Exposure to Ornithine Results in Excessive Accumulation of Putrescine and Apoptotic Cell Death in Ornithine Decarboxylase Overproducing Mouse Myeloma Cells

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Abstract
Ornithine decarboxylase (ODC) is the first key enzyme in the biosynthesis of polyamines, aliphatic polycations that are indispensable for the process of mammalian cell proliferation. The mouse myeloma cell line, 653-1, massively overproduces ODC due to the amplification of an active ODC gene. The addition of ornithine to the growth medium of 653-1 cells results in a massive increase in the intracellular concentration of putrescine, followed by rapid cell death. Ornithine-treated 653-1 cells display fragmented nuclei, chromatin condensation, and an oligonucleosome-sized DNA "ladder"; consequently, their death can be described as apoptosis. Accumulation of putrescine in 653-1 cells is accompanied by a rapid decrease of protein synthesis activity, suggesting that protein synthesis inhibition may be the cause for the apoptotic death of 653-1 cells. However, since the apoptotic death provoked by exposure of 653-1 cells to ornithine reached a maximal level earlier than that caused by cycloheximide, we conclude that protein synthesis inhibition is unlikely to be the direct cause of the observed apoptotic cell death.

Introduction
The naturally occurring polyamines, spermidine, spermine, and their precursor putrescine, are aliphatic polycations present ubiquitously in prokaryotic and eukaryotic cells. Although their absolute requirement in the process of cellular proliferation is well established (1-5), their explicit role in this process, and in other cellular functions, is still obscure. The intracellular concentration of the polyamines is highly regulated by the modulation of enzymes that are involved in both their synthesis and catabolism (6). The two highly regulated enzymes, ODC and S-adenosylmethionine decarboxylase, constitute the control points in the biosynthesis pathway (4, 5), whereas the catabolic pathway is controlled predominantly by the activity of spermidine/spermine N\textsuperscript{1}-acyetyltransferase (7, 8). The concerted action of these enzymes maintains an optimal concentration of polyamines in cells under varying physiological conditions. Under basal conditions, the intracellular concentration of putrescine is usually lower than that of the polyamines spermidine and spermine (4, 5, 9). This is consistent with the widely accepted assumption that the main role of putrescine is to serve as the precursor of spermidine. The range of cellular polyamine concentrations is determined at the lower limit by their absolute requirement for cellular proliferation and at the upper limit by their potential toxicity.

Apoptosis is a normal process involved in the elimination of specific cells during embryonic development and in adult life (10-14). Cells undergoing apoptosis shrink and display nuclear condensation and fragmentation; the DNA within these nuclei is susceptible to cleavage by a nuclease that cleaves chromatin between individual nucleosomes, yielding a typical DNA "ladder." Apoptosis can be induced by a number of physiological and pathological stimuli (10, 15). Induction of apoptosis, and its cellular sensitivity, can be regulated by several cellular genes. For instance, bcl-2 protects cells from induction of apoptosis (16-23), whereas expression of wild-type p53 (24-32) and deregulation of c-myc (33-41) both induce apoptosis.

Here we report that exposing ornithine decarboxylase-overproducing cells to ornithine results in a rapid accumulation of putrescine and induction of cell death. The death of these cells is accompanied by cell shrinkage, nuclear condensation and fragmentation, and DNA degradation into oligonucleosome-length fragments. It can, therefore, be characterized as apoptosis.

Results
Exposure of Mouse Myeloma Cells That Overproduce ODC to Ornithine Results in Their Rapid Killing. We described previously the selection of the mouse myeloma cell line, 653-1, that massively overproduce ODC (42-44). Addition of ornithine (5 mM) to the growth medium of 653-1 cells resulted in massive cell death, whereas the viability of the parental 653 cells remained unaffected (Fig. 1). The selective killing of 653-1 cells by ornithine was rapid; mortality was 20% 4 h after addition and reached 90% by 8 h (Fig. 1). These results suggest that the increased ability of 653-1 cells to metabolize ornithine may result in the accumulation of toxic levels of polyamines in these cells.

Exposure to Ornithine Induces Apoptosis in 653-1 Cells. Cells that undergo apoptosis are characterized by a distinct morphology and a typical pattern of DNA fragmentation (DNA ladder) that results from cleavage of chromosomal DNA in internucleosomal segments (10, 16, 45, 46). To determine whether ornithine induces apoptotic cell death, these features were investigated in 653 and 653-1 cells at various times, following the addition of ornithine. Typical cell shrinkage, nuclear fragmentation, and chromatin condensation were observed in 653-1 within 2 h following their exposure to ornithine (Fig. 2). After 4 h, massive cellular collapse was already observed. Moreover, exposure to ornithine also results in the appearance of the characteristic DNA ladder in 653-1 cells (Fig. 3). None of these apoptotic

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: ODC, ornithine decarboxylase; α-DFMO, α-difluoromethylornithine.

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features was observed in the parental 653 cells (Figs. 2 and 3). We, therefore, conclude that the ornithine-induced death of 653-1 cells can be characterized as apoptosis.

**Exposure to Ornithine Results in Massive Accumulation of Putrescine in 653-1 Cells.** As mentioned above, the selective ability of ornithine to induce apoptotic death of 653-1 cells, but not of the parental 653 cells, suggests that this death may be a result of massive conversion of ornithine to putrescine and/or to the polyamines spermidine and spermine. To determine whether this is indeed the case, polyamine concentrations in 653 and 653-1 cells were determined prior to and following exposure to ornithine. As might have been expected from the lack of effect on their viability, the polyamine concentration in 653 cells was practically unaffected (Fig. 4). As demonstrated in the ODC-overproducing mouse leukemia L1210 cells (47), the basal concentration of putrescine in 653-1 cells (13 nmol/mg protein) was significantly higher then in 653 cells (1.04 nmol/mg protein; Fig. 4). Following the exposure of 653-1 cells to ornithine, the concentration of putrescine increased linearly (during the first hour), reaching 16-fold induction after 1 h (213 nmol/mg protein; Fig. 4). Interestingly, while the concentration of putrescine increased, that of spermidine and spermine remained unchanged (34 and 5.3 nmol/mg protein, respectively; Fig. 4). Cadaverine (represented in Fig. 4 as the spot migrating slightly faster than putrescine), the product of the low-affinity decarboxylation of lysine by ODC (47, 48) is also present in 653-1 cells (at a concentration similar to that of putrescine) but not in 653 cells. In fact, exposure of 653-1 cells to lysine results in the accumulation of cadaverine and in the induction of cell death. However, because the affinity of ODC towards lysine is 100-fold lower then its affinity towards ornithine (48), higher lysine concentrations were required to induce 653-1 cell death. We have attempted to determine whether exposure of 653 and 653-1 cells to putrescine will provoke apoptotic death by adding varying concentrations of putrescine to their growth medium. However, since these cells uptake putrescine very poorly, an extremely high concentration of putrescine (100 mM or higher) was required to induce mortality. Under these conditions, only a minor increase in the intracellular concentration of putrescine was noted (putrescine concentration in 653 cells exposed to 100 mM putrescine was similar to that measured in untreated 653-1 cells). We, therefore, conclude that in this case, cell death is provoked by a mechanism that is different from that manifested following the exposure of 653-1 cells to ornithine.

**The Death of 653-1 Cells Is Not a Result of Toxic Compound Accumulation in the Growth Medium.** Lack of accumulation of spermidine and spermine in the ornithine-treated 653-1 cells does not necessarily mean that these polyamines are not overproduced. Excessive spermidine and spermine may be secreted to the growth medium. In the medium, they can be oxidized by serum amine oxidase to form hydrogen peroxide and aldehydes, compounds that were demonstrated to provoke cell death (49, 50). Three experimental approaches have been used to challenge this possibility: (a) aminoguanidine and β-mercaptoethanol, two potent inhibitors of serum amine oxidase (50, 51), were added to the growth medium of 653-1 cells together with ornithine. Both inhibitors failed to inhibit cell death (Fig. 5A); (b) medium of 653-1 cells was collected 3 h following the addition of ornithine. This conditional medium failed to provoke apoptotic death of 653 cells (Fig. 5B); and (c) we have noted that some 653-1 cells survived the ornithine treatment. Southern analysis performed on genomic DNA isolated from cultures derived from such surviving cells have demonstrated deamplification of the ODC gene (Fig. 5C). Therefore, we concluded that the ornithine-induced death of 653-1 cells is not a consequence of the generation of toxic compounds in the growth medium due to oxidation of secreted polyamines and that survival of individual cells is a direct result of losing their ability to excessively metabolize ornithine.

**Protein Synthesis Inhibition Due to Excessive Accumulation of Putrescine Is Not the Cause of the Apoptotic Death of 653-1 Cells.** Studies using reticulocyte lysate demonstrated that excessive amounts of polyamines dramatically reduce protein synthesis activity (52). Moreover, while in most cell systems apoptosis appears to be an active process that requires de novo RNA and protein synthesis (12, 13, 45, 53, 54), several recent studies have demonstrated that ongoing macromolecular synthesis is not always essential for the induction of apoptosis. In fact, induction of apoptosis by inhibition of macromolecular synthesis has been described in several cellular systems, including myeloma cells (23, 55–58). To determine whether the massive intracellular accumulation of putrescine affected protein synthesis, 653 and 653-1 cells were pulse-labeled with [35S]methionine at various times, following their exposure to ornithine. This has demonstrated that the accumulation of putrescine in 653-1 cells was accompanied by a rapid inhibition of protein synthesis (Fig. 6). Since the addition of ornithine to the growth medium does not inhibit protein synthesis in 653 cells and in α-DFMO-treated 653-1 cells (data not shown), we conclude that the accumulation of putrescine is the cause for the observed inhibition of protein synthesis activity in these cells. This result may suggest that ornithine induced the apoptotic death of 653-1 cells by provoking protein synthesis inhibition. To directly test this possibility, 653 and 653-1 cells were treated with cycloheximide (20 μg/ml), and their viability was determined at

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4 E. Mammroud-kidron and C. Kahana, unpublished results.
Fig. 2. Apoptotic features in ornithine-treated 653-1 cells. Ornithine (5 mM) was added to the growth medium of 653 and 653-1 cells. At the indicated times, cyto-centrifuged preparations of the cells were fixed, stained by May-Grünwald-Giemsa (A) or 4,6-diamidino-2-phenylindole (B), and photographed at a magnification of x 1000.

Fig. 3. DNA fragmentation in ornithine-treated 653-1 cells. Total DNA was isolated from 653 and 653-1 cells (as described in "Materials and Methods") at the indicated times, following the addition of ornithine (5 mM) to their growth medium. The isolated DNA was fractionated by agarose gel electrophoresis. Left, positions of molecular weight markers (in kb).

Fig. 4. Polyamine analysis in ornithine-treated 653 and 653-1 cells. Extracts of ornithine-treated 653 and 653-1 cells were prepared, and the polyamines were dansylated, as described in "Materials and Methods." The dansylated derivatives were fractionated on a G-50 silica plate using ethyl acetate/cyclohexane as a solvent system. Dansylated derivatives of ornithine and putrescine, as well as the polyamines, spermidine and spermine, were used as markers. The duplicate spot comigrating with the putrescine marker represents cadaverine (faster migrating) and putrescine (slower migrating). The plate was photographed under UV illumination. The concentration of the polyamines was determined using a fluorimeter, as described in "Materials and Methods," and expressed as nmol polyamines/mg of protein.

Various times thereafter. As shown in Fig. 7A, cycloheximide provoked the death of both cell types. However, since cycloheximide blocked protein synthesis more rapidly than ornithine (Fig. 6), yet it was slower then ornithine in inducing cell death (Fig. 7A), we conclude that the inhibition of protein synthesis is not the mechanism by which ornithine treatment causes the apoptotic death of 653-1 cells.

Interestingly, although cycloheximide provoked the death of both cell types, it was significantly more potent in killing 653-1 cells (Fig. 7A). The difference between the two cell lines is further emphasized by the 2-h delayed appearance of the apoptotic DNA ladder in 653.
Fig. 5. The ornithine-induced apoptotic death of 653-1 cells is a result of intracellular toxicity. A, 653-1 cells were treated for 8 h with 5 mM ornithine in the presence of 1 mM aminoguanidine or 400 μM β-mercaptoethanol, two potent inhibitors of serum amine oxidase. Cell viability was determined by trypan blue staining. The results are an average of three independent experiments. B, conditional medium was collected from 653-1 cells 3 h following their exposure to ornithine, a stage at which major apoptotic features have been observed. This medium (which contains the added ornithine) was applied to 653 and 653-1 cells, and their viability was determined 8 h thereafter by trypan blue staining. The results are an average of three independent experiments. C, 653-1 cells were treated with ornithine. Foci developed from surviving cells (653-1R) were collected and expanded; then genomic DNA was prepared. The DNA was digested with EcoRI, fractionated in agarose gel (parallel to DNA that was isolated from 653 and 653-1 cells), transferred to nitrocellulose, and probed with radiolabeled ODC cDNA. Left, position of molecular weight markers (in kilobase pairs).

Fig. 6. Inhibition of protein synthesis in ornithine- and cycloheximide-treated 653 and 653-1 cells. 653-1 and 653-1 cells were treated by the addition of 5 mM ornithine or 20 μg/ml cycloheximide. At the indicated times, the cells were pulse-labeled (for 5 min) with [35S]methionine. Incorporation of [35S]methionine into proteins was determined by measuring radioactivity in the trichloroacetic acid precipitated material. The presented data represent an average of three independent experiments; bars, SD.

Discussion

Our present study demonstrates that excessive intracellular accumulation of putrescine in mouse myeloma cells is lethal. We demonstrate here that the addition of ornithine to the growth medium of the mouse myeloma 653-1 cells that massively overproduce ODC (42) results in apoptotic cell death, whereas the viability of the parental 653 cells remains unaffected. This differential effect is a result of the increased ability of 653-1 cells to metabolize ornithine, leading to a rapid and massive accumulation of putrescine. Interestingly, while putrescine accumulated in 653-1 cells to very high levels, the intracellular concentration of the polyamines, spermidine and spermine, remained unchanged.

We noted that exposure of 653-1 cells to ornithine and the subsequent accumulation of putrescine lead to a rapid cessation of protein synthetic activity in these cells. Since it was recently demonstrated that inhibition of gene expression induces apoptotic death of B-cell hybridomas (23, 59), we tested whether the putrescine-induced inhibition of protein synthesis in 653-1 cells may be the direct cause of their apoptotic death. Our analysis has demonstrated that while cycloheximide blocked protein synthesis more rapidly than ornithine, it was slower then ornithine in inducing the death of 653-1 cells. This would suggest that ornithine-induced death may be independent of its effect on translation. Actually, as previously demonstrated (60, 61), inhibition of macromolecular synthesis may be a part of the cell death program.

Polyamines have been associated with both the induction of cell death (49–51, 62) and cell survival (63, 64). Spermine, and to a lesser extent spermidine (but not putrescine), were demonstrated to prevent endonuclease activation and

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cells compared to 653-1 cells ([Fig. 7B]). This suggests that the high basal concentration of putrescine (or the presence of cadaverine) in 653-1 cells may act synergistically with cycloheximide in inducing cell killing. In support of this proposed synergistic action is the identical kinetics of the DNA ladder appearance in 653 and α-DFMO-treated 653-1 cells, following their exposure to cycloheximide (data not shown).
apoptosis in thymocytes, probably by modulating chromatin structure (63). An increase in the intracellular putrescine pool was demonstrated to be essential for the growth of L1210 mouse leukemia cells under conditions of hypoosmotic stress (64). In contrast, intracellular overaccumulation of spermidine, due to its enhanced uptake in the ODC-overproducing L1210 mouse leukemia cells under hypoosmotic stress, evokes a lethal effect (51), which was recently characterized as apoptosis. This lethal effect results only partially from oxidation of N\[^\text{1}\] acetylspermidine, which is produced due to the induction of spermidine/spermine N\[^\text{1}\] acetyltransferase activity (51). Finally, catabolism of spermidine and spermine by amine oxidases generating hydrogen peroxide, aminoaldehydes, and lower order amines in the growth medium and in blastocysts fluid was demonstrated to cause cellular toxicity (49, 60, 62). In these two cases, apoptosis was suggested as the underlying mechanism. Recently, following the demonstration that the ODC gene is a potential target for transactivation by c-Myc (65, 66), it was shown that ODC stimulation mediates the induction of apoptosis by c-Myc (67). As we show here, excessive accumulation of putrescine in mouse myeloma cells results in their apoptotic death. Accumulation of putrescine to such high levels as reported here may displace the polyamines, spermidine and spermine, from their cellular target sites without substituting for their functions. Displacement of polyamines from the cellular protein synthetic apparatus may account for the observed inhibition of protein synthesis. Their displacement from other cellular sites may make an additional contribution to the induction of cell death. In addition, intracellular production of oxidative products of transiently increased concentration of polyamines may also contribute to the establishment of apoptotic features in these cells. Although our results suggest that the accumulated putrescine directly induces apoptotic death, they do not exclude the possibility that products of further metabolism of putrescine is the cause of death.

Deregulated polyamine metabolism may play an important role in carcinogenesis. In fact, there is evidence that an increase in ODC activity is a common property of tumor promotion and that high ODC activity is required to maintain rapid tumor growth (9, 68). These and other observations have led to intensive investigation of the potential use of polyamine biosynthesis inhibitors as therapeutic agents of various proliferative disorders, including cancer (9). The ability of selective accumulation of putrescine in cells exhibiting increased ODC activity by a simple administration of ornithine may be used as an alternative therapeutic approach. As we show here, massive overaccumulation of putrescine is cytotoxic, leading to rapid apoptotic cell death. Moreover, even moderate accumulation of putrescine can synergize with other apoptosis-inducing agents such as cycloheximide. It will be of great interest to determine whether treatment with ornithine, leading to a moderate accumulation of putrescine, will increase the ability of standard chemotherapeutic drugs to kill those malignant cells that exhibit increased ODC activity.

**Materials and Methods**

**Cells and Cell Culture Conditions.** The mouse myeloma ODC-overproducing cell line 653-1 (42) was selected by culturing mouse myeloma 653 cells in the presence of increasing concentrations of the ODC suicide inhibitor, α-DFMO (42). The cells were cultured at 37°C in DMEM containing 10% bovine calf serum (HyClone) and 20 mM α-DFMO. Before experiments, cells were transferred to a medium lacking α-DFMO for at least 7 days. Where indicated, the culture medium was supplemented with ornithine (5 mM) or cycloheximide (20 μg/ml).

**Assays for the Determination of Apoptosis.** Cells were treated as described in each experiment. Cell viability was determined by trypan blue staining. The percentage of viability was determined by comparing the number of viable cells in treated cultures to the number of cells in a parallel, untreated culture. The appearance of apoptotic cells was determined at the indicated times by inspecting May-Grünwald-Giemsa-stained or 4,6-diamidino-2-phenylindole-stained cyt centrifuge preparations. Apoptotic cells were smaller and contained condensed chromatin and fragmented nuclei. DNA fragmentation was determined by isolating DNA from treated and untreated cells using the G NOME DNA isolation kit (Bio 101, La Jolla, CA). Equal portions of DNA were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

**Polyamine Determination.** Polyamines were determined essentially as described by Seiler (69). Cells were collected by centrifugation and resuspended in 500 μl PBS; perchloric acid was then added to a final concentration of 3%. Insoluble material was removed by centrifugation for 5 min in an Eppendorf centrifuge, and 400 μl dansyl chloride (30 mg/ml prepared in acetone) were mixed with a 200-μl portion of the supernatant. Following the addition of 20 mg

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5 R. Poulin and A. E. Pegg, personal communication.
sodium carbonate, the mixture was incubated in the dark. After 12 h of incubation, 100 μl proline (100 mg/ml) were added, and the mixture incubated for an additional hour. Dansylated derivatives were then extracted into 0.5 ml toluene. Portions of 50–100 μl were spotted on silica G-50 plates, and the dansylated derivatives resolved using ethyl acetate/cyclohexane (2/3) as a solvent, with dansylated derivatives of known polyamines serving as markers. The individual polyamines were visualized by UV illumination, scraped off the plate, and extracted into ethyl acetate. Their fluorescence was determined using a fluorometer, at an excitation of 360 nm and emission of 510 nm. The perchloric acid pellet was dissolved in 1 N NaOH, and the protein concentration was determined by the Bradford method (70). The concentration of the polyamines is expressed relative to protein concentration.

**Monitoring Protein Synthesis.** Cells were treated with 5 mM ornithine or 20 μg/ml cycloheximide. Following the indicated times of treatment, the cells were labeled for 5 min with [35S]methionine (200 μCi/ml; 1000 Ci/mmol; Amersham). The cells were then lysed in lysis buffer (100 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% Triton X-100, and 0.1% SDS) by three cycles of freezing and thawing. Rates of protein synthesis were measured by determination of radioactivity in the trichloroacetic acid-insoluble material.

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**References**


