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Ornithine Decarboxylase Is a Mediator of c-Myc-Induced Apoptosis

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c-Myc plays a central role in the regulation of cell cycle progression, differentiation, and apoptosis. However, the proteins which mediate c-Myc function(s) remain to be determined. Enforced c-*myc* expression rapidly induces apoptosis in interleukin-3 (IL-3)-dependent 32D.3 murine myeloid cells following IL-3 withdrawal, and this is associated with the constitutive, growth factor-independent expression of ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis. Here we have examined the role of ODC in c-Myc-induced apoptosis. Enforced expression of ODC, like c-*myc*, is sufficient to induce accelerated death following IL-3 withdrawal. ODC induced cell death in a dose-dependent fashion, and α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC enzyme activity, effectively blocked ODC-induced cell death. ODC-induced cell death was due to the induction of apoptosis. We also demonstrate that ODC is a mediator of c-Myc-induced apoptosis. 32D.3-derived c-*myc* clones have augmented levels of ODC enzyme activity, and their rates of death were also a function of their ODC enzyme levels. Importantly, the rates of death of c-*myc* clones were inhibited by treatment with DFMO. These findings demonstrate that ODC is an important mediator of c-Myc-induced apoptosis and suggest that ODC mediates other c-Myc functions.

Myc family proteins are important regulators of cell growth and are frequently activated in tumorigenesis (26, 28). c-Myc is a transcription factor (1, 5, 7, 23), promotes cell proliferation (2, 12, 13, 15), and inhibits differentiation where this is normally associated with a withdrawal from the cell cycle (10, 17, 33). More recently, a role for c-Myc in the induction of apoptosis has also been demonstrated. Apoptosis is a genetic cell suicide program which plays an essential role in the control of development and hematopoiesis (14, 32, 34, 35, 46, 47). Inappropriate expression of c-*myc* induces apoptosis in fibroblasts (6, 15) and myeloid cells (2, 3), and c-Myc is required for anti-CD3-induced apoptosis in T-cell hybridomas (37). However, the targets regulated by c-Myc which mediate its biological activities remain to be identified.

c-Myc is a sequence-specific transcription factor (1, 5, 7, 23), although the relevance of this activity to c-Myc biologic effects has not been confirmed. We have demonstrated that the ornithine decarboxylase (ODC) gene is a transcriptional target of c-Myc (2, 3, 5). c-Myc is a potent *trans*-activator of the ODC promoter (5), and enforced c-*myc* expression results in constitutive expression of the ODC gene (2, 3, 11). This activation is most likely direct, as it strictly requires conserved consensus c-Myc binding sites (5) and does not require intermediate protein synthesis (45). ODC catalyzes the conversion of ornithine to putrescine, the first and a rate-limiting step in polyamine biosynthesis (31, 41). c-Myc and ODC have similar biologic effects. Both are required for entry into S phase of the cell cycle (8, 18) and are necessary (4, 36) and sufficient (4, 12) for the morphological transformation of fibroblast cell lines. Therefore, ODC is a strong candidate as a mediator of c-Myc functions.

In growth factor-dependent murine myeloid 32D.3 cells, c-*myc* and ODC expression requires interleukin-3 (IL-3) (2, 9). Removal of IL-3 results in a rapid loss of c-*myc* and ODC expression, and cells withdraw from the cell cycle and eventually lose viability through the induction of apoptosis (2). By contrast, enforced c-*myc* expression results in the constitutive, growth factor-independent expression of ODC, suppresses cell cycle arrest, and rapidly induces apoptosis following IL-3 withdrawal (2). In this study we tested the hypothesis that c-Myc-induced ODC expression contributes to the induction of apoptosis by c-Myc in 32D.3 myeloid cells. We demonstrate that enforced ODC expression is sufficient to induce apoptosis following IL-3 withdrawal and that ODC is a mediator of c-Myc-induced apoptosis.

MATERIALS AND METHODS

Cell culture. 32D.3 cells and c-*myc* clones have been previously described (2, 43). To generate 32D.3-derived clones constitutively expressing ODC, we utilized the ODC expression plasmid pMV7-ODC (19). pMV7-ODC contains the murine ODC cDNA under transcriptional control of the Moloney murine sarcoma virus long terminal repeats and the neomycin resistance gene under the control of the simian virus 40 promoter. 32D.3 cells were electroporated with pMV7-ODC DNA (20 μg), and G418-resistant clones were isolated by being cloned in methylcellulose containing IL-3 (40 U/ml) and G418 (active drug, 400 μg/ml). Control *neo* clones were generated in the same fashion by transfecting cells with the neomycin expression plasmid, pMCneoPolyA (Stratagene). Clones were isolated and expanded and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 U of recombinant murine IL-3 per ml, 1% (wt/vol) L-glutamine, and G418 (400 μg/ml). To ensure that all clones were analyzed when in exponential growth phase, cells were set at 0.5×10^6/ml on 2 consecutive days before analysis.

To address the effects of enforced ODC and c-*myc* expression upon viability, cells were washed twice in RPMI 1640 medium lacking IL-3 and resuspended at 10^6 cells per ml in RPMI 1640 medium lacking IL-3. Cell viability was determined

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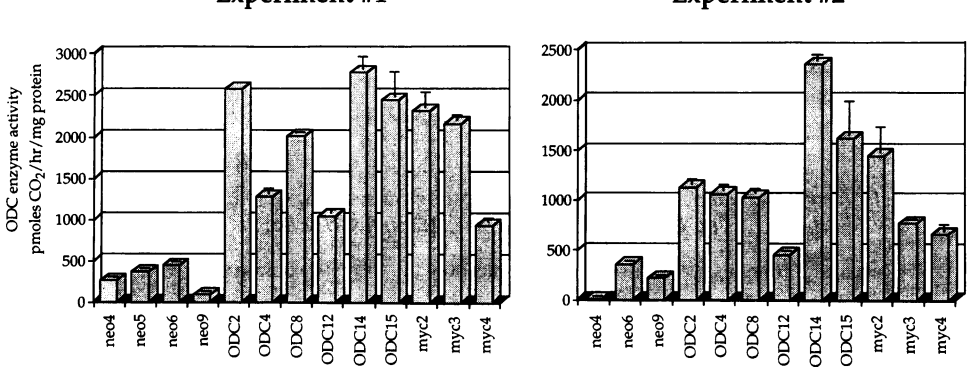

FIG. 1. ODC enzyme activity in *neo*, ODC and c-*myc* clones. Cells (in two separate experiments) were set at 0.5×10^6 cells per ml on 2 consecutive days as described in Materials and Methods. On the third day, cells were recovered and analyzed for ODC enzyme activity. Error bars are derived from the average of duplicate assays. Results shown are representative of four separate experiments.

with a hemocytometer and by trypan blue dye exclusion as an indicator of viability.

To inhibit ODC enzyme activity, cells were incubated with α-difluoromethylornithine (DFMO; kindly provided by Marion Merrill Dow Research Laboratories) at the concentrations indicated in the figure legends. In experiments to determine the effects of DFMO on rates of cell death following IL-3 withdrawal, cells were treated with DFMO for 2 h before removal of IL-3 and throughout the experiment.

ODC enzyme assays. Cells (5×10^6) were collected by centrifugation, washed twice in phosphate-buffered saline, and then resuspended in 500 μl of ODC assay buffer (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 2.5 mM dithiothreitol, 0.2 mM pyridoxal-5-phosphate). Cells were lysed by three cycles of freeze-thawing, and cell debris was removed by centrifugation for 5 min at 13,000 rpm. L-[1-^{14}C]ornithine (0.2 μCi, 57 mCi/mmol; New England Nuclear, Dupont) was then added to the cell extract (200 μl; approximately 200 μg of protein) and incubated in a sealed 6-ml polycarbonate tube for 30 min at 37°C. Trichloroacetic acid (50% [wt/vol], 200 μl) was added through a syringe in the lid, and liberated $^{14}CO_2$ was trapped on a filter suspended above the reaction mixture that had been soaked in Soluene 350 (Packard). After 30 min, filters were recovered, and radioactivity was determined by scintillation counting. ODC assays were standardized for protein content by using the Bio-Rad assay. ODC assays were performed in either duplicate or triplicate.

Apoptosis assays. Cells at the exponential phase of growth were washed twice in medium lacking IL-3 and resuspended at 10^6 cells per ml in medium lacking IL-3. Cell morphology was analyzed following cytospin and staining with Wright-Giemsa stain, and genomic DNA was isolated from 10^6 cells and analyzed on a 2% agarose gel as previously described (2).

RESULTS

Enforced expression of c-*myc* or ODC augments ODC enzyme activity. We have previously demonstrated that enforced c-*myc* expression accelerates apoptosis of 32D.3 myeloid cells following IL-3 withdrawal and that this is associated with deregulation of ODC expression (2). If ODC is to be considered a potential mediator of c-Myc function, then enforced expression of c-*myc* should also deregulate ODC enzyme activity in these cells. To address this issue, c-*myc* and control *neo* clones were set at 0.5×10^6 cells per ml (as described in Materials and Methods), in two separate experiments, and after 24 h were analyzed for ODC enzyme activity (experiments 1 and 2 [Fig. 1]). c-*myc* clones overexpressed ODC activity (3.2- to 8-fold) relative to the average of control *neo* clones (Fig. 1). Therefore, in addition to its effects on ODC RNA expression (2, 3, 11), enforced c-*myc* expression results in augmented ODC enzyme activity. Similar findings have been reported for fibroblasts harboring inducible Myc-estrogen receptor chimeric constructs (45).

To constitutively express ODC in the 32D.3 cell line, we electroporated 32D.3 cells with the pMV7-ODC retroviral expression plasmid (19) and derived individual clones (ODC clones) by cloning in methylcellulose in IL-3 and G418. Clones were expanded in liquid culture containing IL-3 and G418, and ODC RNA expression was determined. All ODC clones expressed long terminal repeat-derived ODC mRNA as determined by Northern (RNA) hybridization analysis of total RNA (data not shown). We then analyzed ODC clones for augmented expression of ODC enzyme activity relative to control *neo* clones as described above. All ODC clones displayed augmented levels of enzyme activity (2.4- to 12.3-fold) relative to those of control cells (Fig. 1).

Enforced ODC enzyme activity is sufficient to induce apoptosis. To determine whether augmented ODC activity was sufficient to induce cell death following IL-3 withdrawal, a sample of the cells from the ODC clones analyzed in the two experiments shown in Fig. 1 were deprived of IL-3 and their viabilities were examined against time and compared with that of control *neo* clones. ODC clones showed accelerated loss of viability relative to *neo* clones and parental 32D.3 cells when deprived of ligand (experiments 1 and 2 [Fig. 2A]). Moreover, rates of cell death in individual ODC clones were dose dependent with respect to ODC enzyme activity. ODC clones which expressed relatively high levels of enzyme activity died more rapidly than clones which expressed lower levels (e.g., compare ODC2 and ODC12 [Fig. 1 and 2A]). Parallel analyses of an additional 12 ODC clones revealed a similar dependence of rates of death on ODC enzyme activity (data not shown). The relative extent of ODC overexpression and the resultant rates of death of individual ODC clones did vary somewhat between experiments (e.g., see ODC4 experiment 1 versus experiment 2 [Fig. 1 and 2A]). However, ODC clones always overexpressed ODC activity and had accelerated death relative to *neo* controls. Analyses of conditioned medium from IL-3-deprived ODC clones demonstrated that they did not secrete

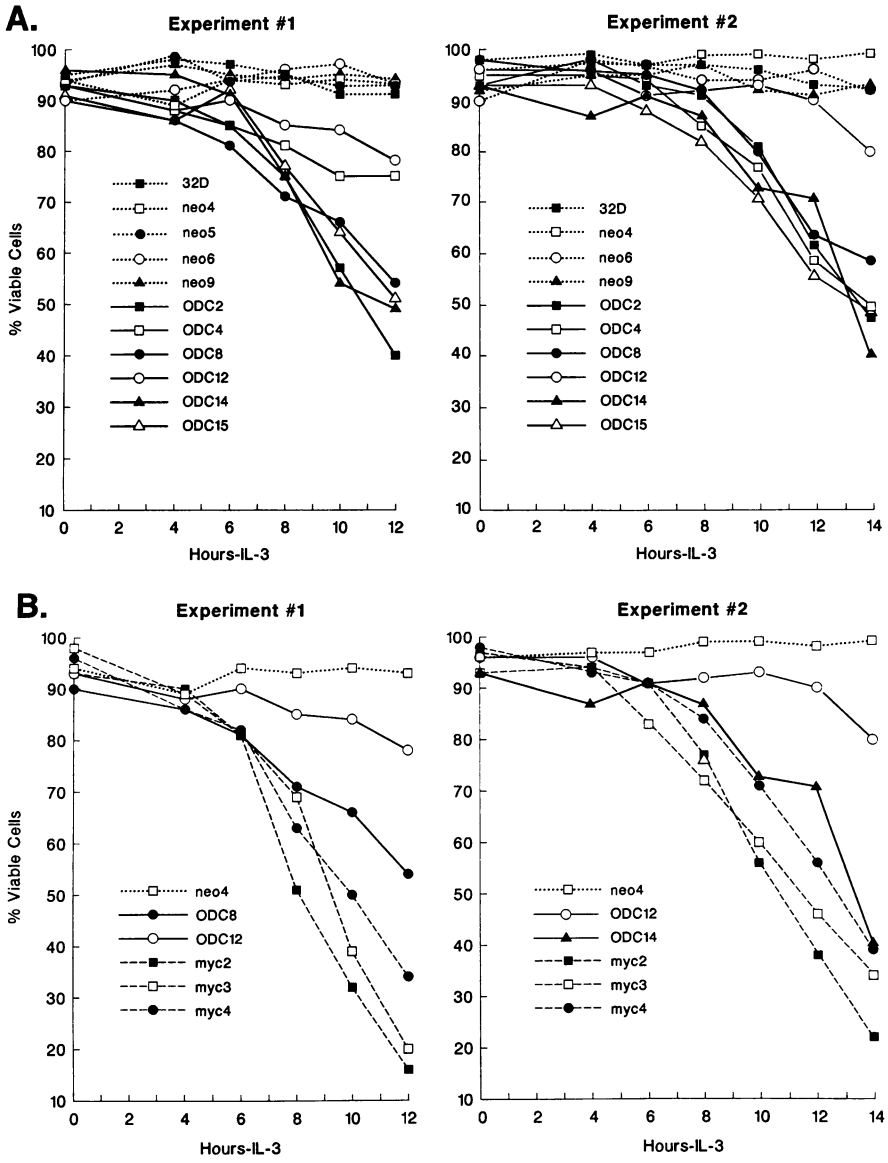

FIG. 2. Enforced expression of ODC and c-*myc* accelerate apoptosis following IL-3 withdrawal. A sample of the cells from the two experiments analyzed in Fig. 1 was thoroughly washed to remove IL-3 and resuspended in medium without IL-3. Cell viability was determined by using trypan blue dye exclusion at the indicated time points following IL-3 withdrawal. (A) Rates of cell death of ODC clones relative to control cells; (B) comparison of rates of death of c-*myc* clones and selected ODC clones with comparable ODC enzyme activities. Results are representative of more than 10 separate experiments. Additional experiments demonstrated that the error for individual viability determinations was less than or equal to 3% (30a).

factors which accelerated the death of parental 32D.3 cells (data not shown).

Enforced expression of both c-*myc* and ODC results in accelerated cell death following IL-3 withdrawal. To directly compare the rates of death of c-*myc* and ODC clones, cells analyzed for Fig. 1 were deprived of IL-3 and their viability was examined against time. The relative rates of death of individual c-*myc* clones were also dependent on their ODC enzyme activity, as c-*myc* clones with lower levels of activity died at a slower rate (cf. myc4 versus myc2 [Fig. 1 and 2B]), suggesting that ODC is an important mediator of apoptosis in these cells. However, a comparison of c-*myc* and ODC clones with equivalent enzyme activities revealed that c-*myc* clones consistently died at a faster rate (Fig. 2B, cf. myc4 versus ODC12 and myc2 versus ODC8 [experiment 1] and ODC14 [experiment 2]).

The cell death induced by enforced expression of c-*myc* following IL-3 withdrawal is due to the induction of apoptosis (2). Therefore, we analyzed whether the accelerated loss of viability of ODC clones was also due to apoptosis. ODC clones underwent distinct changes in cell morphology following IL-3 withdrawal characteristic of apoptosis, including the formation of micronuclei and apoptotic bodies (Fig. 3). Most apoptosis is accompanied by internucleosomal nicking of genomic DNA resulting in the formation of the typical "laddering" of DNA isolated from apoptotic cells. This was clearly evident in ODC clones following IL-3 withdrawal (Fig. 4), though laddering was

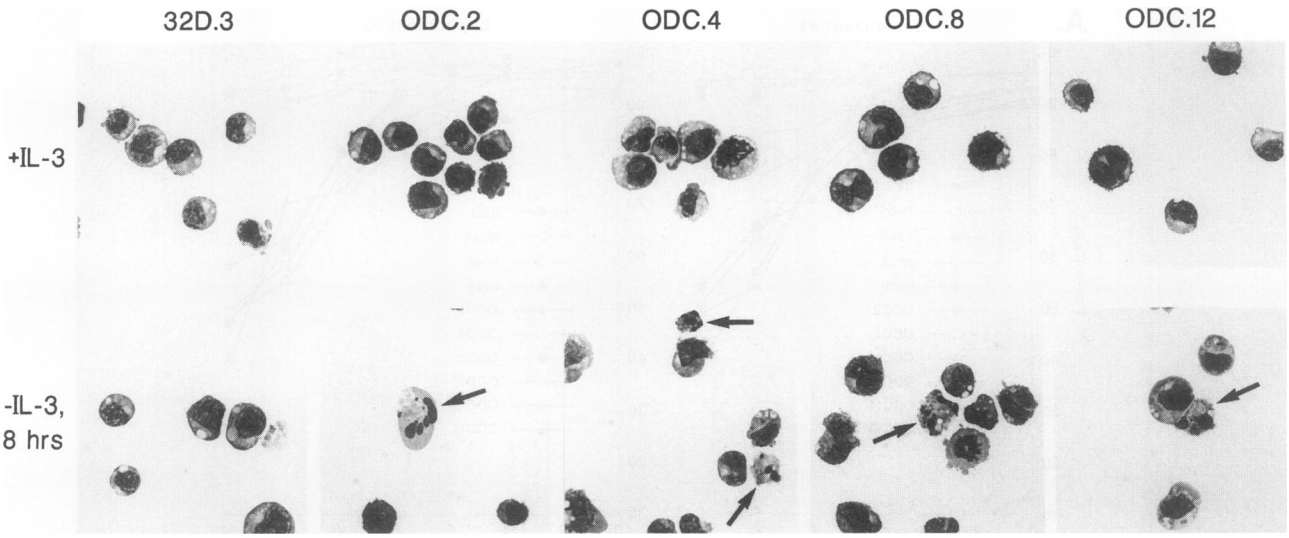

FIG. 3. Morphology of ODC clones following IL-3 withdrawal. Parental 32D.3 and ODC clones were set at 0.5×10^6 cells per ml as described in the legend to Fig. 1 on the day before the experiment. The following day cells were washed thoroughly and resuspended in growth medium without IL-3. Cytospins were prepared 8 h after removal of IL-3, and cells were stained with Wright-Giemsa stain. Cells not washed of IL-3 were analyzed as controls. Arrows indicate cells undergoing apoptosis.

more advanced in c-*myc* clones at this time, consistent with their more rapid cell death. These effects were not observed in parental 32D.3 cells (Fig. 3 and 4) or in *neo* clones (30a).

ODC is a mediator of c-Myc-induced apoptosis. To directly assess the contribution of ODC enzyme activity to c-Myc-induced apoptosis, we used DFMO, a specific, irreversible, inhibitor of ODC enzyme activity (31). To establish the effectiveness of this inhibitor in 32D.3 cells, we treated cells grown in IL-3 with DFMO and measured enzyme activity with time. DFMO rapidly inhibited ODC enzyme activity, and within 30 min only 10% of the activity seen in untreated cells remained (Fig. 5A). Therefore, DFMO is an effective enzyme inhibitor in 32D.3 cells.

To determine the effect of DFMO on rates of apoptosis following IL-3 withdrawal, parental 32D.3 cells and ODC and c-*myc* clones were treated with DFMO (as described in Materials and Methods) and their rates of death were compared with those of untreated cells (Fig. 5, two experiments shown). As expected, DFMO was an effective inhibitor of apoptosis in ODC clones, as DFMO-treated ODC clones died at rates comparable to that of parental 32D.3 cells (Fig. 5B). Importantly, DFMO also inhibited the rates of death of c-*myc* clones (Fig. 5C). DFMO inhibited cell death of c-*myc* clones for 6 h following IL-3 withdrawal, although at later time points, inhibition of rates of cell death was less complete. By contrast, the protracted loss of viability of 32D.3 cells and *neo* clones was not inhibited by DFMO (Fig. 5D and data not shown).

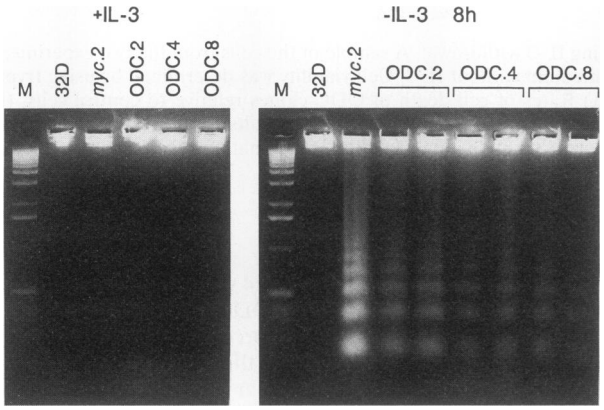

FIG. 4. Analysis of genomic DNA of ODC clones following IL-3 withdrawal. Parental 32D.3, ODC, and c-*myc* clones were set at 0.5×10^6 cells per ml as described in the legend to Fig. 1. The following day, cells were washed thoroughly and resuspended in growth medium without IL-3. Genomic DNA was analyzed at 8 h following IL-3 withdrawal. Cells not washed of IL-3 were analyzed as controls. Duplicate cultures of ODC cells deprived of IL-3 were analyzed. Markers (lanes M) are 1-kb DNA ladders (GIBCO).

DISCUSSION

Enforced c-*myc* expression in 32D.3 myeloid cells induces apoptosis and the constitutive, growth factor-independent, expression of ODC (2, 3). c-Myc appears to directly regulate expression of ODC (2, 5, 45), and here we have demonstrated that c-Myc regulation of ODC contributes to the induction of apoptosis by c-Myc. ODC is generally associated with cell proliferation, as it is required for cell cycle progression (8) and for the morphologic transformation of fibroblast cells by the v-*src* tyrosine kinase oncogene (4). More recently, however, the precise regulation of ODC enzyme activity has been proposed to prevent ODC-induced cell toxicity (29). Our data clearly support this concept, since under conditions in which ODC expression is normally down-regulated, enforced ODC enzyme activity is sufficient to induce apoptosis. ODC, like c-Myc, is therefore associated with both cell growth and cell death. Moreover, rates of death of ODC clones were a function of their ODC enzyme activity, as clones with higher levels of ODC activity always died at faster rates. Thus, similar to the effects of c-Myc in fibroblast cells (15), rates of apoptosis induced by ODC are also dose dependent.

Two lines of evidence suggest that ODC is downstream from c-Myc in the induction of apoptosis. First, a specific and irreversible inhibitor of ODC enzyme activity, DFMO, inhibits

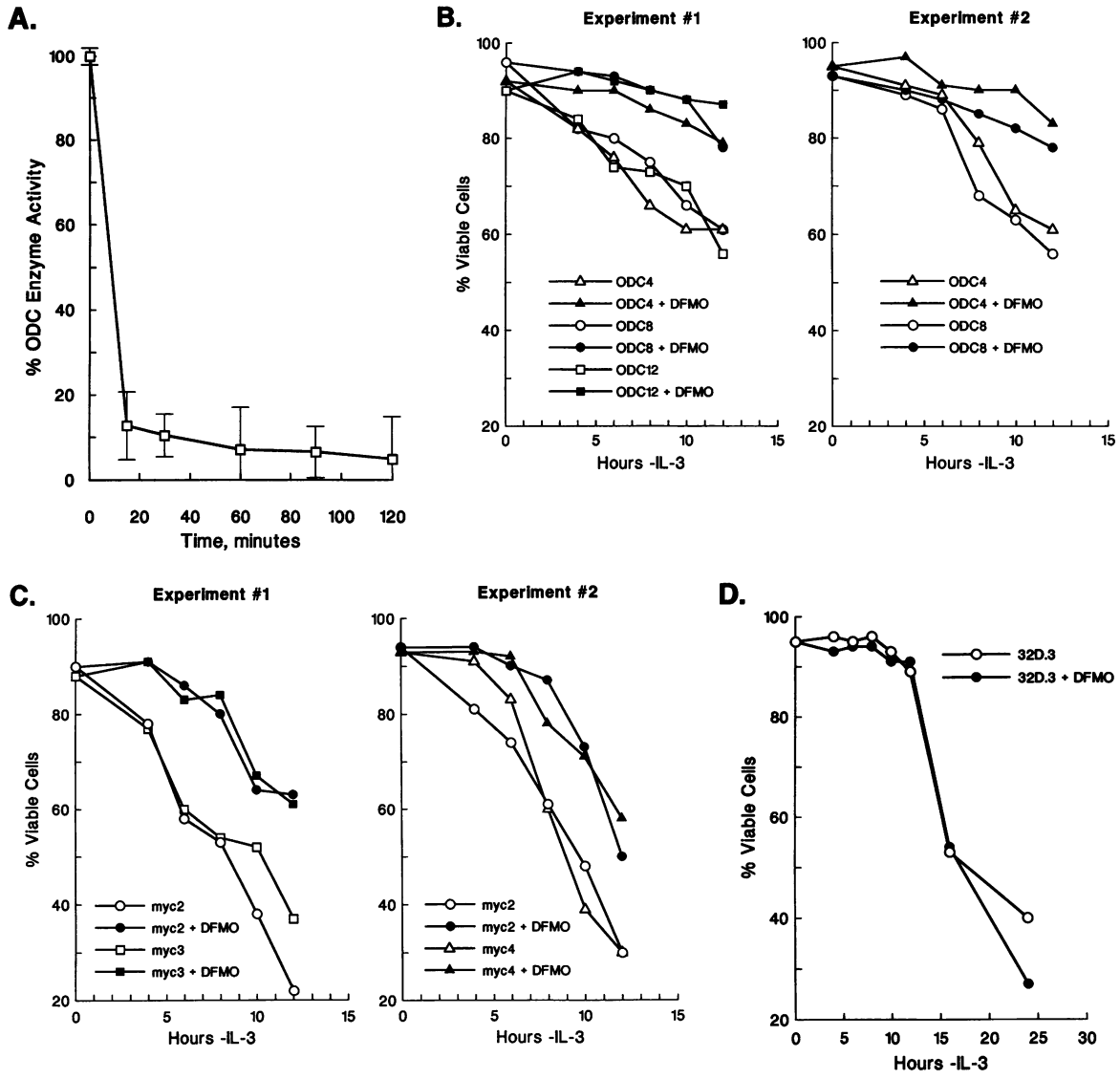

FIG. 5. Effects of the ODC enzyme inhibitor DFMO on ODC enzyme activity (A) and the rates of cell death of ODC (B) and c-*myc* clones (C) (two experiments) and parental 32D.3 cells (D) following IL-3 withdrawal. (A) Exponentially growing 32D.3 cells were treated with DFMO (10 mM) and analyzed for ODC enzyme activity at the indicated times. (B to D) Cells were set at 0.5×10^6 cells per ml on 2 consecutive days as described in Materials and Methods. On the third day, cells were treated with DFMO (10 mM) for 2 h before being deprived of IL-3 and after ligand removal, and their rate of loss of viability was compared with that of IL-3-deprived cells not treated with DFMO. Similar results were obtained in four separate experiments.

the rate of c-Myc-induced apoptosis. Second, the accelerated apoptosis in ODC clones is not c-Myc dependent, as c-*myc* expression cannot be detected in these cells following IL-3 withdrawal (30a). Therefore, the results demonstrate that ODC is an effector of c-Myc-induced apoptosis. Importantly, this is the first demonstration that a gene shown to be a direct transcriptional target of c-Myc mediates a c-Myc function, proving that c-Myc transcriptional activity is relevant to its biologic effects.

The results also strongly suggest that there are other c-Myc mediators, in addition to ODC, which participate in the induction of apoptosis. A direct comparison of rates of death of ODC and c-*myc* clones expressing equivalent amounts of ODC enzyme activity demonstrated that c-*myc* clones always died more rapidly. Therefore, the rapid rate of death of c-*myc* clones cannot solely be accounted for by ODC levels. In addition, DFMO treatment did not completely inhibit apoptosis in c-*myc* clones but was effective at inhibiting the death of ODC clones over the same time period. Thus, the induction of apoptosis by c-Myc involves both ODC-dependent and ODC-independent pathways.

Clearly our data support the emerging hypothesis that apoptosis can be induced by multiple, independent pathways (2, 34, 37, 47). Although either c-Myc or ODC is sufficient to induce apoptosis, the induction of apoptosis in parental 32D.3 cells is likely independent of both c-Myc and ODC, as their expression is rapidly down-regulated prior to the commitment of these cells to apoptosis, and DFMO did not alter their rate of death. c-Myc-dependent and -independent pathways of apoptosis have been similarly demonstrated with T cells (37).

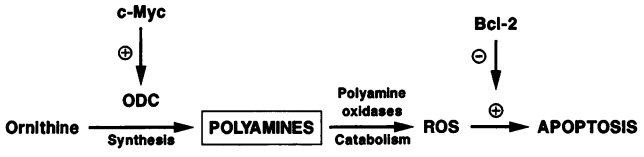

FIG. 6. A speculative model for c-Myc-induced apoptosis. c-Myc-induced ODC enzyme activity would be predicted to increase both synthesis and catabolism of intracellular polyamine pools. Excess polyamines are catabolized by intracellular polyamine oxidases, resulting in the production of ROS, which mediate apoptosis. Bcl-2 prevents ROS-mediated damage and therefore inhibits c-Myc-induced apoptosis.

However, apoptosis initiated by each pathway is accompanied by apparently identical morphological changes, suggesting that each pathway feeds in to a common effector.

DFMO-mediated cell cycle arrest of 32D.3 cells (and other cell types) by polyamine depletion requires over 3 days of treatment with the inhibitor and must be performed in medium containing serum depleted of polyamines (27, 30a, 40). Under these conditions, the effect of DFMO is reversed by the addition of putrescine, as cells can recover polyamines from the medium (4, 8). However, the rates of death of c-*myc* and ODC clones are inhibited by a short treatment with DFMO, even when performed in complete medium. Therefore, in contrast to the effect of DFMO on cell cycle, inhibition of rates of cell death is unlikely to be mediated by simple depletion of intracellular polyamine pools. In agreement with this concept, we have not been able to reverse the beneficial effects of DFMO on cell survival of ODC and c-*myc* clones by the addition of putrescine (30a).

These observations have led us to propose the following speculative model (Fig. 6). We suggest that c-Myc-induced ODC enzyme activity results in the generation of reactive oxygen species (ROS) which mediate apoptosis. Augmented ODC enzyme activity would result in the increased rate of synthesis of polyamines. Intracellular polyamine levels are tightly regulated, and to offset this increased rate of synthesis, excess polyamines might be catabolized by intracellular polyamine oxidases (38). There are at least three polyamine oxidases present in cells (38), all of which may generate ROS in response to elevated polyamine levels, resulting in apoptosis. We speculate that, in our system, DFMO does not significantly deplete polyamines but rather inhibits excess rates of synthesis and catabolism of these compounds. We have not found evidence for serum amine oxidase, which is present in fetal calf serum (42), as a mediator of cell death, as the accelerated rates of death of ODC clones were not influenced by treatment with the serum amine oxidase inhibitor aminoguanidine and were comparable in serum-free medium (30a). Bcl-2 prevents many forms of apoptosis (20–22, 30, 44), including c-Myc-induced apoptosis (6, 16), and can function as an antioxidant to protect cells from apoptosis initiated by the activity of ROS (21, 22). Bcl-2 would prevent cell damage from intracellular ROS and thus block apoptosis. The role of intracellular oxidases in c-Myc-induced apoptosis is currently being evaluated.

We have shown that ODC is an effector of c-Myc-induced apoptosis. Both c-*myc* and ODC cooperate with activated *ras* in transformation of fibroblasts (19, 24, 25, 39), and as with c-Myc (18), ODC is required for entry into the S phase of the cell cycle (8, 30a). Furthermore, the transformation of fibroblasts by tyrosine kinase oncogenes, which has been shown to be c-Myc dependent (36), is inhibited by treatment with DFMO (4). Therefore, in addition to its role in c-Myc-induced apoptosis, these observations suggest that ODC mediates additional c-Myc functions.

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