Physiol. Res. 46: 161 – 164, 1997

SHORT COMMUNICATION

Estimation of Apoptosis in C6 Glioma Cells Treated with Antidepressants

A. ŠPANOVÁ[1], H. KOVÁŘŮ[2], V. LISÁ[3], E. LUKÁŠOVÁ[4], B. RITTICH[5]

[1]Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Brno, [2]First Medical Faculty, Charles University, Prague, [3]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, [4]Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno and [5]Faculty of Chemistry, Technical University, Brno, Czech Republic

Received June 14, 1996
Accepted December 20, 1996

Summary

Gel electrophoresis of DNA was used for estimation of DNA changes caused in C6 glioma cells by treatment with psychotropic drugs (imipramine, amitryptiline and fluoxetine). Some discrete bands containing a population of short DNA fragments appeared after 1 and 5 days of cultivation. Apoptotic DNA breaks were verified at single cell level using the TUNEL test in cells treated with fluoxetine.

Key words

Glioma cells – Antidepressants – DNA fragmentation – Apoptosis

Necrosis and apoptosis are two fundamentally different modes of cell death (Kerr *et al.* 1972). Necrosis occurs following major intoxications or extensive tissue trauma. Apoptosis, on the other hand, is a more physiological mode of cell death observed during processes such as tissue organization. Cell dying *via* apoptosis requires activation of specific genes. It can be induced by non-physiological stimuli (such as irradiation and anticancer drugs) and also by physiological stimuli such as cytokines (Hacker and Vaux 1994, Musilová *et al.* 1995, Kozubík *et al.* 1995). The production of many cytokines in the brain is dramatically changed by various stimuli during systemic or CNS diseases (Rothwell and Hopkins 1995, Kozáková *et al.* 1996). For this reason, we were interested in the question of whether antidepressant drugs can influence cell death in glioma cells.

A fundamental biochemical marker of apoptosis is the change in chromatin structure, often associated with fragmentation into oligonucleosomal fragments of about n x 180 base pairs in length. Apoptosis is a highly ordered process and consists of several successive steps (Walker *et al.* 1993). A major step in apoptosis is the destruction of the higher structure of chromatin by the introduction of double-strand breaks into DNA at 300 kb and 50 kb intervals. The 50 kb fragments are further degraded, in some but not all cells, to smaller fragments and release small oligonucleosome fragments that are recognized as the characteristic DNA ladder on conventional agarose gels (Skalka *et al.* 1976, Allen *et al.* 1993, Sgove and Wick 1994).

Results were preliminary presented at "CNS – Advance in Research of Normal and Neoplastic Cells" which was held in Brno (April 25, 1996) as the satellite minisymposium of the 42nd International Congress of the European Tissue Culture Society (Španová et al. 1996).

Other techniques can be used for the detection of chromatin destruction besides agarose electrophoresis of DNA – e.g. ELISA or terminal transferase-mediated dUTP nick-end labelling – the TUNEL method (Gavrieli *et al.* 1992). The TUNEL method allows qualitative analysis of DNA fragmentation on microscopic slides. It enables to determine apoptosis in single cells. The aim of this work was to study if the antidepressant drugs generated DNA-linked changes in cells (i.e. apoptosis or necrosis).

The C6 glioma cells of rat origin were grown on a D-MEM medium complemented with 5 % of foetal bovine sera. Drugs were added to the medium to a final concentration $1x10^{-6}$ M and/or $5x10^{-6}$ M. The cells were cultivated for 1 day or 5 days (in the presence of the above mentioned drugs) at 37 °C. Microscopic cover slips were immersed into the medium and cultivated together with the cells. The cells grown on the surfaces of the slips were used in the TUNEL test. DNA was isolated from approximately $1x10^7$ cells using a lysis buffer (50 mM Tris, pH 8.0, 10 mM NaCl, 100 mM EDTA pH 8.0, 1 % SDS, 10 μg proteinase K). The lysis was carried out overnight at 37 °C (Špánová *et al.* 1995).

Gel electrophoresis was carried out in 1.8 % agarose at 1.5 V/cm using TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) as buffer (Maniatis *et al.* 1982). The gels were photographed after staining with ethidium bromide (1 μg/ml). In the TUNEL test, the slides with fixed cells were treated with terminal transferase according to the manufacturer and counterstained with propidium iodide (0.1 μg/ml) after washing. The slides with stained cells were observed under a Jenalumar microscope (Carl Zeiss, Jena, Germany) with dual band filters constructed for propidium iodide. The image was captured by a colour chilled high-resolution CCD camera C5310 (Hamamatsu, Japan) and digitized with a frame-grabber (MuTech, Japan).

Antidepressants (amitryptiline, imipramine and fluoxetine) as well as proteinase K were obtained from Sigma (St. Louis, Mo, USA). The chemicals for DNA isolation and electrophoresis (Tris-base, EDTA, SDS, agarose, EtBr) were obtained from Amresco (Solon, USA). The kit for the TUNEL test was from Boehringer (Mannheim, Germany).

DNA was isolated from C6 glioma cells treated with psychotropic drugs as described above and separated using gel electrophoresis. Agarose gel electrophoresis of DNA showed broad but discrete bands which contained a population of DNA fragments of 200–300 bp or 400–500 bp in length (Fig. 1). The DNA fragmentation was the same after 1 day and 5 days of cultivation. This type of DNA fragmentation did not correspond to the typical apoptotic or necrotic pattern: neither the characteristic apoptotic "ladder" (180 bp and their multiples) nor a necrotic smear of many fragments of different lengths were detected. The relative discrete character of bands serves as evidence against necrosis.

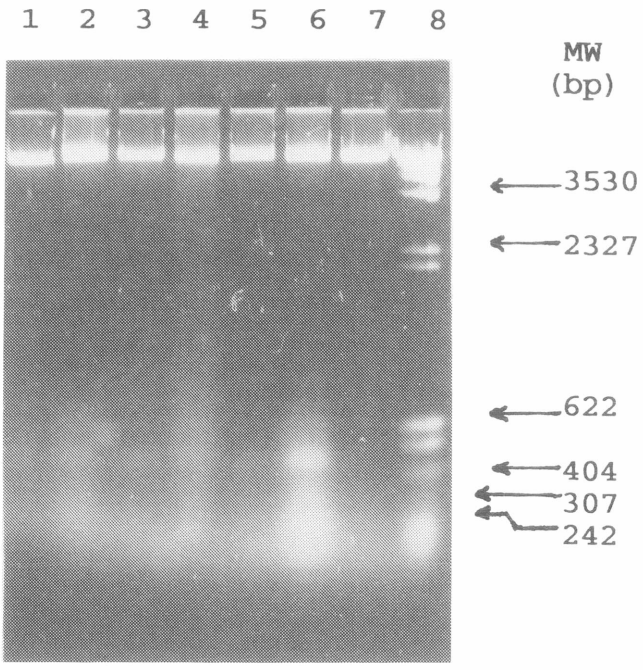

Fig. 1. *DNA fragmentation detected in C6 glioma cells after cultivation with imipramine ($5x10^{-6}$ M and $1x10^{-6}$ M, lanes 1 and 2), amitryptiline ($5x10^{-6}$ M and $1x10^{-6}$ M, lanes 3 and 4) and fluoxetine ($5x10^{-6}$ M and $1x10^{-6}$ M, lanes 5 and 6). Lane 7 contains DNA from control untreated cells. Lane 8 contains DNA molecular weight standards (λ/EcoRI, pBR322/MspI).*

In the literature, no-standard cases of apoptosis have been described. According to Collins *et al.* (1992), the apoptotic process was not always associated with oligonucleosomal DNA fragmentation in cells detected as apoptotic by morphological criteria. On the other hand, DNA fragmentation is random and smear of fragments occurs on agarose gel in necrosis. The absence of a DNA ladder may be influenced by the fact that the amount of fragmented DNA in the sample is below the detection limit of the analytical technique (electrophoresis on agarose gel). The amount of apoptotic fragments in the analysed sample is also influenced by the procedure employed for the lysis of the cells (Walker *et al.* 1993). In our procedure, SDS effectively deproteinated the DNA and enabled to reveal double stranded fragments.

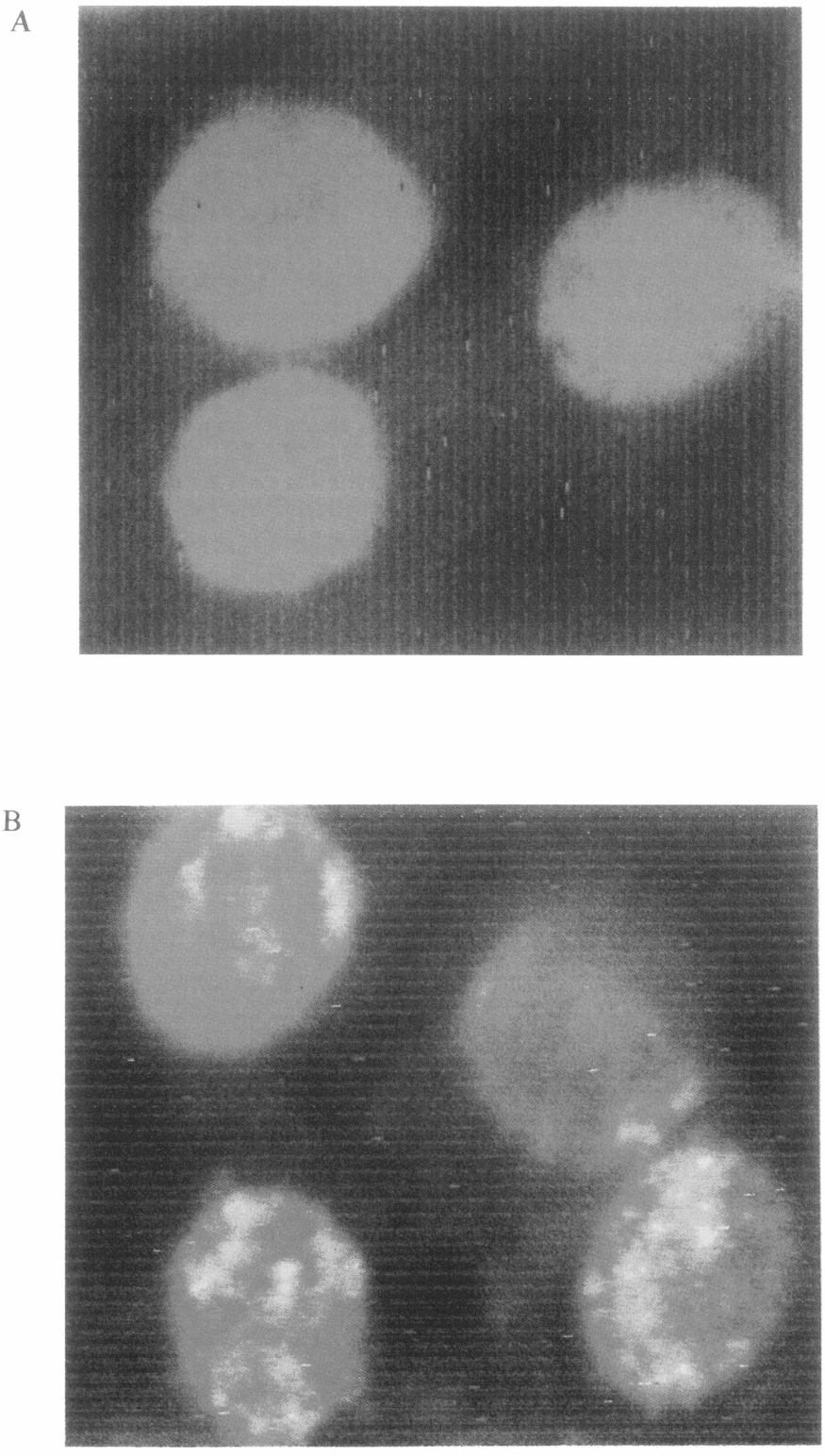

Fig. 2. *A. Control C6 glioma cells. B. Apoptotic DNA fragmentation detected by the TUNEL test in C6 glioma cells treated with fluoxetine (5 days, 5.10^{-6} M).*

The most pronounced DNA fragmentation was detected in C6 glioma cells treated with fluoxetine. From this reason, the apoptotic character of cell death in these cells was also confirmed at single cell level by the TUNEL method (Fig. 2). Positive labelling was detected in 2–3 % of treated cells as compared to the control cells (with no positive labelling in 200 cells).

The TUNEL method allows only a qualitative analysis of apoptosis. Another methods must be used for specification of necrotic DNA degradation.

It follows from the above mentioned results that psychotropic drugs provoked some DNA changes in C6 glioma cells. These changes were verified as apoptotic in cells treated with fluoxetine.

Acknowledgements
We would like to thank Mr. L. Červený for the language corrections of the manuscript. These results were partially supported by the grants of GA CR No. 309/95/1121 and 505/95/0357.

References

ALLEN P.D., BUSTIN S.A., MACEY M.G., JOHNSTON D.H., WILLIAMS N.S., NEWLAND A.C.: Programmed cell death (apoptosis) in immunity and haematological neoplasia. *Br. J. Biomed. Sci.* **50:** 135–149, 1993.

COLLINS R.J., HARMON B.V., GOBÉ C.C., KERR J.F.R.: Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Rad. Biol.* **61:** 451–453, 1992.

GAVRIELI Y., SHERMAN Y., BEN-SASSON S.A.: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119:** 493–501, 1992.

HACKER G., VAUX D.L.: Viral, worm and radical implications for apoptosis. *Trends Biochem Sci* **19:** 99–100, 1994.

KERR J.F.R., WYLLIE A.H., CURRIE A.R.: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26:** 239–257, 1972.

KOZÁKOVÁ H., KOVÁŘŮ H., MAREŠ V., KOVÁŘŮ F.: Low molecular cytokines of CNS and immune system. *Physiol. Res.* **45:** 3P, 1996.

KOZUBÍK A., HOFMANOVÁ J., MUSILOVÁ E., ŠIMEK V., VALETTE A.: The effects of transforming growth factor-β 1 on the condition of inhibited lipoxygenase metabolic pathway, on cell proliferation and differentiation. *J. Exp. Clin. Cancer Res.* **14:** 203–204, 1995.

MANIATIS, T., FRITSCH E.F., SAMBROOK J.: *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, 1982.

MUSILOVÁ E., ŠPANOVÁ A., HOLÁ J., KOZUBÍK A.: Changes in the cell cycle induced by phorbolesters, retinoic acid, dimethyl sulfoxide, transforming growth factor-1 and starvation can influence the response of promyelocytic HL-60 cells to apoptosis after γ-irradiation. *Proc. Symp. Rad. Biol. Appl. Space Res.*, Brno, 1995, pp. 50–58.

ROTHWELL N.J., HOPKINS S.J.: Cytokines and the nervous system II: Actions and mechanisms of action. *Trends Neurosci.* **18:** 130–136, 1995.

SGOVE R., WICK G.: Methods for the detection of apoptosis. *Arch. Allergy Immunol.* **105:** 327–332, 1994.

SKALKA M., MATYÁŠOVÁ J., ČEJKOVÁ M.: DNA in chromatin of irradiated lymphoid tissues degrades in vivo into regular fragments. *FEBS Lett.* **72:** 271–275, 1976.

ŠPANOVÁ A., KOZUBEK S., PIPALOVÁ I., RAVČUKOVÁ B., VOJTÍŠKOVÁ M.: Programmed cell death detected in mouse embryos and thymocytes after gamma and fast neutron irradiation. *Proc. Symp. Rad. Biol. Appl. Space Res.* Brno, 1995, pp. 44–49.

ŠPANOVÁ A., KOVÁŘŮ H., LISÁ V., LUKÁŠOVÁ E., RITTICH B.: Cell death detected by DNA fragmentation in C6 glioma cells. *Physiol. Res.* **45:** 6P, 1996.

WALKER P.R., KOKILEVA L., LEBLANC J., SIKORSKA M.: Detection of the initial stages in DNA fragmentation in apoptosis. *BioTechniques* **15:** 1032–1040, 1993.

Reprint requests
B. Rittich, Faculty of Chemistry, Technical University, Veslařská 230, 637 00 Brno, Czech Republic.