

<

ANNEXIN V AND COMET ASSAY DETECTION OF VIOLACEIN-INDUCED APOPTOSIS

Pedro H.P.A. Schildknecht* and Benedicto C. Vidal

¹*Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, Campinas, SP 13085-970, Brazil.*

* To whom correspondence should be addressed: pedroh@unicamp.br or pedroh@operamail.com.
Phone +551937886124, Fax +551937886111.

Abstract

The apoptosis triggered by violacein in V79 cells was assessed using annexin V/propidium iodide labeling and the Comet assay. No necrosis was detected by propidium iodide staining. Apoptotic nuclei were labeled by annexin V, and the results agreed with those with the Comet assay in weak alkaline solution. Characteristic apoptotic nuclear phenotypes were obtained from violacein-treated cells just lysis step. The use of annexin V/propidium iodide staining together with the Comet assay provided a fast, reliable means of characterizing cell death. Chromatin fragmentation and the externalization of phosphatidylserine were visualized by annexin V/propidium iodide staining after less than 48 h of exposure to violacein.

Keywords: annexin-v, apoptosis, comet assay, single cell gel eletrophoresis (SCGE), violacein.

Introduction

The morphological characteristics of apoptosis include cell shrinkage, membrane blebbing, nuclear condensation and the emergence of apoptotic bodies (Wyllie I *et al* 1981, Palardy *et al* 1997, Raffray & Cohen 1997, Maria *et al* 2000). In contrast to necrosis, apoptosis is an active process with well organized, regulated biochemical events, involving cell signaling and ordered enzyme cascades (Raffray & Cohen 1997).

The treatment of currently incurable human cancers and the improvement of therapy for other diseases, such as Chagas' disease, represent important applications for knowledge about apoptosis. Rather than promoting a general, irretrievable collapse of cellular homeostasis and necrosis, as usually occurs with chemotherapeutics, apoptosis-triggering drugs such as violacein (Melo *et al* 2000) induce less pronounced tissue damage but with a similar therapeutic effect .

Apoptosis has been detected by agarose gel electrophoresis , as well as histochemical (Wyllie I *et al* 1980, Koopman *et al* 1994, Mello 1999), cytochemical (Singh *et al* 1988, Singh 2000) and immunochemical (Gavrieli *et al* 1992, Mello *et al* 2000) methods as discussed by Singh (2000). In this work, violacein-induced apoptosis was detected using the Comet assay and annexin V labeling. This approach provided a fast, accurate method for assessing chromatin fragmentation and biochemical modifications present in apoptotic cells.

Results and Discussion

Violacein, a pigment produced by *Chromobacterium violaceum*, is a potential drug for treating leukemia, lymphoma cells and Chagas' disease (Melo *et al* 2000). The antitumoral and apoptotic-promoting activities, as well as the National Cancer Institute assays for validation of violacein were described by Melo *et al* (2000). Based on these results, violacein is a useful tool for studying apoptosis.

The labeling of apoptotic cells with annexin V conjugated to fluorescein isothiocyanate is very useful for detecting apoptosis (Koopman *et al* 1994). Annexin V is a Ca^{2+} -dependent protein with a strong affinity for phosphatidylserine (PS), which is externalized in the early stages of apoptosis (Koopman *et al*, 1994). In non-apoptotic cells, PS is present only on the inner surface of the membrane and false positive results may occur if the membrane is damaged, as occurs in necrotic cells. Cell damage and false positive PS labeling were assessed by staining with propidium iodide which does not enter an intact cell.

Positive labeling by annexin V (indicative of apoptotic cells) was rare in control culture. The apoptotic cells were usually detached from the coverslip, probably as a result of cell-cell interactions (Park *et al* 1999, Maria *et al* 2000). Violacein-treated cells were round and smaller than control cells, and showed no cytoplasmic protrusions (Fig. 1). The cell shrinkage observed was typical of apoptotic cells (Loo & Rillena, 1998, Maeno *et al* 2000). In agreement with a previous report (Melo *et al* 2000), no micronuclei was seen; this absence appears to be peculiar to violacein toxicity. About 50% of the cells treated with violacein

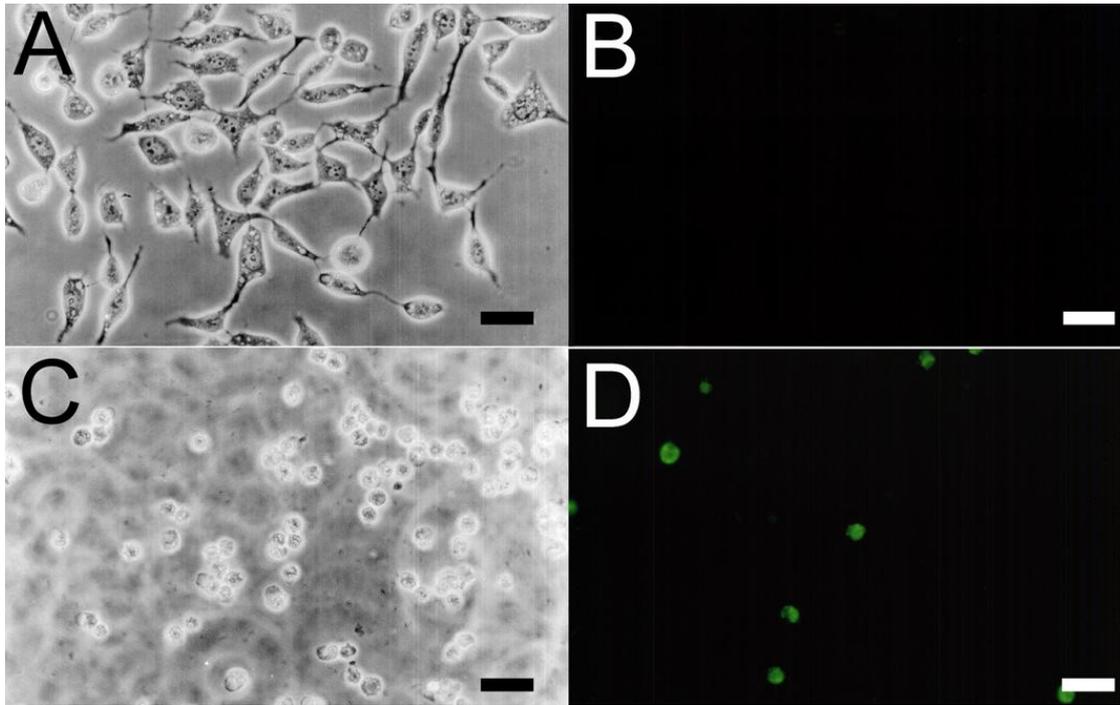


Figure 1: Annexin V / propidium iodide labeled V79 cells under phase contrast (A, C) and fluorescence (B, D) microscopy for apoptosis detection . Apoptotic cells were not detected in control cells (A, B), but only in violacein–treated cells (C, D). Necrosis was not observed in both control and treated cells. Bar = 50 μ m.

were positive for annexin V labeling, but no necrosis was observed after propidium iodide staining of control or violacein–treated cultures. The possibility of triggering apoptosis without necrosis reinforces the usefulness of violacein as a therapeutic agent and a tool in apoptosis research.

The Comet assay (single cell gel electrophoresis) is a fast, reliable method for detecting DNA damage in individual cells (Singh 1988, Olive *et al* 1992, Klaude *et al*, 1996). Alkaline lysis followed by a weak alkaline medium for DNA unwinding and electrophoresis enhanced the sensitivity of the test (data not shown), in agreement with Olive *et al* (1992). Good results were also obtained if TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8) was used instead of alkaline buffer during electrophoresis (data not shown).

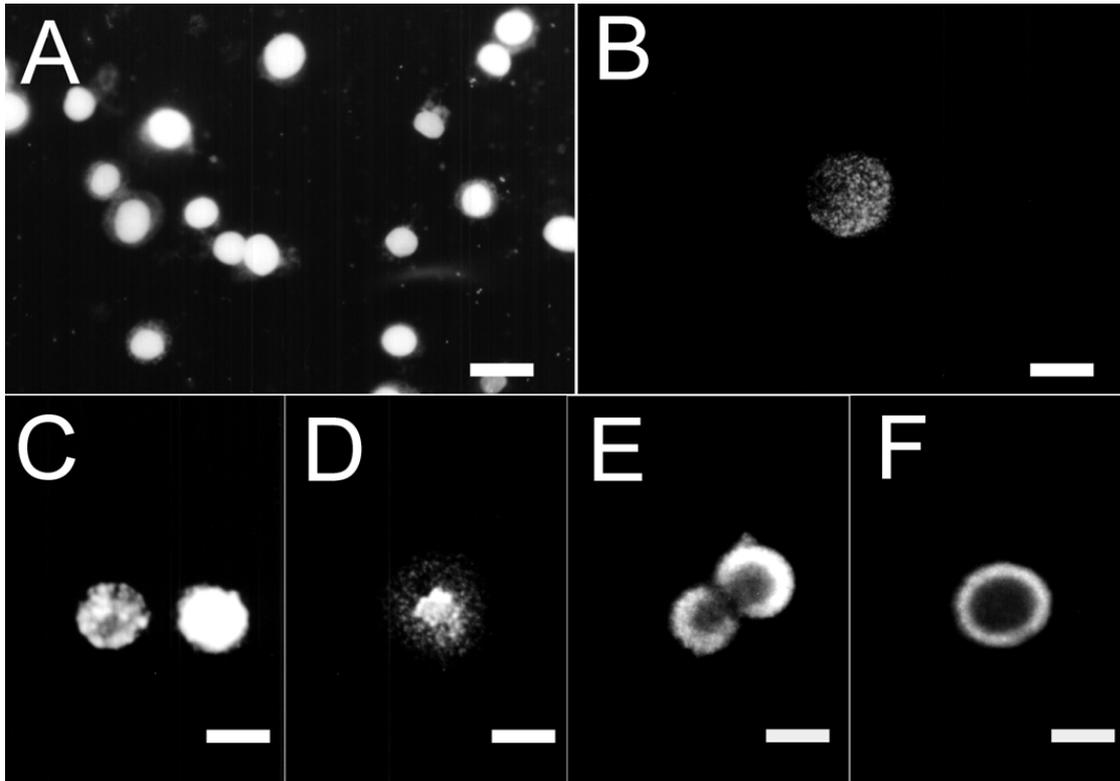


Figure 2: V79 cells after the alkaline lysis step stained with propidium iodide. In control cells (A), a DNA halo is observed around some nuclei while in violacein-treated cells (B–F) only condensed chromatin could be seen displayed in small granules or in nuclear periphery. Bar = 20 μ m.

After lysis and chromatin unwinding, the nuclei of control cells were uniformly stained by propidium iodide, with small "halos" of DNA (Fig. 2). The "halo" phenotype resulted from the removal of histone by the high-salt concentration lysis solution which allowed chromatin loops to disperse within and outside the nuclei while still attached to nuclear matrix proteins and to the nuclear envelope (Olive *et al* 1992, Vidal 2000). Violacein-treated cells had a granular appearance and the higher level of DNA condensation meant there were no halos (Fig. 2). The apoptotic nature of violacein-treated cells was clearly seen in some nuclei showing the marginalization of chromatin at the periphery of the nuclear envelope (Fig. 2). The extent of chromatin diffusion in agarose gels was also useful for distinguishing healthy cells from apoptotic and necrotic cells, as reported by Singh (2000).

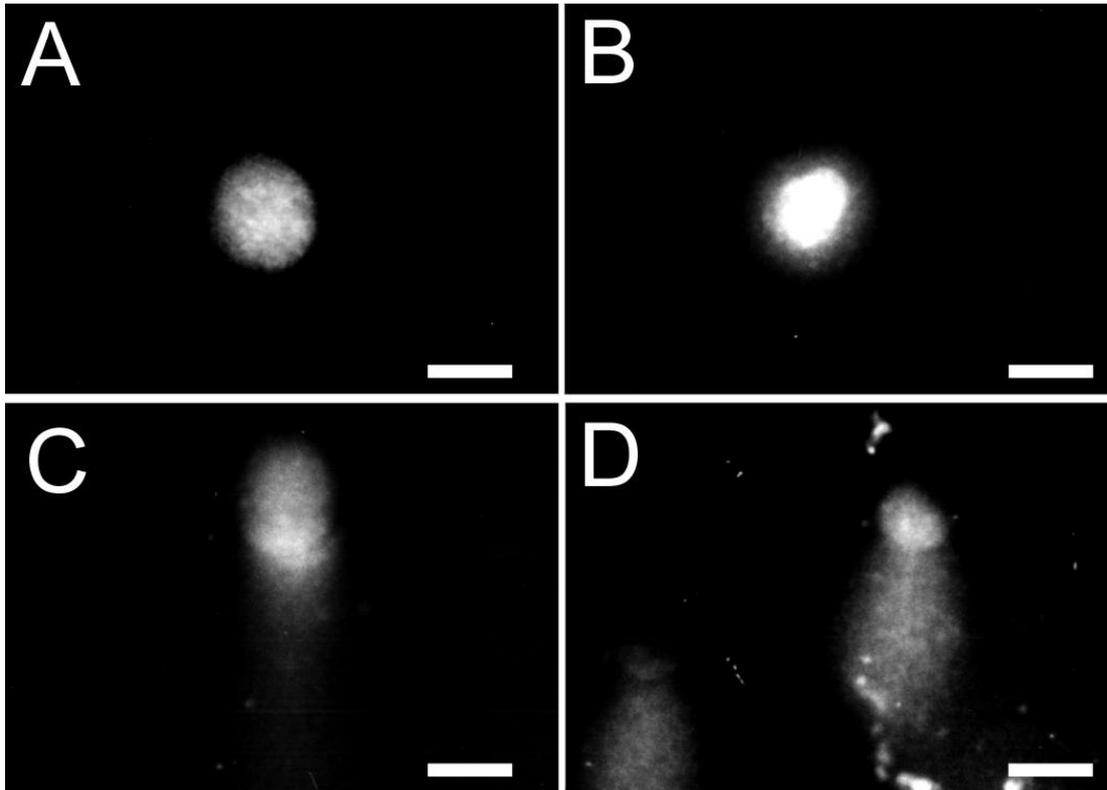


Figure 3: V79 nuclei subjected to Comet test and stained with propidium iodide. The nuclei can be classified by the phenotypes: type 1 (A) and type 2 (B) retract healthy nuclei while type 3 (C) and type 4 (D) are regarded to apoptotic nuclei. Bar = 20 μ m.

Apoptosis was also assessed after electrophoresis with the cells being classified according to their tail extent, as described in experimental procedures (Fig. 3). Although some subjectivity is involved in classifying nuclei as type 1 or 2 or as type 3 or 4, it is unlikely that a normal nucleus (type 1 and 2) was mistakenly identified as apoptotic (type 3 or 4) or vice versa, because of the marked difference in the nuclear appearance of these two groups. Thus, the interpretation of the Comet assay without the need for image analysis makes this a fast, reliable and non-expensive method.

The Comet assay showed that control cells had few nuclei with fragmented chromatin whereas a high number of nuclei had no detectable tail (type 1 and 2) (Fig. 4). Apoptotic nuclei (type 3 and 4) were more frequent in violacein-treated cells. These results reinforce those obtained with annexin V labeling and confirm previous data suggesting that the expressive chromatin fragmentation in violacein-treated cells was a result from apoptosis (Melo *et al* 2000).

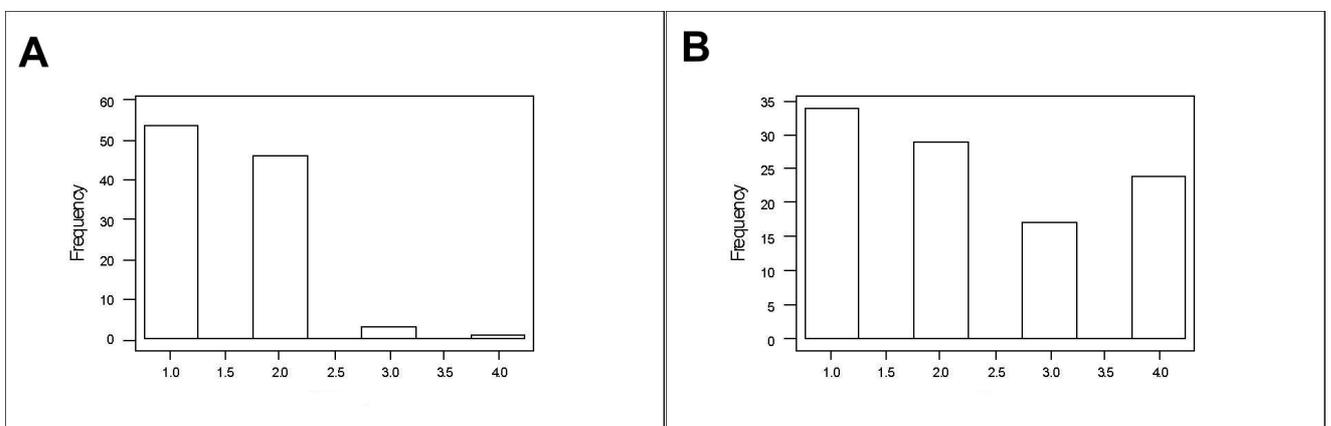


Figure 5: Frequency histogram of V79 cells subjected to Comet test and classified by phenotypes. Control cells (A) showed a low frequency of apoptotic nuclei (types 3 and 4), in contrast to violacein treated cells (B).

Conclusions

Our results showed that violacein was a potent inducer of apoptosis and its use in apoptosis research is recommended. Annexin V and the Comet assay provide a fast and efficient tools for studying apoptosis in animal cells and may be of diagnostic use in the future.

Experimental procedures

Cell culture. For annexin V labeling, V79 Chinese hamster lung fibroblasts (clone M-8) were plated on coverslips in 5 ml of Dulbecco's modified Eagle medium containing antibiotics (100 U penicillin/ml, 100 µg streptomycin/ml) and supplemented with 10% fetal calf serum, in a 5% carbon dioxide-humidified atmosphere at 37° C. After 48 h, the cells were treated with 2 µM violacein, (generously supplied by Dr. N. Durán, Institute of Chemistry, UNICAMP) for 24 h. For further details, see Melo *et al* (2000).

For the Comet assay, V79 cells were grown in culture bottles and treated with violacein in the same conditions as described above.

Annexin V labeling. The coverslips with V79 cells were washed twice in 0.05 M phosphate-buffered saline (PBS) and immediately incubated with annexin V and propidium iodide in HEPES buffer as recommended by the manufacturer (Boehringer-Mannheim, Germany). The observations were done using live cells in an Axiophot fluorescence microscope (Zeiss, Germany) with an excitation filter of 515–560 nm and a barrier filter of 590 nm. For longer analysis, the cells were maintained in a humidified chamber for up to 1 h.

Comet (SCGE) assay. After treatment, the cells were trypsinized, washed in ice-cold PBS containing calcium and resuspended in calcium-free ice-cold PBS. The cell suspension was mixed with an equal amount of 1% low melting point agarose kept at 37° C. Immediately after mixing, 100 µl of the suspension was pipetted on to microscope slides pretreated according to Klaude *et al* (1996), then covered with a 25 mm x 50 mm parafilm coverslip and placed on a glass tray on ice. The parafilm was removed after the agarose had set and the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with freshly added 1% Triton X-100 and 2% DMSO) followed by incubation at 4° C for at least 1 h. The electrophoresis in weak alkali (0.03 M NaOH, 1 mM EDTA, pH 12.1) at 1 V/cm and 30 mA for 15 min was preceded by a 20 min immersion of the slides in the electrophoresis buffer to promote chromatin unwinding.

After electrophoresis, the slides were neutralized in 0.05 M Tris buffer pH 8.0, rinsed in distilled water, fixed in methanol, air dried and stored. DNA was stained with propidium iodide (20 µg/ml) for 10 min, washed in distilled water and examined in a fluorescence microscope (Zeiss Axiophot).

Apoptosis was estimated by analyzing 200 nuclei from each treatment (50 nuclei per slide). Nuclear phenotypes identified according to their tail length (Fig. 3): type 1—no tail or DNA diffusion halo, type 2—no tail but with a DNA diffusion halo, type 3—a tail and a DNA diffusion halo, and type 4—a tail and not a DNA diffusion halo. Type 3 and 4 were markers for the early and late apoptotic stages, respectively.

Acknowledgements

This work was supported by grants from FAPESP (98/00471-1 and 00/01658-0). The authors thank Dr. N. Durán (IQ-UNICAMP) for kindly providing the violacein, Dr. Haun and Dr. Melo (IB-UNICAMP) for the cell culture maintenance, and Dr. S. Hyslop for revising the language of the manuscript.

References

- A.H. Wyllie, G.J. Beathe, A.D. Hargreaves.** Chromatin changes in apoptosis. *Histochem. J.* 13 (1981) 681–692.
- S.S. Maria, B.C. Vidal, M.L.S. Mello.** Image analysis of DNA fragmentation and loss in V79 cells under apoptosis. *Gen. Mol. Biol.* 23 (2000) 109–112.
- M. Pallardy, M. Perrin–Wolff, A. Biola.** Cellular stress and apoptosis. *Toxicol. In vitro* 11 (1997) 573–578.
- M. Raffray, G.M. Cohen.** Apoptosis and Necrosis in Toxicology: A continuum or distinct modes of cell death? *Pharmacol. Ther.* 75 (1997) 153–177.
- P.S. Melo, S.S. Maria, B.C. Vidal, M. Haun, N. Duran.** Violacein cytotoxicity and induction of apoptosis in V79 cells. *In Vitro Cell Dev. Biol. Animal* 36 (2000) 539–543.
- A.H. Wyllie, J.F.R. Kerr, A.R. Currie.** Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68 (1980) 251–306.
- L.F. Barbisan, M.L.S. Mello, J. Russo, B.C. Vidal.** Apoptosis and catastrophic cell death in benzo[a]pyrene–transformed human breast epithelial cells. *Mutat. Res.* 431 (1999) 133–139.
- N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider.** A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* (1988) 184–191.
- N.P. Singh.** A simple method for accurate estimation of apoptotic cells. *Exp. Cell Res.* 256 (2000) 328–337.
- M.L.S. Mello, S.S. Maria, P.H.P.A. Schildknecht, N.A. Grazziotin.** DNA fragmentation in programmed cell death in nucleate erythrocytes: a cytochemical analysis. *Acta Histochem. Cytochem.* 33 (2000) 355–359.
- Y. Gravieli, Y. Sherman, S.A. Bem–Sasson.** Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119 (1992) 493–501.
- M.L.S. Mello.** Discrimination of Feulgen–stained apoptotic nuclei by image analysis. *Proc. Am. Assoc. Cancer Res.* 40 (1999) 691 (Abstract).
- G. Koopman, C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals.** Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 85 (1994) 332–340.
- M. Klaude, S. Eriksson, J. Nygren, G. Ahnström.** The comet assay: mechanisms and technical considerations. *Mutat. Res.* 363 (1996) 89–96.
- P.L. Olive, D. Wlodek, R.E. Durand, J.P. Banáth.** Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp. Cell Res.* 198 (1992) 259–267.
- B.C. Vidal.** Extended chromatin fibres: crystallinity, molecular order and reactivity to concanavalin–A. *Cell Biol. Int.* 24 (2000) 723–728.