Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 84-90

# BCL-2 OVEREXPRESSION ABOLISHES EARLY CALCIUM WAVING PRECEDING APOPTOSIS IN NIH-3T3 MURINE FIBROBLASTS

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Received July 29, 1994

**SUMMARY:** Overexpression of the apoptosis protection gene bcl-2 abolished the earliest response to serum withdrawal in NIH-3T3 murine fibroblasts, *i.e.*, the abrupt cytoplasmic free calcium drop. This phenomenon, also observed in a myeloid cell line, led us to propose this ionic waving as an early apoptotic signal. Its abolition by bcl-2 overexpression suggests that this gene plays a role also on early events "priming" apoptosis. 

**INTRODUCTION.** Bcl-2 is the prototype member of a family of genes crucial to the decision between cell life or death (1), as they exhibit the property of protecting from apoptosis, which is conserved in evolution from nematodes to man (2).

An intence research, stimulated by the extensive incidence of bcl-2 overexpression in human follicular lymphomas (3), provided evidences for a number of different mechanisms of action of bcl-2. The p26 bcl-2-encoded protein (p26) exhibits two main influences on cell metabolism, *i.e.* involvement in calcium homeostasis (4,5) and free radicals scavenging and antioxidant activity (6,7). The two activities might be cooperative, under the light of the relationships between oxidants and calcium release from mitochondrial stores (8); this proposal is also supported by the preferential mitochondrial distribution of the p26 (9).

Data on calcium waving so far collected are very hardly comparable, due to the different role of this ion in different apoptotic systems. For instance, the rapid apoptotic death induced by corticosteroids in thymocytes is accompanied by a very

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0006-291X/94 $5.00

early and sustained calcium rise (10), whereas the apoptosis induced by trophic factors withdrawal in nervous tissue (11,12,13) is suppressed by cytoplasmic calcium elevation. These apparently controversial evidences, led to the proposal of a "calcium set point hypothesis" (14) and stimulated the extension of studies on calcium waving to a variety of apoptotic systems (reviewed in 15). We recently found that apoptosis inducted in 32D murine myeloid cells by interleukin-3 (IL-3) withdrawal was preceded by an early drop of intracellular free calcium level, followed by a progressive and marked rise accompaning the fatal final events (cell shrinkage, nuclear fragmentation, apoptotic bodies, DNA ladder, etc.) (2). On this basis, we proposed this early calcium drop as an event "priming" 32D cells to apoptosis.

In this report, we show that in a NIH-3T3 clone very sensitive to apoptosis following serum withdrawal, the latter induces an early calcium drop (similar to that observed in 32D cells), which is completely suppressed when bcl-2 is overexpressed. This suppression supports the hypotesis that this ionic drop may be involved in the apoptotic "priming" in the same cells.

**METHODS.** NIH-3T3 cells were obtained by Dr. S.A. Aaronson, Bethesda, Maryland, USA. Clones were selected for accelerated apoptosis following serum withdrawal. The clones used for this studies (LM) undergoes apoptosis within 20 hours of starvation, as revealed by characteristic morphological changes. Agarose DNA electrophoresis did not show any DNA degradation, as visualized by either ladder or smear.

Cell fractionation was performed as reported (16)

Endocellular free calcium was measured as reported (4). The human bcl-2 construct was a gift of Dr. Y. Tsujimoto, University of Osaka, Japan. Transfection, antibiotic selection and cloning were carried out as reported (17). The overexpression of the gene was confirmed by western blot analysis (17) using a monoclonal antibody anti human p26, a gift of Dr. Delia (Istituto Nazionale dei Tumori, Milano, Italy) and a chemioluminescent system (Amersham, U.K.).

**RESULTS AND DISCUSSION.** Fig.1 shows the expression of the human bcl-2 gene product, as revealed by western blot in different subcellular fractions (nucleus, mitochondria, microsomes) of four independently selected LM clones (numbered 1 to 4). Clones 3 and 4 showed the highest expression of p26, without preferential localization to any of the cellular fractions studied. Clones 1 and 2 exhibited a much lower and uneven expression of Bcl-2. Interestingly, this expression appeared highest in the microsomes and lowest in the nucleus, suggesting that the microsomal fraction may be the first one in which p26 is accumulated.

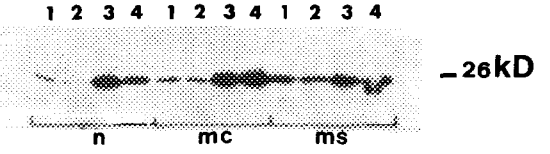


Figure 1. Western blot analysis of p26 expression in various cellular fractions of different (1 to 4) LM-bcl-2 clones. **n**: nucleus; **mc**: mitochondria; **ms**: microsomes. Cell fractionation was carried out as described (19).

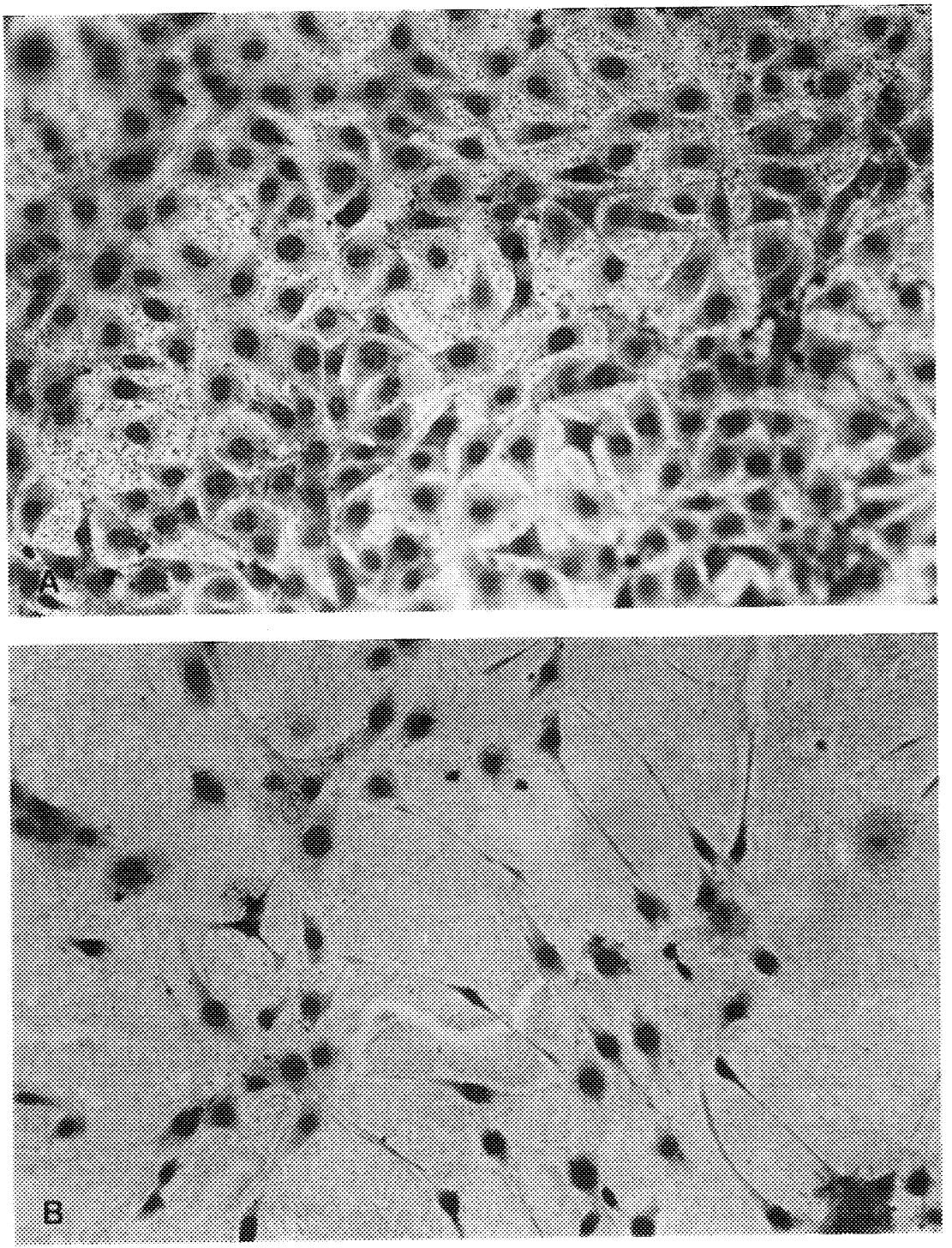


**Figure 2.** Morphological features of LM wt cells in the presence of 10% serum (**A**) and following 20 hs of serum withdrawal (**B**).

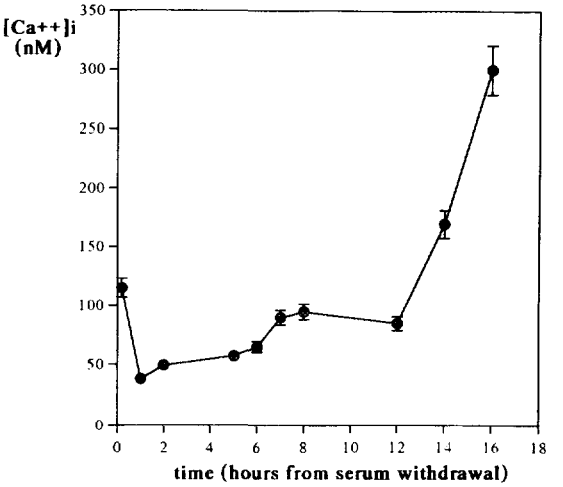


**Figure 3.** Intracellular calcium waving in FCS-starved LM cells. LM cells were grown routinely (DMEM supplemented with 10% FCS). At time 0 cells were washed and incubated with the standard medium. Endocellular calcium was measured at the indicated times in triplicate samples.

Clones 3 and 4 were used to study the effects of serum withdrawal on the induction of morphological signs of apoptosis and intracellular free calcium levels.

Fig.2 shows that LM cells clearly develop, after 18/20 hours of serum withdrawal, morphological features of apoptosis (revealed by Giemsa staining), including shrinking, nuclear picnosis and fragmentation, cell blebbing and appearance of apoptotic bodies. In spite of a clear transglutaminase increase, DNA degradation did not occur (data not shown). However, recent views on the multiplicity of the apoptotic pathways have stressed that DNA degradation is not an obligated feature of apoptosis in eukariotic cells (15).

Fig.3 shows that in response to serum withdrawal (1hr), intracellular free calcium decreased, from 120 to 40 nM. This drop was followed by a progressive rise, so that calcium concentration approaching the micromolar range when cells showed evident signs of apoptosis (data not shown). On the contrary, bcl-2-LM cells were not only protected from apoptosis (Fig.4), but also insensitive to serum withdrawal in terms of early calcium waving (Fig.5).

On the whole, the free calcium drop observed in murine myeloid (4), as well as in fibroblast-like cell lines (this report), in response to growth factor withdrawal, is very likely to represent an important early apoptotic signal. This proposal is in agreement with the finding that calcium waving is completely abolished by the overexpression of the apoptosis protector gene bcl-2. This finding also allows to hypothesize that the role of Bcl-2 may not be limited to late antioxidant and calcium increasing roles. On the contrary, Bcl-2 is likely to interfere with the generation of early signals priming cells to apoptosis. Interestingly, very recently Fernandez-Sarabia and Bischoff (1994) (18) showed that p26 associates with the *ras*-related protein $p23^{R\text{-}ras}$. The finding would very interestingly link the effect of p26 with the block of G protein-mediated signals,

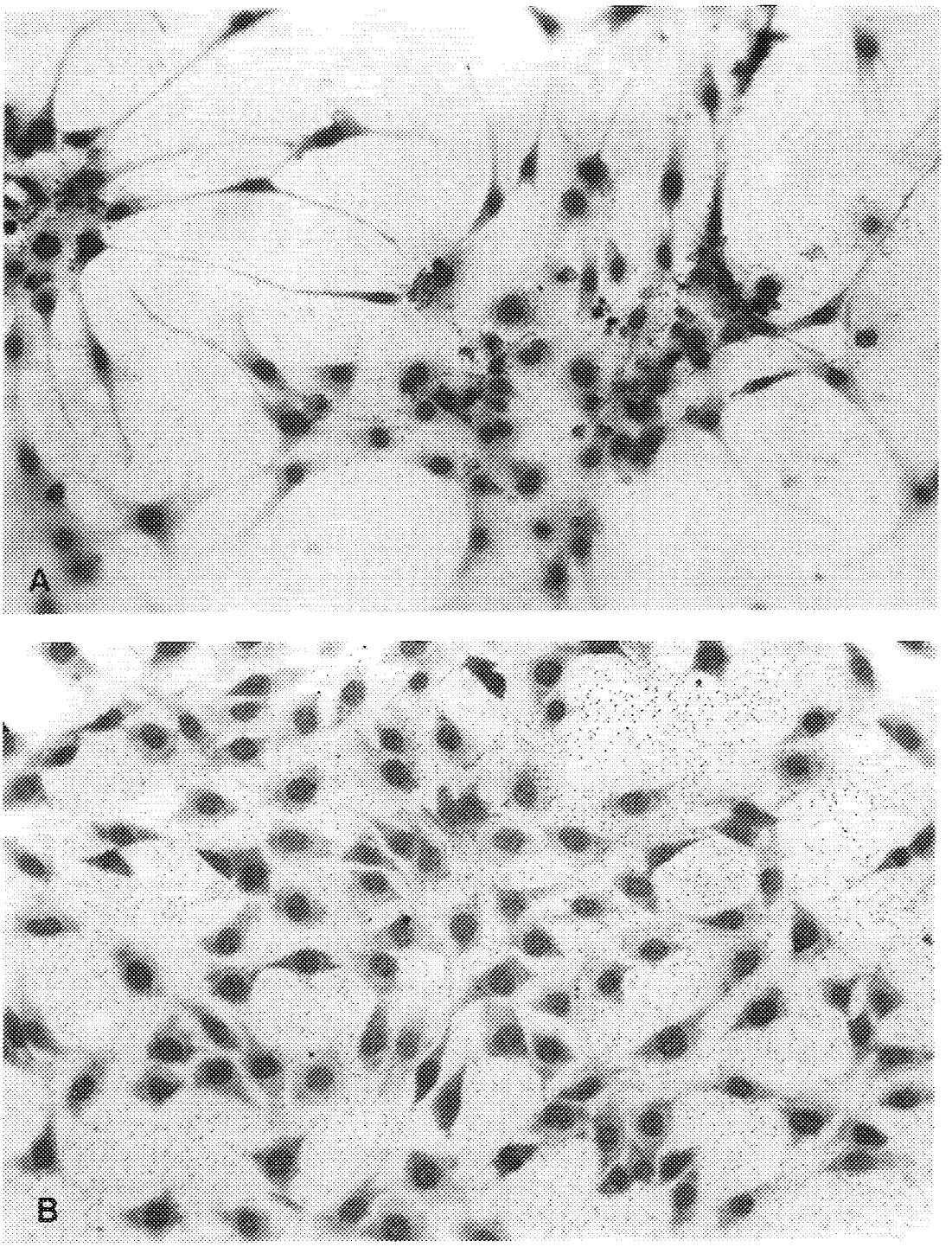


**Figure 4.** Morphological features of control LM (**A**) and bcl-2-LM cells (**B**) following 24 hs of serum withdrawal.

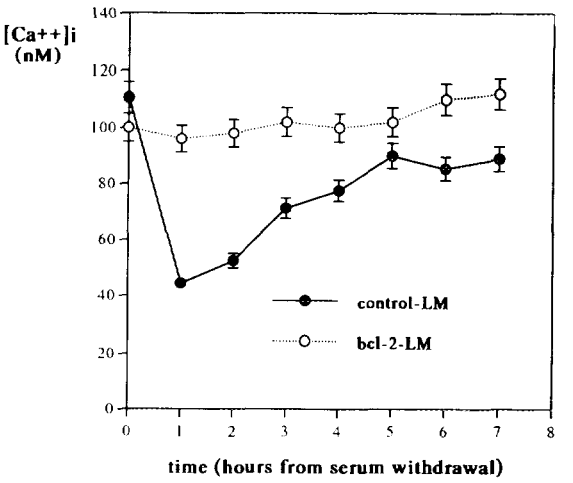


Figure 5. Intracellular calcium waving in FCS-starved control LM (●) and bcl-2-LM cells (○). Points in plot are means of triplicate samples. Data on bcl-2-LM cells were obtained from two different (4 and 5) clones.

which are of basic importance in the regulation of mitogenesis and differentiation. Moreover, a further relationship between "the *ras* affair" and apoptosis has been shown by Fath et al. (1994) (19). The Authors isolated a $GRB_2$ (the $SH_2/SH_3$-containig domains adaptor of GF receptors to SOS/Ras) isoform, called $GRB_3$, which induces apoptosis when microinjected into fibroblasts.

Therefore, it is not to be excluded that, in the still misterious mechanism of action of bcl-2, the very early apoptosis-inducing effect of Bcl-2 is G protein-mediated and that the other effects of p26 on calcium and antioxidant activity are secondary cell responses.

**ACKNOWLEDGMENTS.** This work was supported by ACRO (CNR, Rome, Italy) and AIRC (Milan, Italy). Lucia Magnelli has an AIRC fellowship, Marina Cinelli and Alessandra Turchetti have FIRC fellowships.

## REFERENCES

1. Williams, G.T. and Smith, C.A. (1993) Cell. 74: 777-779.
2. Vaux, D.L. (1993) Proc. Natl. Acad. Sci. U.S. 90: 786-789.
3. Korsmeyer, S. J. (1992) Blood 80: 879-886.
4. Magnelli, L.,Cinelli M.,Turchetti,A. and Chiarugi,V.(1993) Bioch. Biophys.Res.Comm.194: 1394-1397.
5. Baffy, G., Miyashita, T., Williamson, J.R. and Reed, J.C. (1993) J.Biol.Chem. 268: 6511-6519.5.
6. Hockembery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L. and Korsmeyer, S.J.(1993) Cell. 75: 241-251.
7. Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord,T.and Bredesen , D.E. (1994) Science.262:1274-1277.

8. Richter, C. (1993) FEBS lett. 325: 104-107.
9. Hockembery, D.M., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Nature. 348: 334-336.
10. McConkey, D.J., Hartzell, P., Nicotera, P. and Orrenius,S.(1989) FASEB J. 3: 1843-1849.
11. Koike, T., Martin, D.P. and Johnson, E.M. (1989) Proc. Natl. Acad. Sci. U.S. 86: 6421- 6425.
12. Koike, T. and Tanaka, S. (1991) Proc. Natl. Acad. Sci. U.S. 88: 3892-3896.
13. Koh, J.Y. and Cotman, C.W. (1992) Brain Res. 587: 233-240.
14. Cambray-Deakin,M.A.and Burgoye,R.D.(1992) Dev.Brain. Res. 66:25-32.
15. Reed, J.C. (1993) J.Cell.Biol. 124:1-6.
16. Reid, E. and Williamson, R. (1974) Methods in Enzymology. XXXI:713-733.
17. Tsujimoto, Y. and Croce, C. (1986) Proc. Natl. Acad. Sci. U.S. 83: 5214-5218.
18. Fernandez-Sarabia, M.J. and Bischoff, J.R. (1994) Nature. 366: 274-275.
19. Fath, I., Schweighoffer, F., Rey, I., Multon, M.C., Boiziau, J., Duchesne M.and Tocquè, B. (1994) Science. 264: 971-974.