Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 23-29

EARLY GENE SIGNALLING-DEPENDENT AND -INDEPENDENT INDUCTION OF APOPTOSIS IN RAMOS HUMAN B CELLS CAN BE INHIBITED BY OVER-EXPRESSION OF BCL-2

Zhi-Qiang Ning[1], John D. Norton[2], Diane Johnson[2] and John J. Murphy[1,*]

[1]Infection and Immunity Research Group, Division of Life Sciences, King's College London, London W8 7AH, UK

[2]CRC Department of Gene Regulation, Paterson Institute for Cancer Research, Christie Hospital, Manchester, U. K.

Received August 21, 1995

Summary: We have previously shown that calcium ionophore-induced apoptosis of Ramos human B cells is preceded by the induced expression of early response genes, implying a requirement for new gene expression in this mode of programmed cell death. We have found in the present studies that inhibitors of macromolecular synthesis, cycloheximide and actinomycin D, are also potent inducers of apoptosis in the same Ramos cell model. These drugs trigger apoptosis through apparently early gene signalling-independent pathways. Although different mechanisms for induction of apoptosis exist in Ramos cells, enforced over-expression of Bcl-2 protects cells from apoptosis induced in response to different agents, demonstrating that Bcl-2 blocks a final common pathway for programmed cell death in the Ramos cell model.

Apoptosis is an important mechanism for eliminating cells and is essential for normal development and homeostasis. The widely accepted view is that cellular signalling cascades and new gene expression are required for this process (1). Indeed, the encoded products of a number of early response genes (ERG) have been implicated as positive regulators of apoptosis. These include c-Myc (2), c-Fos (3), and Nur77 (4). A number of gene products, such as those encoded by the prototype *bcl-2* family have also implicated in promoting cell survival (5-7).

Although cellular signalling and induced gene expression is in many instances required for apoptosis to occur and indeed inhibition of either protein or RNA synthesis can inhibit apoptosis in some cell types (8, 9), there are also reports demonstrating induction of apoptosis in response to inhibition of macromolecular synthesis (10, 11). This implies that

***Corresponding author. Fax:**(44) 171 333 4500.

0006-291X/95 $12.00

the apoptotic machinery can be triggered through quite diverse pathways. Although several speculations concerning the nature of apoptotic pathways have been made with regard to different cell types (1, 12), relatively little is known about these processes within any one single cell type.

We have recently reported that calcium ionophore-induced apoptosis of Ramos human B cells is preceded by the induced expression of a distinct set of ERGs, implying a requirement for new gene expression in this mode of programmed cell death (13). In the course of investigating this further we have now found that inhibitors of macromolecular synthesis themselves also induce apoptosis in Ramos B cells. We report here on the results of evaluating the different pathways of apoptosis induction by different agents in Ramos B cells and the effects of ectopic over-expression of Bcl-2 on these diverse apoptotic pathways.

Materials and Methods

Cells and reagents. All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) except where otherwise indicated. The EBV negative human Burkitt's lymphoma cell line was obtained from The European Collection of Animal Cell Cultures (Poole, Dorset, UK). Cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics and maintained at 37°C in a humidified 5% CO_2 atmosphere. For all experiments exponentially growing cells (5×10^5 cells/ml) were used. Monoclonal antibody to human Bcl-2 was a kind gift from Dr. David Mason (Haematology Department, John Radcliffe Hospital, Oxford, UK).

Induction and assessment of apoptosis. For apoptosis induction, cells were placed in 96-well culture plates and treated with different concentrations of A23187, cycloheximide, or actinomycin D. Cells were collected at various times shown in the text and apoptosis assays were carried out using acridine orange staining as described in a previous report (13).

RNA isolation and hybridisation analysis. Ramos cells were harvested at various times before and after addition of different agents as indicated in the text. Total RNA was isolated from cells by lysis in RNAzol solution (Biogenesis Ltd., Bournemouth, UK) and purification of RNA was carried out according to the manufacturers instructions. Samples of RNA were electrophoresed through a denaturing 1.0% agarose-formaldehyde gels and transferred onto nylon membranes (Genescreen Plus, NEN Research Products, Boston, USA). Blots were hybridised to ^{32}P-labelled probes and washed as described previously (14).

Transfection of Ramos cells with the *bcl-2* gene. Ramos cells were electroporated with either the *bcl-2*-expression vector, pZen *bcl-2* SV neo (15), or with the same vector lacking a *bcl-2* coding sequence by using a Biorad gene pulser apparatus (Bio-Rad Richmond, CA, USA). Approximately 10^7 cells in 350 µl of standard growth medium were electroporated with 10-20 µg DNA at 300 V and 960 µF. After a further two days of growth, individual *bcl-2* overexpressing or control cell clones were isolated following G418 selection (0.5mg/ml for two weeks) and limiting dilution.

Western blot analysis. Cell lysates of wild-type cells and cells transfected with empty vector or the *bcl-2* gene were obtained by lysis of Ramos cells in sample buffer (100 mM

Tris-Cl, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.2% Bromophenol blue). Samples were electrophoresed through 12% SDS-polyacrylamide gels and proteins were electrotransferred on to nitrocellulose membranes. Filters were blocked for 1 h with PBS containing 5% FCS and incubated with anti-human Bcl-2 mAb for 1 h. Blots were washed and reacted with horseradish peroxidase-labelled rabbit anti-mouse Ig (Dakopatts, Denmark) for another 1 h. Signal detection of Immunoblots were carried out using the enhanced chemiluminescence protocol according to the manufacturers instructions (Amersham, UK).

Results

In experiments designed to examine whether calcium ionophore (A23187)-induced apoptosis in Ramos cells could be blocked by inhibitors of macromolecular synthesis, the drugs cycloheximide (CHX) and actinomycin D (ActD), known to inhibit translation and transcription respectively, were incubated with Ramos cells in the presence of A23187. We found that neither CHX nor ActD inhibited A23187-induced apoptosis (data not shown). In contrast, both these agents were potent inducers of apoptosis. Fig. 1 shows the dose-dependent induction and time course of apoptosis induced by these agents in Ramos cells, as assessed by acridine orange staining of the cells with condensed/fragmented nuclei (13). At concentrations as low as 5 μg/ml (CHX) or 0.5 μg/ml (ActD) these drugs could induce more

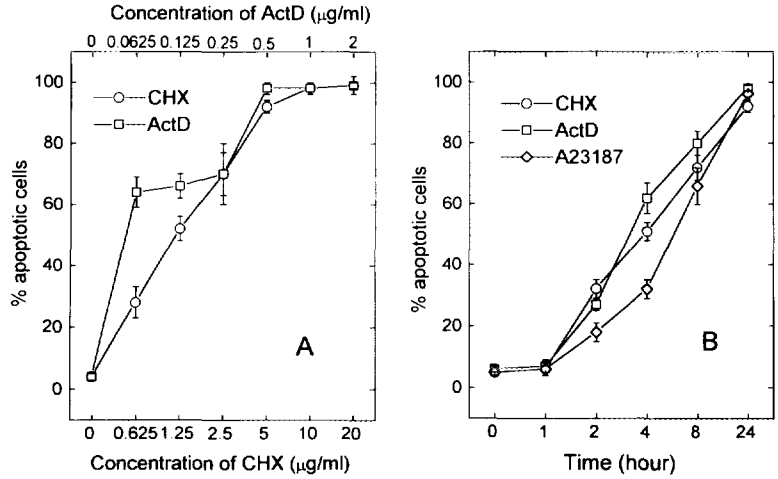

Fig. 1. Dose-dependence (A) and time course (B) of apoptosis induced in response to different agents in Ramos B cells.

A. Ramos cells were incubated with different concentrations of CHX or ActD. 24 h later the percentage of cells with nuclear condensation/fragmentation was assessed by acridine orange staining. The results are expressed as the mean ± SEM of three independent experiments.

B. Ramos cells were treated with either CHX (5 μg/ml), ActD (0.5 μg/ml), or A23187 (0.5 μM) for the times shown. After acridine orange staining, the percentage of cells with nuclear condensation/fragmentation was assessed. The results are expressed as the mean ± SEM of three independent experiments.

than 90% of the cells to become apoptotic by 24 h (Fig. 1A) and the apoptotic cells could be observed as early as 2 h after addition of these agents (Fig. 1B). Indeed, the kinetics of apoptosis induced in response to inhibitors of macromolecular synthesis was similar to that observed for calcium ionophore (Fig. 1B).

Expression of a number of ERGs (c-*fos*, *krox20* and an anonymous ERG, *10A*) has been shown to precede the onset of apoptosis induced in response to calcium ionophore in Ramos cells (13). To exclude the possibility that inhibitors of macromolecular synthesis may activate a similar early gene signalling pathway we compared the expression of these ERGs in Ramos cells in response to the three agents, A23187, CHX and ActD, with particular interest in the *10A* gene which we have shown by gene transfer to be involved in positive regulation of calcium ionophore-induced apoptosis in Ramos cells (Ning et al., submitted). Fig. 2 shows, as expected, that basal expression of the *10A* gene was dramatically induced by A23187 treatment and either not changed or inhibited by CHX and ActD treatment respectively. Neither CHX nor ActD induced expression of ionophore-induced ERGs, c-*fos* and *krox20* (data not shown). These results demonstrate that in the Ramos cell model, in spite of the similar kinetics of apoptosis induced in response to either calcium ionophore or inhibitors of macromolecular synthesis, the mechanisms for triggering apoptosis are apparently distinct for these different agents.

The product of the protooncogene *bcl-2* is well documented as an inhibitor of apoptosis in many cell types including B cells (16). However, not all mechanisms of apoptosis appear to be susceptible to suppression by Bcl-2 (17, 18). To gain further insight into the diversity of

Fig. 2. Distinct patterns of ERG *10A* expression in Ramos B cells treated with different agents.

Ramos cells were incubated with either A23187 (0.5 μM), CHX (5 μg/ml), or ActD (0.5 μ g/ml) and total RNA was isolated from cells at the times indicated. Northern blot analysis was carried out as described in the Materials and Methods.

Fig. 3. Western blot analysis of Bcl-2 expression in transfected Ramos B cells.

Ramos B cells were transfected with the human *bcl-2* gene and stable clones of transfectants were isolated. The expression of Bcl-2 was checked by using the lysate of 2×10^5 cells per lane. Lanes 1 and 2 show level of Bcl-2 in wild type and a clone of vector alone transfected cells respectively. Faint bands showing low level of Bcl-2 protein were visible on longer exposure in these lanes. Lanes 3-5 show level of Bcl-2 expression in 3 clones of bcl-2 positive transfectants.

pathways mediating programmed cell death in Ramos B cells, we investigated to what extent enforced over-expression of the *bcl-2* gene could protect these cells from apoptosis induced in response to different agents. Ramos cells were transfected with an expression vector encoding the Bcl-2 protein and a number of transfected cell clones were isolated in parallel with cells transfected with the same vector but lacking *bcl-2* coding sequence. As shown in Fig. 3 for three Bcl-2 over-expressing clones examined in detail, levels of this protein were around 20-fold higher, as measured by densitometry, than those seen in control cells which express low levels of this protein (Ning et al., submitted). Following incubation with different inducers of apoptosis, all these three Bcl-2-over-expressing clones were resistant to induction of cell death irrespective of the agent used (Fig. 4). Thus, despite the apparently diverse pathways coupled to calcium ionophore and the two inhibitors of macromolecular synthesis, apoptosis induced by these three effectors is susceptible to blockade by Bcl-2 over-expression.

Discussion

Several early response genes have been identified as positive regulators of apoptosis in different cell types (2-4). We have previously reported that expression of a B cell early response gene, *10A*, precedes the onset of apoptosis induced by calcium ionophore in Ramos B cells (13). Our current investigation of the *10A* gene has revealed that blockade of 10A expression can inhibit apoptosis induced by the ionophore in Ramos cells (Ning et al., submitted), demonstrating that the Ramos cell line can be specifically induced to undergo programmed cell death through an early gene signalling-dependent pathway.

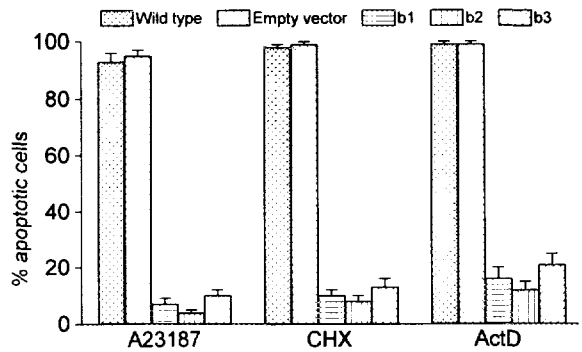

Fig. 4. Over-expression of Bcl-2 protects Ramos B cells from undergoing apoptosis in response to different agents.

Wild type, vector alone transfected and clones of Bcl-2 positive transfectants (b1, b2, b3) were treated with either A23187 (0.5 μM), CHX (5 μg/ml), or ActD (0.5 μg/ml). 24 h later cells were stained with acridine orange and the percentage of cells with nuclear condensation/ fragmentation was assessed. The results are expressed as the mean ± SEM of three independent experiments.

Inhibitors of macromolecular synthesis can inhibit apoptosis in some circumstances (for example, see reference 9) and fail to do so or even actually promote apoptosis in other situations (for example, see reference 10). The inconsistent effects of these agents on apoptotic cell death reflect the diverse mechanisms of apoptosis induction in different cell types. Our results show that CHX and ActD induce a strong apoptotic cell death in the same Ramos cell model. In contrast to ionophore-induced apoptosis which triggers an early gene signalling cascade, these drugs induce apoptosis apparently through different pathways.

The present studies have also shown that over-expression of Bcl-2 can inhibit apoptosis of Ramos B cells induced in response to different apoptosis-inducing agents, consistent with the view that Bcl-2 blocks a final common pathway for programmed cell death (16). It is possible that Bcl-2 plays a role as one of "survival" factors which negatively control the pre-existent apoptotic machinery. The apoptosis-inducing effect of the macromolecular synthesis inhibitors may be mediated through inhibiting Bcl-2 production, at least as one of their major targets. This view is supported by the observations that CD40-mediated protection of apoptosis induced by inhibitors of macromolecular synthesis is correlated closely with the up-regulation of Bcl-2 expression in Ramos B cells (Ning et al., submitted).

Thus, a simple model for controlling apoptotic cell death in the Ramos B cell line can be drawn from the present studies: The molecular machinery required for apoptosis is constitutively expressed in overtly healthy cells. This machinery is normally negatively controlled by "survival" factors and can be activated in other circumstances by triggering signals coming from outside or inside the cell. Calcium ionophore triggers an early gene signalling cascade, which activates the apoptotic machinery and leads the cell to die by apoptosis (13, and Ning et al., submitted). On the other hand, inhibitors of macromolecular synthesis induce cell apoptosis by blocking or lowering the concentration of "survival" factors, such as Bcl-2, which normally inhibit the apoptotic machinery.

Acknowledgments: We are grateful to Dr. S. Cory for the bcl-2 vector, p Zen *bcl-2* SVneo. We also thank Dr. L. Fairbain for helpful discussions and advice. This work was supported by the U.K. Medical Research Council and Cancer Research Campaign. Z.N. holds a K.C. Wong scholarship.

References

1. Cohen, J. J. (1993) Immunol. Today 14, 126-130.
2. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992). Cell 69, 119-128.
3. Smeyne, R. J., Vendrell, M., Hauward, M., Baker, S. J., Miao, G. G., Schilling, K., Robertson, L. M., Curran, T., and Morgan, J. I. (1993). Nature 363, 166-169.
4. Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994). Nature 367, 277-281.
5. Bolse, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson C. B. (1993). Cell 74, 587-608.

6. Lin, E., Orlovsky, A., Berger, M. and Prystowsky, M. (1993). J. Immunol. 151, 1979-1988.
7. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993). Proc. Natl. Acad. Sci. USA, 90, 3516-3520.
8. Martin, D. P., Schmidt, R. E., DiStefano, P. S., Lowry, O. H., Carter, J. G., and Johnson, E. J. (1988). J. Cell Biol. 106, 829-844.
9. McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H., and Orrenius, S. (1989). Arch. Biochem. Biophys. 269, 356-370.
10. Martin, S. J., Lennon, S. V., Bonham, A. M., and Cotter, T. G. (1990). J. Immunol. 145, 1859-1876.
11. Collins, R. J., Harmon, B. V., Souvlis, T., Pope, J. J., and Kerr, J. F. R. (1991). Br. J. Cancer 64, 518-522.
12. Jacobson, M. D., Burne, J. F., and Raff, M. C. (1994). EMBO J. 13, 1899-1910.
13. Ning, Z.-Q., and Murphy, J. J. (1993). Eur. J. Immunol. 23, 3369-3372.
14. Murphy, J. J., and Norton, J. D. (1990). Biochim. Biophys. Acta 1049, 261-271.
15. Vaux, D. L., Cory, S., and Adams, J. M. (1988). Nature 335, 440-442.
16. Reed, J. C. (1994). J. Cell Biol. 124, 1-6.
17. Vaux, D. L., Aguila, H. L., and Weissman, I. L. (1992). Int. Immunol. 4, 821-826.
18. Cuende, E., Ales-Marines, J., E., Ding, L., Gonzalez-Garcia, M., Martinez-A, C., and Nunez, G. (1993). EMBO J. 12, 1555-1560.