Biochemical and Biophysical Research Communications **260,** 256–264 (1999)
Article ID bbrc.1999.0852, available online at http://www.idealibrary.com on IDEAL®

Apoptosis and Mitotic Arrest Are Two Independent Effects of the Protein Phosphatases Inhibitor Okadaic Acid in K562 Leukemia Cells

Ana Lerga,* Carlos Richard,† M. Dolores Delgado,* Matilde Cañelles,* Pilar Frade,* M. Angeles Cuadrado,† and Javier León*,[1]

**Grupo de Biología Molecular del Cáncer, Departamento de Biología Molecular-Unidad Asociada al Centro de Investigaciones Biológicas, Facultad de Medicina, Universidad de Cantabria, Santander, Spain; and †Servicio de Hematología, Hospital Universitario Marqués de Valdecilla, Santander, Spain*

Received May 10, 1999

Treatment of human myeloid leukemia K562 cells with the serine/threonine protein phosphatases inhibitor okadaic acid induced mitotic arrest followed by apoptosis in a synchronized manner. The effect was observed at drug concentrations that inhibited the protein phosphatase type 2A but not type 1. We investigated whether apoptosis was a consequence of the preceding mitosis arrest or was induced independently by okadaic acid. We found that (1) apoptosis, but not mitotic arrest, was inhibited in cells with constitutive expression of Bcl-2; (2) pretreatment of cells with the DNA synthesis inhibitor hydroxyurea blocked the mitotic arrest but not the apoptosis mediated by okadaic acid; (3) down-regulation of c-*myc* gene was associated with apoptosis, but not with mitotic arrest; and (4) inhibition of protein synthesis abrogated mitotic arrest, but not apoptosis. The results suggest that inhibition of protein phosphatase 2A by okadaic acid provokes mitotic arrest and apoptosis of leukemia cells by independent mechanisms. © 1999 Academic Press

The molecular mechanisms involved in execution of apoptosis and mitosis are different, but they share many morphological changes such as chromatin condensation and molecular events as the induction of mitotic cyclins and unscheduled activation of cyclin-dependent kinases, observed in some apoptosis models (1–5). However, apoptosis can take place in the absence of $p34^{cdc2}$ activation in other systems (6–8). Although it has been established that prolonged cell arrest in mitosis by drugs (e.g. taxol, vinblastine and colcemide) leads to apoptosis, whether the apoptosis is a necessary consequence of a mitotic arrest or whether both effects are triggered by independent mechanisms is still under controversy.

Okadaic acid (OA) is an inhibitor of the serine/threonine protein phosphatase types 1 (PP1) and 2A (PP2A), the IC_{50} for PP2A being 0.1-1 nM, while for PP1 it is 100 times higher (9). OA has been shown to induce mitotic arrest or premature chromosome condensation in different of cell types. These include mouse oocytes and embryos (10, 11), HeLa cells (12, 13), fibroblasts (14), pituitary cells (15), spermatocytes (16), neuronal cells (17, 18), myeloid cells (19) and lymphocytes (20). It must be noted that most of these data were obtained with OA concentrations much higher than the IC_{50} for PP2A and PP1, which may result in pleiotropic effects, thus making a mechanistic explanation of OA activity in those models difficult.

OA has been reported to inhibit apoptosis in some instances (21–22). However, OA induces apoptosis in a wide variety of human and rodent cell lines (17–20, 23–25). An obvious possibility is that mitotic arrest is a prerequisite for the OA-mediated apoptosis, which would occur after an abortive mitotic attempt. The mechanism by which OA provokes mitotic arrest is not well understood, although PP2A activity appears to be required for the metaphase-anaphase transition, an effect thought to be mediated through Cdc25A phosphatase activation (reviewed in 26). Another mechanism recently proposed for *Xenopus* eggs is that OA induces an activity that prevents cyclin B ubiquitination and subsequent degradation, thus blocking exit from mitosis (27).

The K562 cell line was derived from a human chronic myeloid leukemia in blastic crisis and is very resistant

[1] To whom correspondence should be addressed at Departamento de Biología Molecular, Facultad de Medicina, 39011 Santander, Spain. Fax: 34-942-201945. E-mail: leonj@medi.unican.es.

0006-291X/99 $30.00

Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

to treatments that induce apoptosis in other human myeloid leukemia cell lines (28–31). Possible reasons for this resistance to apoptosis are the lack of p53 (32) and the expression of the Bcr-Abl kinase (29). However, OA induces apoptosis in K562 (33, 34) and we have shown that the OA-mediated apoptosis of K562 takes place concomitantly with the inhibition of PP2A—the most sensitive phosphatase isoform—but not PP1 (34). Interestingly, it has been reported that OA also induces mitotic arrest in K562 (35), although the temporal and mechanistic relationship between both phenomena has yet to be established. Therefore, the K562 model system is very well suited to investigate the connection between abortive mitosis and apoptosis. In the present paper we show that in K562 cells OA-induced mitotic arrest precedes the onset of apoptosis, but that both effects occur independently.

METHODS

Cell culture, cell cycle analysis, and p34^{cdc2} activity. K562 cells (from ATCC) were grown in RPMI 1640 medium (Gibco-Life Technologies) supplemented with 8% fetal calf serum and gentamycin (80 μg/ml). When indicated, exponentially growing cells at a concentration of 2.5×10^5 cells/ml were treated with OA (Boehringer Mannheim) or hydroxyurea (Boehringer Mannheim). Cell morphology was analyzed using May-Grünwald-Giemsa stain of cytocentrifuge preparations. Cell growth and viability were assayed by hemocytometer and the trypan blue exclusion test. DNA synthesis was monitored by [^{3}H]thymidine incorporation as described (36). Cell cycle distribution was determined by fluorescent-activated cell sorting. K562 cells were harvested, washed with PBS, fixed and stained with propidium iodide in a DNA-Prep apparatus following the manufacturer's instructions (Coulter). DNA content was determined in a Coulter Epics flow cytometer. For p34^{cdc2} activity determination, cells were lysed and immunoprecipitated with anti-p34^{cdc2} antibodies as described (37). The kinase activity of the immunoprecipitates was determined using histone H1 as the substrate.

Determination of DNA fragmentation. Fragmented cytoplasmic DNA was prepared essentially as described (34) and analyzed electrophoretically on 1% agarose gels containing 0.1 μg/ml of ethidium bromide. For the *in situ* end-labeling of genomic DNA, cytocentrifuge preparations of K562 cells were fixed in 4% paraformaldehyde. The assay was performed using digoxigenin-dUTP (Boehringer Mannheim) essentially as described (38) but using T7 DNA polymerase (Pharmacia, 10 U/ml) as the labelling enzyme.

Retroviral infection of K562. Retrovirus expressing human Bcl-2 were produced from the amphotropic packaging cell line PA317 (39) transfected with the plasmid LBcl2-SN (40) (provided by S. Collins, Fred Hutchinson Cancer Research Center, Seattle). To produce control viruses, the empty vector LXSN (39) was electroporated into PA317 cells and virus-producing cells selected with 500 μg/ml of G418 (Geneticin, Life Sci. Technologies). To infect K562 cells, 2×10^3 viruses of each virus stock were added to 10^6 cells in the presence of 4 μg/ml polybrene. After 48 hours the cells were washed and incubated with selecting media containing G418. Resistant cells were selected and the cells were designated KLXSN (infected with the retroviral vector) and KLBcl2v (infected with Bcl-2 retroviruses).

Analysis of mRNA and protein. Total RNA was prepared using the acid guanidine method (41) and Northern blots were prepared as described (42). The probes for human c-*myc* and *max* were as described (34). Probes were labelled with [^{32}P]αdCTP by random priming (Ready-to-Go labeling kit, Pharmacia) and the blots were hybridized as described (42). The expression of Bcl-2 was determined by immunoblot as previously described (43). Expression of cyclin A was determined with a monoclonal antibody (Santa Cruz Biotechnology) and expression of cyclin B1 with a rabbit polyclonal antibody (provided by X. Graña, Fels Institute, Philadelphia).

RESULTS

Mitotic Arrest and Apoptosis Are Consecutively Induced by Okadaic Acid in K562 Cells

To set up the experimental conditions to investigate the relationship between mitotic arrest and apoptosis in K562, we first carried out a kinetic and dose-response studies of the growth-inhibiting effect of OA on K562 cells. We found that OA, at concentrations of 10 nM and above induced growth arrest, as determined by cell count (Fig. 1A). We used 15 nM OA for further experiments, as we demonstrated previously that this OA concentration is sufficient to inhibit PP2A but no other phosphatases in K562 (34). In agreement with the cell counts, the [^{3}H]thymidine incorporation into DNA was also abolished by 14 nM OA after 48 hours of treatment (Fig. 1B). We then determined the reversibility of the growth arrest mediated by OA. Cells were treated with 15 nM OA for different periods of time, washed, and the growth was determined for up to 3 days. Exposure for 18 h and longer to 15 nM OA resulted in irreversible growth arrest (Fig. 1C). Morphological analysis showed that most of the cell treated for 3 days were apoptotic. Some binucleated cells (about 15%) were also detected after 48 h of treatment (not shown), indicating that some cells successfully completed anaphase but did not divide.

We next analyzed the development of mitotic arrest and apoptosis in K562 cells after different times of exposure to 15 nM OA. Cytological staining confirmed that after 12-24 hours of treatment about 50% of the cells showed metaphasic figures (Fig. 2A). This fraction decreased thereafter with a concomitant increase in cells showing the typical morphological features of apoptosis, e.g. cell shrinkage and nuclear condensation and fragmentation. The fraction of apoptotic cells reached about 60% after 48 hours and 80% after 72 h (Fig. 2A). Endonucleolytic cleavage characteristic of apoptosis was assayed *in situ* by DNA end-labeling using T7 DNA polymerase and digoxigenin-labelled deoxynucleotides. About 15% of positive nuclei were detected after 24 hours of exposure to 15 nM OA, and the fraction of labelled nuclei increased up to about 50% after 48 hours of treatment (Fig. 2B). The apoptotic nature of these morphological changes was confirmed by the presence of internucleosomal DNA fragmentation, the biochemical hallmark of apoptosis. There was a marked DNA laddering after 48 hours and 72 h of treatment with 15 nM OA (Fig. 2C). Okadaic

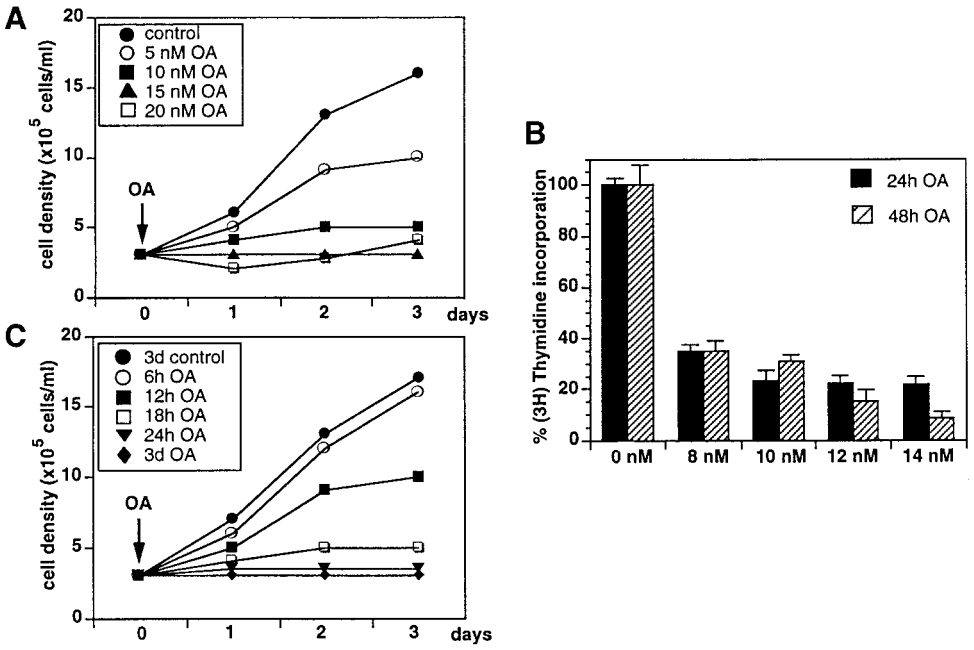


FIG. 1. (A) Growth of K562 in the presence of the indicated OA concentrations. The arrow indicates the time of OA addition. (B) DNA synthesis after 24 and 48 hours of treatment with the indicated OA concentrations, determined by [^{3}H]-thymidine incorporation. (C) Growth of K562 cells after transient exposure to OA. Cells were treated for 6 to 72 hours with 15 nM OA, washed and incubated up to 3 days in fresh media without OA. Cell densities were scored with a cytometer chamber.

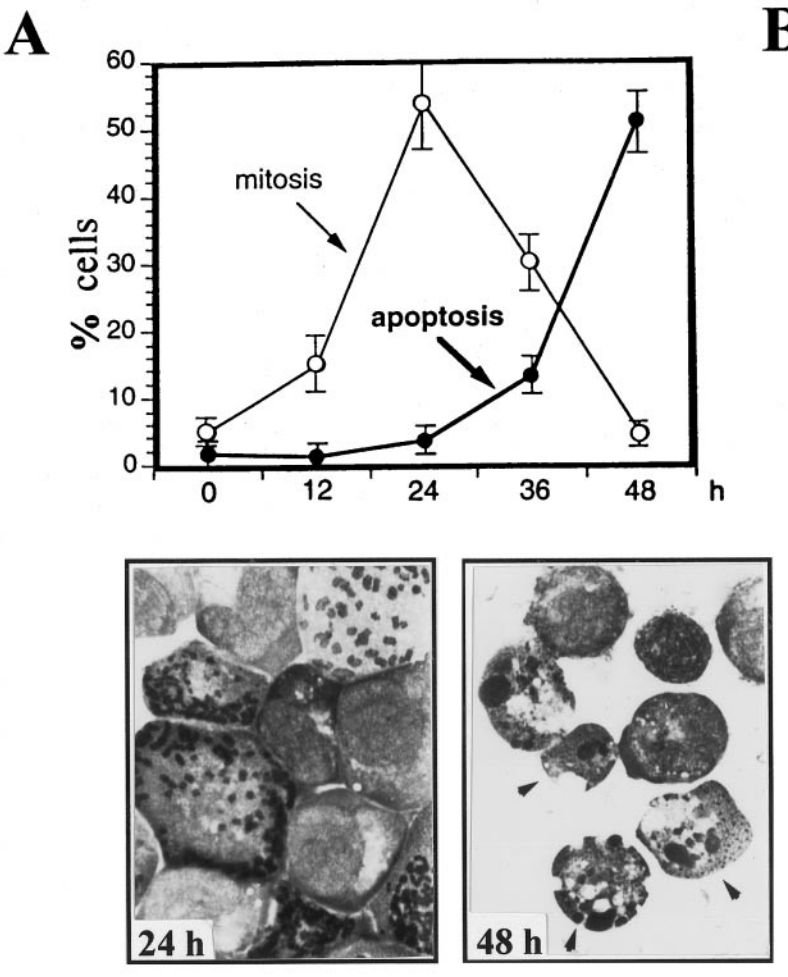
acid tetra-acetate, an OA derivative that does not inhibit protein phosphatases, did not retard K562 cell growth and did not induce apoptosis at concentrations up to 30 nM (not shown).

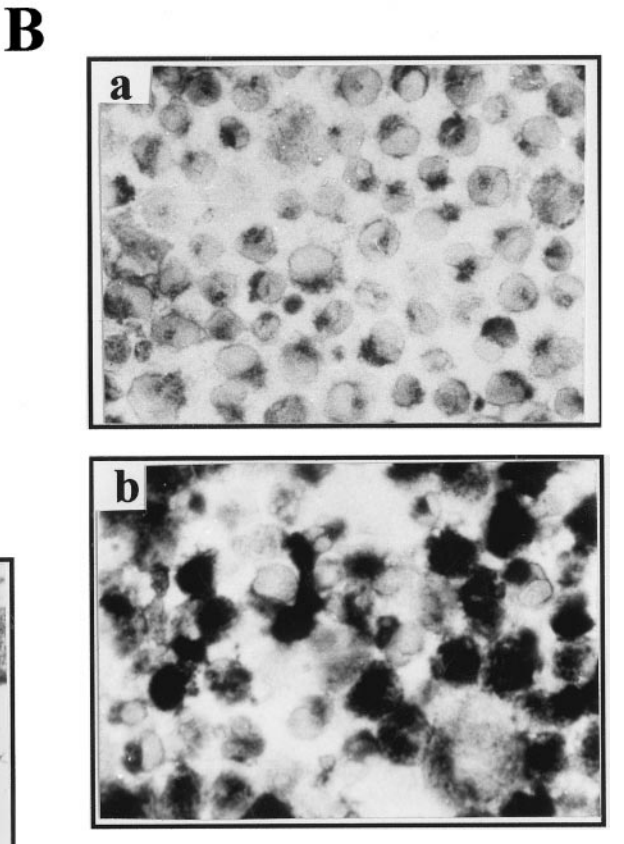
We investigated whether OA induced the onset of normal mitosis in K562 or rather a premature chromosome condensation. We performed analysis of DNA content by propidium iodide staining and flow cytometry. The cytometry profiles (Fig. 2D) revealed that most of the cells contained 4C amount of DNA after 24 h of treatment, appearing at a G2/M peak. This timing coincided with the peak of cells showing mitotic arrest after Giemsa staining (Fig. 2A), suggesting that the metaphase state induced by the drug corresponded to a mitotic arrest rather than unscheduled chromosome condensation. The cytometry profile of DNA content of OA-treated cells also showed an increasing number of cells appearing in the sub-G0/G1 region, which corresponds to apoptotic cells (44). Consistent with the hypothesis that arrest occurs after initiation of a normal mitosis, there was an activation of p34^{cdc2} during the treatment with OA (Fig. 3A), in keeping with previous results (14, 45). Also, expression of cyclin A and B1 was elevated during up to the first 12 h of treatment, dropping thereafter, with faster down-regulation observed for cyclin B1. Cyclin levels were down once the cells were committed to apoptotic death, and undetectable 48 h after OA addition (Fig. 3B). As a control, we determined cyclin A and B1 expression in a parallel experiment using cells treated with 10 μM nocodazole, a drug that reversibly arrests cells in metaphase, with a concomitant cyclin A and B accumulation (Fig. 3B).

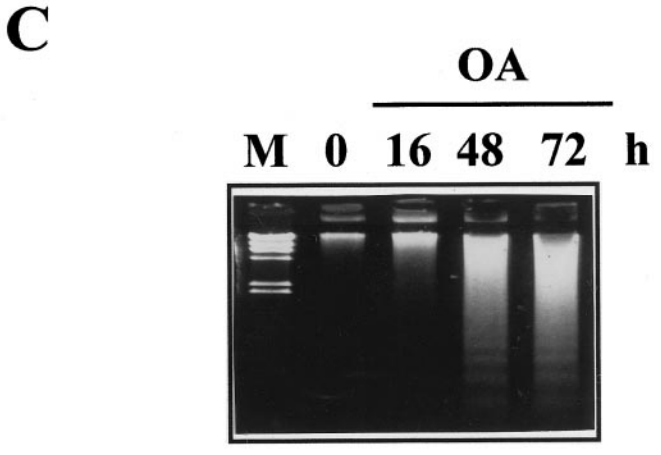
Mitosis and Apoptosis Induced by OA Are Uncoupled in K562 Cells

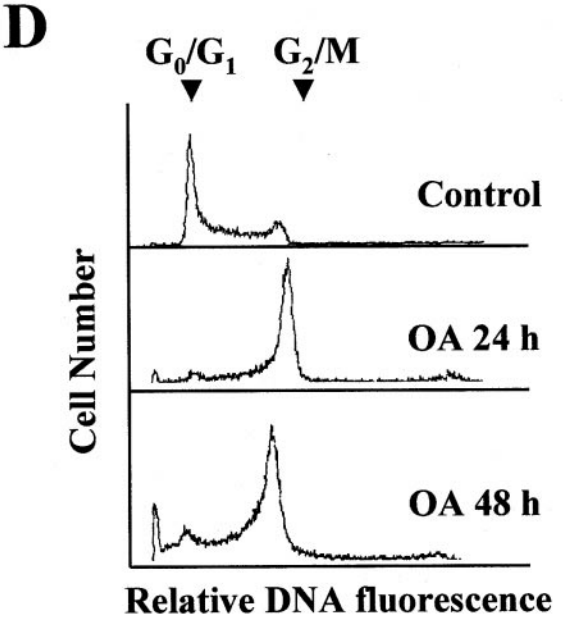
We first generated by retroviral infection a K562 sub-line with constitutive expression of Bcl-2, designated KLBcl2v. As shown in the immunoblot of Fig.

FIG. 2. Mitotic arrest and apoptosis are sequentially induced by OA in K562 cells. (A) Fraction of metaphasic and apopotic and cells during treatment with 15 nM OA. Data are mean values from four separate experiments with standard deviations. Pictures of Giemsa-stained cells collected 24 and 48 hours after OA addition are shown below; arrowheads indicate apoptotic cells. (B) Nuclear DNA breaks detected by in situ end-labeling in control cells (a) and cells treated with 15 nM OA for 48 hours (b). (C) Fragmentation of genomic DNA in cells treated with 15 nM OA for the indicated periods of time. Lane M shows DNA size markers (phage λ DNA digested with *Hind*III). (D) Cell cycle distribution of K562 cells treated with OA. The histograms show the DNA content analyzed by flow cytometry of cells untreated or treated for 24 and 48 hours with 15 nM OA.


A
% cells
mitosis
apoptosis
0
12
24
36
48
h
24 h
48 h


B
a
b


C
OA
M 0 16 48 72 h


D
G0/G1
G2/M
Control
OA 24 h
OA 48 h
Cell Number
Relative DNA fluorescence

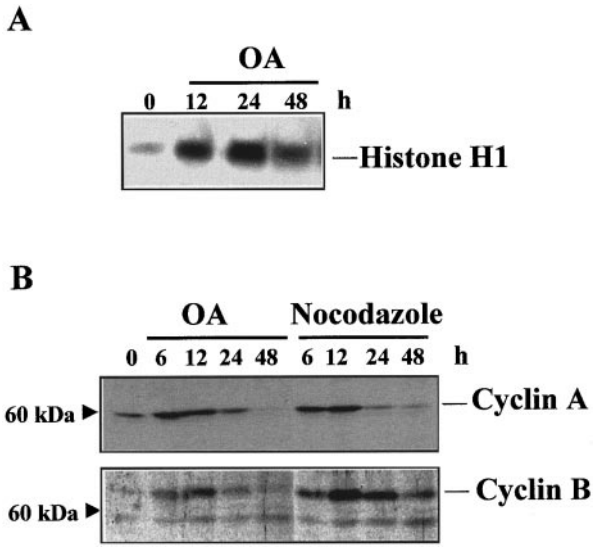


FIG. 3. OA induces $p34^{cdc2}$ activation and mitotic cyclins expression. (A) $p34^{cdc2}$ activity during OA treatment. Cells extracts were prepared at the indicated times and assayed for $p34^{cdc2}$ kinase activity on histone H1. (B) Cyclin A and B expression during OA treatment of K562. Cell extracts were prepared at the indicated times after OA addition (15 nM) and the levels of cyclin A and B were analyzed by immunoblot. Parallel samples were treated with 10 μM nocodazole. The position of cyclin A and B1 are indicated at the right. Arrowheads indicate the position of the 60 kDa protein marker.

4A, KLBcl2v cells expressed high levels of Bcl-2 protein whereas it was undetectable in vector-transfected cells (KLXSN), as expected from previous reports indicating that K562 does not express Bcl-2 (43). We used KLBcl2v cells to test whether mitosis could occur in the absence of subsequent apoptosis in this system. The apoptosis induced by 15 nM OA was dramatically reduced in KLBcl2v cells compared with control cells, but OA still induced a clear mitotic arrest (Fig. 4B). Thus, most cells in mitotic arrest provoked by OA did not progress to apoptosis.

To investigate whether apoptosis was a consequence of the mitotic arrest, we followed two approaches. First, we analyzed OA-induced apoptosis in cells pretreated with hydroxyurea, which reversibly inhibits DNA synthesis (36). The effect of hydroxyurea in K562 was confirmed by flow cytometry of cells stained with propidium iodide. Cells treated with 1 mM hydroxyurea for 24 h did not progress to G2/M phase (Fig. 5A). The effect of hydroxyurea pre-treatment on OA-mediated apoptosis was studied by counting Giemsa-stained cells. As shown in Fig. 5B, when cells were treated for 24 hours with 1 mM hydroxyurea prior to the addition of OA, very few mitotic figures were detected, compared with cells treated with OA alone (compare Fig. 5B with Fig. 2A). However, a high fraction of apoptotic cells (80%) was found 48 h after adding OA. This result was confirmed by DNA fragmentation analysis. Unlike cells treated with hydroxyurea alone, those treated with hydroxyurea plus OA showed the DNA laddering typical of apoptosis (Fig. 5C). Similar results were found when the hydroxyurea was washed out before OA addition (not shown). Therefore

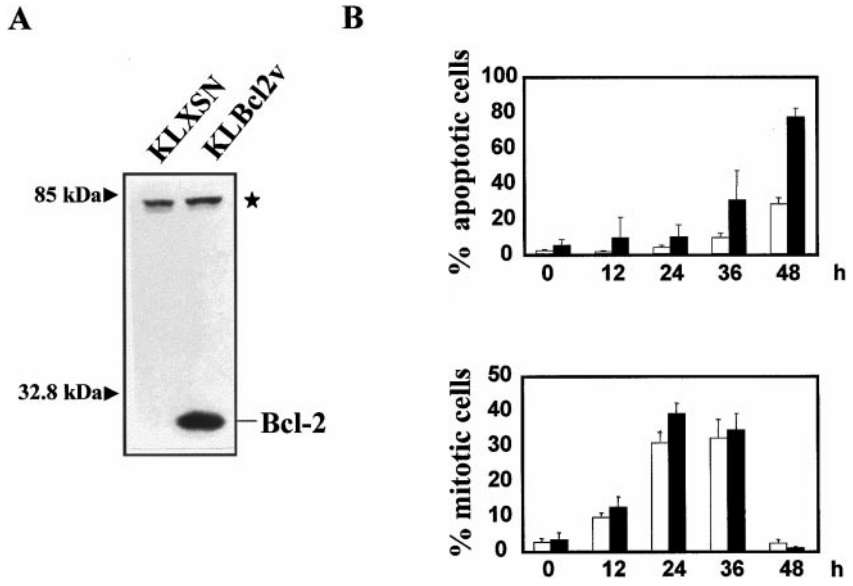


FIG. 4. OA induces mitotic arrest in K562 expressing Bcl-2. (A) Immunoblot analysis of Bcl-2 expression in KLXSN and KLBcl2v cells. A cross-reacting protein (indicated by *) is shown to assess the similar protein loading in both samples. The position of the molecular weight markers are shown at the left. (B) Fraction of cells with apoptotic morphology ("apoptotic cells") and with metaphasic morphology ("mitotic cells") during the treatment with 15 nM OA. White bars represent KLBcl2v cells and black bars control KLXSN cells. Data are mean values from three separate experiments and bars represent standard deviations.

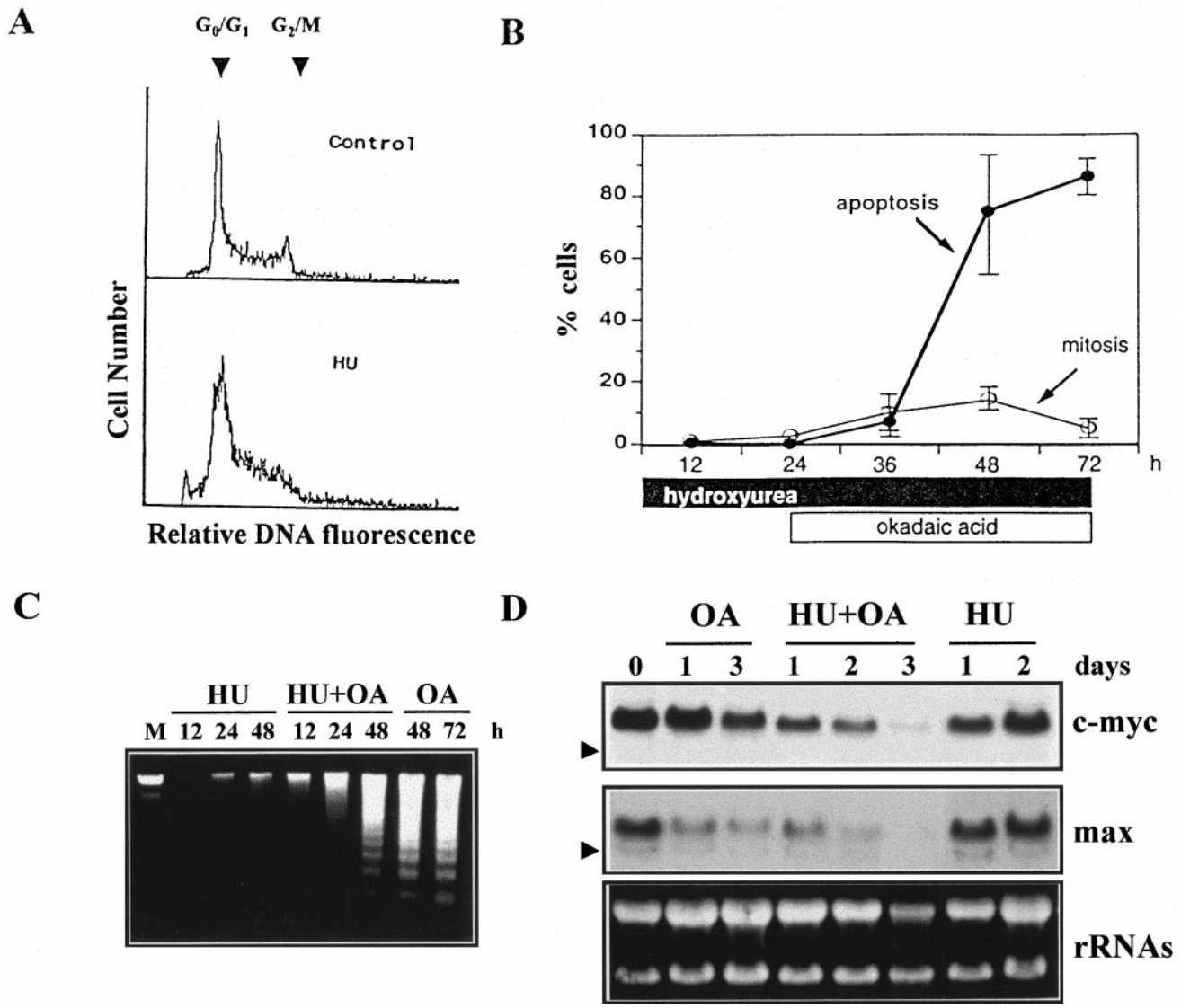


FIG. 5. Hydroxyurea inhibits the mitosis but no apoptosis induced by OA. (A) Flow cytometry analysis of DNA content of untreated K562 cells ("control") and cells treated with hydroxyurea for 24 hours (HU). (B) Fraction of cells with metaphasic ("mitosis") and apoptotic morphology ("apoptosis") during treatment with hydroxyurea and OA. Cells were treated with 1 mM hydroxyurea for 24 hours and OA was then added to a concentration of 15 nM. Data are mean values from three independent experiments and bars represent standard deviations. (C) DNA fragmentation induced by hydroxyurea and OA treatments. K562 cells were incubated with hydroxyurea (HU) or OA for the indicated periods of time, or pretreated 24 hours with hydroxyurea and then with OA for 12, 24, and 48 hours (HU+OA). (D) c-*myc* and *max* down-regulation does not depend on the previous mitotic arrest. Cells were treated with 15 nM OA or 1 mM hydroxyurea for the indicated periods of time and c-*myc* and *max* expression was determined by Northern analysis. Lanes labeled HU+OA refer to cells treated for 24 h with 1 mM hydroxyurea and then for 24, 48, and 72 h with 15 nM OA. The filter was consecutively hybridized to c-*myc* and *max* probes. Arrowheads indicate the position of the 18S rRNA. A picture of the filter with the rRNAs stained with ethidium bromide is shown to assess the loading and integrity of the RNAs.

OA still induced full apoptosis when mitosis was prevented by hydroxyurea.

It has been reported in many cell systems that high c-*myc* expression is linked to a high proliferative state of the cells and decreases when proliferation is inhibited. Consistent with this finding, we had previously shown that OA-mediated apoptosis of K562 is accompanied by down-regulation of c-Myc and its dimerization partner Max (34). Therefore, we investigated whether the down-regulation of c-*myc* was a consequence of OA-induced mitotic arrest. We determined by northern analysis the expression of c-*myc* in cells exposed to OA after treatment with hydroxyurea. c-*myc* expression did not change significantly with the hydroxyrea treatment, but decreased when OA was added (Fig. 5D). The extent of OA-mediated down-

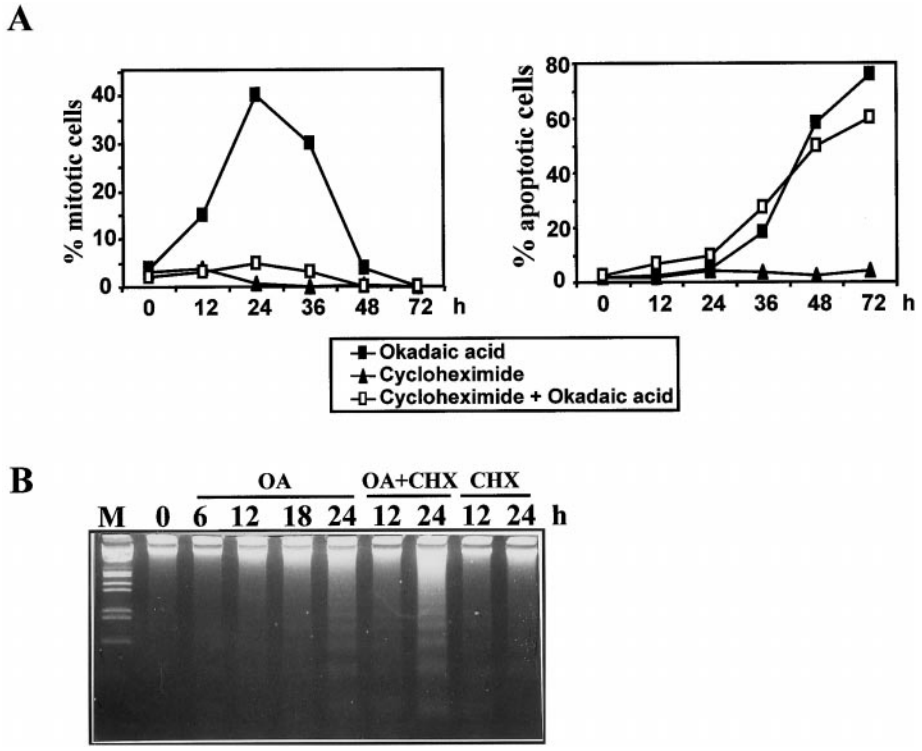


FIG. 6. Cycloheximide inhibits mitotic arrest but not apoptosis induced by OA. (A) Fraction of mitotic cells and apoptotic cells exposed to 15 nM OA, 10 μg/ml cycloheximide, or both drugs, as indicated. (B) DNA fragmentation of K562 cells treated for the indicated times with 15 nM OA (lanes OA), 10 μg/ml cycloheximide (CHX), or both drugs (OA+CHX).

regulation was similar to that observed without pretreatment with hydroxyurea. We found that *max* was also down-regulated during OA-mediated apoptosis, but not with growth arrest mediated by hydroxyurea alone (Fig. 5D). Thus, the down-regulation of c-Myc/Max mediated by OA in K562 is associated with apoptosis but not with mitotic arrest.

As a second approach to exploring the relationship between mitosis and apoptosis, we used the protein synthesis inhibitor cycloheximide. Cycloheximide inhibited K562 cell growth, as expected, but no mitotic arrest or apoptosis was detected during 72 h of treatment. When the cells were treated with OA and cycloheximide (10 μg/ml) the mitotic arrest was completely inhibited, as no cells showing metaphasic figures were detected. This would be the expected result if OA initiated a normal mitosis, which requires synthesis of cyclins. However, the extent of apoptosis in cells exposed to cycloheximide plus OA was similar to that of cells treated with OA alone (Fig. 6A). The same result was observed in the analysis of DNA fragmentation, because cycloheximide did not reduce the laddering of DNA mediated by OA (Fig. 6B). Thus, OA-induced apoptosis does not require protein synthesis and takes place in the absence of previous mitotic arrest.

DISCUSSION

In the present study we explored the functional relationship between mitotic arrest and apoptosis induced in the K562 model. OA induces an accumulation of cells in a metaphasic state which precedes the accumulation of cells with apoptotic morphology. It has been reported that OA induces premature abnormal chromatin condensation in some cell types (11, 13, 20, 46). However, the up-regulation of mitotic cyclins, $p34^{cdc2}$ activity and the 4C DNA content of K562 cells arrested by OA indicates that OA induces normal mitotic onset. It is noteworthy that cyclin A and B are down-regulated after 24 h of treatment, when the cells are irreversibly committed to apoptosis.

OA-induced mitotic arrest was followed by apoptosis. An obvious possibility is that the apoptotic program was activated after a failure to complete mitosis, as already proposed for OA-mediated apoptosis (17, 18) and for other drugs that provoke mitotic blocking. As expected, enforced expression of Bcl-2 rescued cells from OA-mediated apoptosis, while mitotic arrest was still observed. This result could be explained if the mitotic arrest triggered an apoptotic signal and Bcl-2 acted downstream in the apoptosis pathway. However, our results are also compatible with the hypothesis

that OA induce mitotic arrest and apoptosis through separate pathways and Bcl-2 would only block the latter. Surprisingly, our results suggested that, in this model, OA-mediated mitotic arrest is not necessary for apoptosis to occur, and that in K562 cells mitosis arrest and apoptosis are independent effects of protein phosphatases inhibition by OA. Both approaches followed in our experiments lead to this conclusion. Firstly, apoptosis takes place even when the DNA synthesis and subsequent mitosis have been blocked by hydroxyurea. The expression of c-*myc* proto-oncogene is down-regulated during OA-mediated apoptosis of K562. In agreement with the association between high c-*myc* expression and proliferation (47) it is conceivable that the observed c-*myc* down-regulation was a consequence of the mitotic arrest, rather than of apoptosis. However, c-*myc* expression is kept elevated when mitotic arrest is prevented by hydroxyurea. Thus, c-*myc* down-regulation exerted by OA is not a result of the mitotic arrest but linked to the apoptotic process in K562. Using the second approach, we found that inhibition of protein synthesis inhibited mitosis (as expected), but not OA-mediated apoptosis. This approach is feasible in K562 cells due to their resistance to apoptosis mediated by cycloheximide (32 and the present paper). In summary, our results indicate that, conversely to a widely accepted view, apoptosis is not necessarily a consequence of OA-mediated mitotic arrest.

Although the IC50 of OA for inhibition of PP1 and PP2A falls within the nanomolar range (9), many of the effects on mitotic arrest and apoptosis reported on other cell systems are achieved by much higher OA concentrations much higher. On the other hand, mitotic arrest and apoptosis of K562 occur at OA concentrations (15 nM) that, as we have previously shown, inhibit PP2A but not PP1 activity in K562 (34). PP2A plays multiple roles in the cell, and our findings suggest that its function in cell cycle control may be independent from that required for cell viability. Finally, the finding that apoptosis is not necessarily linked to previous mitotic arrest may contribute to understanding the cytotoxic effect of mitotic blockers used in cancer chemotherapy.

ACKNOWLEDGMENTS

We thank Rosa Blanco for technical assistance, Jorge Martín-Perez and David Aaronson for critical review of the manuscript, Steve Collins, Robert Eisenman, Xavier Graña, Carmela Calés, and Jose L. Fernández-Luna for cell lines, antibodies, and probes, and Neus Agell and Oriol Bachs for advice in p34 activity determination. This work was supported by grants SAF96-0083 and PM98-0109 from the Spanish Ministery of Education and Culture.

REFERENCES

1. Meikrantz, W., and Schlegel, R. (1995) *J. Cell. Biochem.* **58,** 160–174.
2. Shimizu, T., O'Connor, P., Kohn, K. W., and Pommier, Y. (1995) *Cancer Res.* **55,** 228–231.
3. Meikrantz, W., and Schlegel, R. (1996) *J. Biol. Chem.* **271,** 10205–10209.
4. Shen, S. C., Huang, T. S., Jee, S. H., and Kuo, M. L. (1998) *Cell Growth Differ.* **9,** 23–29.
5. Levkau, B., Koyama, H., Raines, E. W., Clurman, B. E., Herren, B., Orth, K., Roberts, J. M., and Ross, R. (1998) *Mol. Cell.* **1,** 553–563.
6. Norbury, C., MacFarlane, M., Fearnhead, H., and Cohen, G. M. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1400–1406.
7. Guo, K. W., Th'ng, J. P., Swank, R. A., Anderson, H. J., Tudan, C., Bradbury, E. M., and Roberge, M. (1995) *EMBO J.* **14,** 976–985.
8. Martin, S. J., McGahon, A. J., Nishioka, W. K., La Face, D., Guo, X., Th'ng, J., Bradbury, E. M., and Green, D. R. (1995) *Science* **269,** 106–107.
9. Mumby, M. C., and Walter, G. (1993) *Physiol. Rev.* **73,** 673–699.
10. Alexandre, H., van Cauwenberge, A., Tsukitani, Y., and Mulnard, J. (1991) *Development* **112,** 971–980.
11. Dyban, A. P., De Sutter, P., and Verlinsky, Y. (1993) *Mol. Reprod. Dev.* **34,** 402–415.
12. Paulson, J. R., Ciesielski, W. A., Schram, B. R., and Mesner, P. W. (1994) *J. Cell Sci.* **107,** 267–273.
13. Ghosh, S., Schroeter, D., and Paweletz, N. (1996) *Exp. Cell Res.* **227,** 165–169.
14. Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990) *EMBO J.* **9,** 4331–4338.
15. Van Dolah, F. M., and Ramsdell, J. S. (1992) *J. Cell Physiol.* **152,** 190–198.
16. Wiltshire, T., Park, C., Caldwell, K. A., and Handel, M. A. (1995) *Dev. Biol.* **169,** 557–567.
17. Inomata, M., Saijo, N., Kawashima, K., Kaneko, A., Fujiwara, Y., Kunikane, H., and Tanaka, Y. (1995) *J. Cancer Res. Clin. Oncol.* **121,** 729–738.
18. Nuydens, R., de Jong, M., Van Den Kieboom, G., Heers, C., Dispersyn, G., Cornelissen, F., Nuyens, R., Borgers, M., and Geerts, H. (1998) *J. Neurochem.* **70,** 1124–1133.
19. Ishida, Y., Furukawa, Y., DeCaprio, J. A., Saito, M., and Griffin, J. D. (1992) *J. Cell. Physiol.* **150,** 484–492.
20. Gotoh, E., Asakawa, Y., and Kosaka, H. (1995) *Biomed. Res.* **16,** 63–68.
21. Song, Q., Baxter, G. D., Kovacs, E. M., Findik, D., and Lavin, M. F. (1992) *J. Cell. Physiol.* **153,** 550–556.
22. Ohoka, Y., Nakai, Y., Mukai, M., and Iwata, M. (1993) *Biochem. Biophys. Res. Commun.* **197,** 916–921.
23. Boe, R., Gjertsen, B. T., Vintermyr, O. K., Houge, G., Lanotte, M., and Doskeland, S. O. (1991) *Exp. Cell Res.* **195,** 237–246.
24. Kiguchi, K., Glesne, D., Chubb, C. H., Fujiki, H., and Huberman, E. (1994) *Cell Growth Diff.* **5,** 995–1004.
25. Gjersten, B. T., Cressey, L. I., Ruchaud, S., Houge, G., Lanotte, M., and Doskeland, S. O. (1994) *J. Cell Sci.* **107,** 3363–3377.
26. Wera, S., and Hemmings, B. A. (1995) *Biochem. J.* **311,** 17–29.
27. Vorlaufer, E., and Peters, J.-M. (1998) *Mol. Biol. Cell* **9,** 1817–1831.

28. Martin, S., Lennon, S., Bonham, A., and Cotter, T. (1990) *J. Immunol.* **145,** 1859–1867.
29. McGahon, A., Bissonette, R., Schmitt, M., Cotter, K. M., Green, D. R., and Cotter, T. G. (1994) *Blood* **83,** 1179–1181.
30. Ritke, M. K., Rusnak, J. M., Lazo, J. S., Allan, W. P., Dive, C., Heer, S., and Yalowich, J. C. (1994) *Mol. Pharmacol.* **46,** 605–611.
31. Benito, A., Grillot, D., Nuñez, G., and Fernández-Luna, J. L. (1995) *Am. J. Pathol.* **146,** 481–490.
32. Law, J. C., Ritke, M. K., Yalowich, J. C., Leder, G. H., and Ferrell, R. E. (1993) *Leuk. Res.* **17,** 1045–1050.
33. Zheng, B., Chambers, T. C., Raynor, R. L., Markham, P. N., Gebel, H. M., Vogler, W. R., and Kuo, J. F. (1994) *J. Biol. Chem.* **269,** 12332–12338.
34. Lerga, A., Belandia, B., Delgado, M. D., Cuadrado, M. A., Richard, C., Ortiz, J. M., Martín-Perez, J., and León, J. (1995) *Biochem. Biophys. Res. Commun.* **215,** 889–895.
35. Zheng, B., Woo, C. F., and Kuo, J. F. (1991). *J. Biol. Chem.* **266,** 10031–10034.
36. Gómez-Casares, M. T., Delgado, M. D., Lerga, A., Crespo, P., Quincoces, A. F., Richard, C., and León J. (1993) *Leukemia* **7,** 1824–1833.
37. Draetta, G., and Beach, D. (1988) *Cell* **58,** 17–26.
38. Edwards, S. N., and Tolkovsky, A. M. (1994) *J. Cell. Biol.* **124,** 537–546.
39. Miller, D., Miller, D. J., Garcia, J. V., and Lynch, C. M. (1993) *Meth. Enzymol.* **217,** 581–599.
40. Park, J. R., Robertson, K., Hickstein, D. D., Tsai, S., Hockenbery, D. M., and Collins, S. J. (1994) *Blood* **84,** 440–445.
41. Chomczynski, P., and Sacchi, N. (1988) *Anal. Biochem.* **162,** 156–159.
42. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
43. Benito, A., Lerga, A., Silva, M., León, J., and Fernandez-Luna, J. L. (1997) *Leukemia* **11,** 940–944.
44. Nicoletti, I., Migliorati, G., Pagliacci, M. C., and Riccardi, C. A. (1991) *J. Immunol. Methods* **139,** 271–279.
45. You, J., and Bird, R. C. (1995) *J. Cell. Physiol.* **164,** 424–433.
46. Ajiro, K., Yoda, K., Utsumi, K., and Nishikawa, Y. (1996) *J. Biol. Chem.* **27,** 13197–13201.
47. Henriksson, M., and Lüscher, B. (1996) *Adv. Cancer Res.* **68,** 109–182.