vitamin D3-induced Differentiation of HL-60 Cells**—To date, no molecular connection has been found between differentiation and autophagy. Our data show that knockdown of Beclin1 by RNA interference reduced both the vitamin D3-induced expression of cell surface differentiation marker CD14 and functional differentiation determined by NBT reduction assay (Fig. 4I), whereas knockdown of Beclin1 enhanced cell cycle arrest, determined by the percentage of cells in G1 phase (Fig. 4B). These data indicate that knockdown of Beclin1 impairs vitamin D3-induced differentiation but does not impair vitamin D3-induced cell cycle arrest, suggesting that differentiation and cell cycle arrest can be uncoupled in certain circumstances, and cell differentiation depends on Beclin1 presence. It, thus, appears that Beclin1 not only links to autophagy and apoptosis but also to differentiation.

**Enhanced Autophagy, but Not Apoptosis, Enhances Vitamin D3 Suppression on HL-60 Leukemia Cells**—Our above data show that vitamin D3 inhibits leukemia cells by triggering both autophagy and differentiation. Thus, we hypothesize that combinatorial stimuli with an enhanced mechanism complementary to differentiation may increase the potency of vitamin D3 in inhibiting leukemia cells. To test if additional autophagy stimuli or up-regulation of autophagy enhances the potency of
vitamin D3 on suppression of leukemia cells, HL-60 cells were either treated with rapamycin, the autophagy inducer, or transfected with siRNA targeting mTOR in combination with or without vitamin D3 treatment. Vitamin D3 down-regulated mTOR in a significant manner, whereas rapamycin did not inhibit mTOR expression (Fig. 5A). Unlike rapamycin, which
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FIGURE 5. Up-regulation of autophagy, but not apoptosis, enhances vitamin D3 suppression on leukemia cells. A, vitamin D3, but not rapamycin (Rap), down-regulates mTOR determined at 72 h of treatment by Western blotting. B, knockdown of mTOR by siRNA targeting mTOR gene determined at 72 h of transfection by Western blotting. C, combinatory autophagy and differentiation stimuli enhance the suppression on leukemia cells. The HL-60 cells were treated with vitamin D3, rapamycin (0.1 μM), or transfected with mTOR siRNA individually or in combination for 72 h. Cell growth was examined at 72 h of the treatment. D, combinatory apoptosis and differentiation compromises vitamin D3 suppression on leukemia cells. HL-60 cells were treated with vitamin D3 or the apoptosis inducer Cay10443 (1 μM) or Cay 10443 in combination with vitamin D3. Cell growth was determined at 72 h of the treatment. E, down-regulation of mTOR by siRNA knockdown (mTOR Kd) cooperates with vitamin D3 in enhancing Beclin1 expression and LC3 processing, but an apoptosis inducer attenuates Beclin1 expression and LC3 processing determined by autophagic flux assay. One group of the HL-60 cells was treated as described in C. Another group of the HL-60 cells were treated with pepstatin A1 (10 μg/ml) at 60 h of the vitamin D3 treatment. The above Western results are representative of three independent repeats for each experiment. β-Actin is the loading control in panels A, B, and E. The above cell counting data are represented as mean ± S.E. from three independent repeats for each experiment. *, p < 0.05; **, p < 0.01.

primarily induces autophagy by inhibiting the mTORC1 complex (54), vitamin D3 causes autophagy via down-regulation of mTOR protein levels. Rapamycin alone did not efficiently cause autophagic death possibly because full action of mTOR in regulating autophagy may not be sufficiently down-regulated by targeting only on the mTORC1 complex. Down-regulation of mTOR by vitamin D3 treatment (Fig. 5A) or RNA interference (Fig. 5B) suppressed leukemia cells more significantly than inhibition of mTORC1 complex by rapamycin (Fig. 5C). Combinatorial treatment of the leukemia cells with vitamin D3 and rapamycin strengthened vitamin D3 inhibition on leukemia cell growth, but vitamin D3 treatment in combination with global down-regulation of mTOR by RNA interference more significantly enhanced vitamin D3 inhibition on the leukemia cell growth (Fig. 5C), indicating that enhanced global down-regulation of mTOR more efficiently strengthens vitamin D3 suppression on the leukemia cells. Our studies, thus, propose that combinatory activation of differentiation and autophagy machineries result in significantly enhanced inhibition of leukemia cells. This is especially important because a significant supraphysiological concentration of vitamin D3 is required for antineoplastic effects in current pre-clinical treatment, and such concentrations easily result in hypercalcemia and hypercalcuria. The combinatorial therapy via complementary mechanisms may provide a new strategy for a more efficient combat against leukemia.

To examine if apoptotic stimuli cooperate with differentiation or autophagy in suppression of the leukemia cells, a compound, Cay10443, which causes mitochondrial release of cytochrome c to trigger apoptosis, was tried in combination with vitamin D3. The results show that apoptosis inducer Cay10433 did not improve but significantly compromised vitamin D3 suppression on the leukemia cells (Fig. 5D).

The immunoblotting results show that global down-regulation of mTOR by siRNA significantly strengthened vitamin D3-enhanced Beclin1 expression and significantly increased LC3-II determined by detection of autophagic flux, but treatment with apoptosis inducer Cay10443 significantly weakened vitamin D3-enhanced Beclin1 expression and vitamin D3-induced LC3 processing (Fig. 5E).

In summary, our data demonstrate that vitamin D3 inhibits human myeloid leukemia cells by triggering both autophagy and differentiation. Vitamin D3 up-regulates Beclin1 to trigger autophagy. Eliminating Beclin1 abolishes autophagy, activates apoptosis, and impairs differentiation but does not impair cell cycle arrest, suggesting that differentiation and cell cycle arrest can be uncoupled under certain circumstances, and autophagy cooperates with differentiation but excludes apoptosis in the myeloid leukemia cells. Global down-regulation of mTOR significantly strengthens vitamin D3-enhanced Beclin1 expression, resulting in enhanced autophagic death, but treatment with an apoptosis inducer does the opposite. The above data suggest an interesting role of Beclin1 in linking autophagy, differentiation, and apoptosis. This study, thus, provides the first evidence to our knowledge that Beclin1 may act as an interface for these three different cellular cascades, autophagy, differentiation, and apoptosis, and vitamin D3 exerts potent suppression on leukemia cells via its action not only on differentiation but also on autophagy.

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Vitamin D3 Induces Autophagy of Human Myeloid Leukemia Cells

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doi: 10.1074/jbc.M801716200 originally published online July 15, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M801716200

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