

TIME DEPENDENT APPEARANCE OF SELECTED APOPTOTIC MARKERS AND USEFULNESS OF THEIR DETECTION *IN VITRO*

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Summary: Many experiments have demonstrated that some cell lines are resistant to chemically induced apoptosis *in vitro*, and that apoptosis itself is far from being a homogenous phenomenon. Here we show that 10 µg/ml etoposide elicited only minor changes in Bowes human melanoma cells (temporary decrease in cell viability and proliferation, transient phosphatidylserine externalization and caspase-3 activation), which weren't clearly capable to start apoptotic pathway in the entire treated population. On the other hand, potassium chromate at concentration of 150 µg/ml executed cell death bearing some features of apoptosis (cell blebbing, caspase-3 activation and cytoskeletal changes) but lacking or showing weakly others (DNA fragmentation and phosphatidylserine externalization). Our results suggest that in detecting apoptosis several fault-proof detection systems are to be used to avoid misleading results and conclusions in each experimental setting.

Key words: Melanoma; Caspase-3; Chromate; Apoptosis; Detection; Phosphatidylserine; Etoposide; *In vitro*

Introduction

The phenomenon of cell death, in particular apoptosis, has justifiably attracted numerous scientists both in basic as well as clinical disciplines. Due to this ongoing interest we have learned many useful details concerning its various forms, consequences and regulatory pathways (23). Beside other aspects associated with cell death, it is now chiefly regulatory pathways governing the cell entry into self-destructing process which inspire intensive studies, mainly due to the enormous clinical potential which modulation of these pathways holds (8,12,20,21).

To study individual apoptosis features and mechanisms, one has to determine whether studied cells are actually undergoing apoptosis or any other type of cell death. Furthermore, since it has become known that the initial (and frequently crucial) steps in apoptosis pathway may occur very quickly and discreetly, thereby often escaping our attention, efforts are to be paid to single out these events and reveal their timing. Until recently, researchers conveniently relied upon an established sequence of observable traits, and it seemed that apoptosis represents comparatively compact end point easily characterized by these universal features (morphological, biochemical and molecular) detectable in majority of *in vitro* and *in vivo* models. Nevertheless, with increasing number of experimental approaches and conditions where the course of apoptosis has been studied there appeared circumstances where the principle of universal appearance of apoptosis seemingly failed (15). Many studies have since shown that different

cell lines display a wide heterogeneity in their genotypic and phenotypic makeup, and thereby they are predisposed to the heterogeneous behavior upon triggering of apoptosis pathway. In addition, as the apoptosis cascade and its regulation are quite intricate, individual apoptosis instigators employing variable mechanisms might provoke slightly diverse cellular responses (1,19). On the other hand, the need for a rapid and reliable method(s) for positive detection of apoptosis has prompted efforts to find such a test which would be applicable in most cases. During the past 20 years, approximately three groups of tests have been proposed and subsequently verified in detection of apoptosis. The first group includes the tests visualizing the changes in apoptotic membrane such as loss of its asymmetry, increased permeability, formation of blebs and activation of death receptors. In the second group there are assays exploring the apoptotic nucleus, i.e. morphological detection of chromatin shrinkage and clumping, visualization of the typical DNA fragmentation or measurement of subG1 DNA content in cells. The presence of various apoptosis-specific molecules (caspases, DNAses, cytosolic cytochrome c and so forth) along with measurement of functional state of some intracellular organelles and structures (mitochondria or cytoskeleton) represent the third group of tests (2,13,25,26).

The special place in the studies focusing on apoptosis is reserved for assays exploring various genes with which it is assumed nowadays lies the ultimate control of cell suicide process (7,20). Unlike the above-mentioned approaches, the gene-aiming tests are neither rapid nor easy to carry out however specific and valuable they prove to be. This is

probably the main reason why these tests are used only when other approaches have already hinted at apoptosis in the studied model.

In our previous works we have shown that different xenobiotics induce apoptosis in Hep-2 cell line (6,19). Despite general similarities, time course and particular appearance of dying cells upon treatment with these xenobiotics varied, and that is why we wanted to verify whether the time aspect and usefulness of selected apoptotic markers will be similar in alternative experimental system - Bowes human melanoma cell line.

Materials and Methods

Chemicals

Potassium chromate was purchased from Sigma-Aldrich (Prague, Czech Republic), dissolved in a serum-free Dulbecco's modified Eagle's medium - DMEM, (Sevapharm, Prague, Czech Republic), sterilized by ultrafiltration and kept at low temperature as 1.5 mol/l stock solution. Prior each experiment, the stock solution was diluted with DMEM to the final concentration of 150 µg/ml.

Etoposide (Vepesid inj., Bristol-Myers Squibb, New York, U.S.A.) was diluted from the original ampoules supplied by manufacturer in a serum-free DMEM to the tested concentration of 10 µg/ml.

WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was purchased from Boehringer Mannheim-Roche (Basel, Switzerland), DAPI (4', 6-diamidino-2-phenylindole), Triton-X was obtained from Sigma-Aldrich (Prague, Czech Republic). Annexin V-FITC was purchased from Bender MedSystems Diagnostics GmbH (Vienna, Austria). Monospecific antiserum for the detection of activated caspase-3 was obtained from New England Biolabs, Inc. (New England Biolabs, Beverly, U.S.A.). Secondary antibodies were from Molecular Probes, Inc. (Molecular Probes, Eugene, U.S.A.) and from EXBIO (EXBIO- Institute of Molecular Genetics, Prague, Czech Republic). All other chemicals were from Sigma-Aldrich (Prague, Czech Republic).

Cell line

Bowes human melanoma cell line (ATCC, No. CRL - 9607, Manassas, United States) was grown as adherent cell culture in DMEM PAN (Aidenbach, Germany) supplemented with 10 % fetal bovine serum (Aidenbach, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in an incubator at 37 °C and 5 % CO₂ atmosphere and were passaged two times a week using 0.05 % trypsin/EDTA.

Cell proliferation

Bowes cells in 200 µl of DMEM PAN medium containing 10 % fetal bovine serum were seeded in four 96 well microtiter plates (Nunclon, Roskilde, Denmark) with the first column of wells without cells (blank). After incubation

(24 hours at 37 °C and in 5 % CO₂), cells were treated with 10 µg/ml etoposide and 150 µg/ml potassium chromate for 4, 6, 8, 10, 12, 18, 24, 48, 72 and 96 hours. After each time interval, 100 µl of WST-1 (Boehringer Mannheim-Roche, Cat. No. 1 644 807) was added. The cells were further incubated for 2 hours in an incubator. The absorbance was read at 450 nm with 650 nm of reference wavelength by a scanning multiwell spectrophotometer Titertek Multiscan MCC/340 (Huntsville, U.S.A.). All experiments were repeated at least three times.

Time-lapse video microscopy

Observation of Bowes cells dynamic morphology after treatment with 10 µg/ml etoposide and 150 µg/ml potassium chromate was carried out as described elsewhere (19). In brief, 24 hours old cell cultures were treated with the above-mentioned chemicals, and after 20 minutes of temperature adjustment in an incubator were transferred to a 37 °C heated chamber under inverted microscope Olympus IX-70 (Olympus Optical CO, Ltd., Tokyo, Japan) equipped with a long-working distance condenser, a 20 x phase contrast lens and Mitsubishi CCD-100E camera (Mitsubishi Corporation, Tokyo). The recording was done with Mitsubishi video recorder HS-S5600 (Mitsubishi Corporation, Tokyo) using recording mode 480 for up to 96 hours. The resulting records were digitalized by means of Adobe premiere 6.0 program and analyzed.

Labeling of externalized phosphatidylserine with Annexin V-FITC

Bowes cells in cytospin chambers (Hettich, Tuttlingen, Germany) were centrifuged for 5 minutes at 500 rpm (JOUAN, Nantes, France) at room temperature (RT), the medium was carefully aspirated and the cells were rinsed with phosphate saline buffer (PBS). To each chamber 1 ml of Annexin-binding buffer with 20 µl of Annexin V-FITC (A) and 20 µl of propidium iodide (PI) was added, and chambers were left at dark for 15 min. After second centrifugation (conditions see above), labeling medium was aspirated and 1 ml of Annexin-binding buffer was added for 1 min. Following third centrifugation the buffer was aspirated, cytospin chambers were disassembled and the cells on a slide mounted into a special anti-bleaching medium and examined under fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 450-490 nm (emission filter 520 nm). The results were quantified by cell counting in 100 visual fields. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. All the experiments were done in triplicate.

Nuclear chromatin fragmentation staining

Bowes cells in modified cytospin chambers (Hettich, Tuttlingen, Germany) were centrifuged for 5 minutes at 500

rpmi (JOUAN, Nantes, France), washed with cold PBS and air-dried. The cells were then labeled with DAPI (5 min) and mounted for examination. Examination was done under fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 330–380 nm and emission filter 420 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. The results were quantified by cell counting in 100 visual fields. Experiments were done in triplicate.

DNA fragmentation assay

Isolation of DNA and investigation of its appearance in agarose gel was described elsewhere (19).

Immunocytochemical detection of activated caspase-3

Bowes cells in cytospin chambers (Hettich, Tuttlingen) were centrifuged for 5 minutes at 500 rpmi (JOUAN, Nantes) at RT, the medium was carefully aspirated and the cells were fixed with 1 ml of 4% formaldehyde. Cytospin chambers were disassembled and the cells on a slide were air-dried. The slides with cells were rinsed three times with phosphate saline buffer with Triton X (PBS-T) and then treated to skimmed milk for 30 min at RT. Next, a primary antibody was added to the cells and the slides were left overnight in a cultivation chamber MIST (The Binding Site Ltd., Birmingham) at 4 °C. The slides were then rinsed three times with PBS-T buffer, a secondary antibody (anti-mouse conjugated with Alexa 488) was added, and the cells were incubated in the cultivation chamber MIST for 90 min at RT. The specimens were optionally post-labeled with DAPI, mounted into a special anti-bleaching medium and examined under a fluorescence microscope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa) equipped with the digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 330–380 nm and emission filter 420 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. In all immunofluorescence experiments (see below), the system of immunological control was employed to avoid false positive or negative staining reactions. All the experiments were done in triplicate.

Immunocytochemical staining of F-actin

Bowes cells were cytopspined, fixed with 1 ml of 2% paraformaldehyde and permeabilized with 1 ml of 0.1% Triton X solution. The cells were then stained with FITC conjugated phalloidin at concentration of 10 µg/ml for 30 minutes. After staining, the specimens were rinsed three times with cold PBS, post-labeled with DAPI and mounted into a special antibleaching medium. The localization and status of F-actin cables were examined under fluorescence micro-

scope Nikon Eclipse E 400 (Nikon Corporation, Kanagawa, Japan) equipped with digital color matrix camera Basler A113CP (Basler, Ahrensburg, Germany), using an excitation filter 450–490 nm and emission filter 520 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed.

Statistics

Statistical analysis was carried out with a statistical program GraphPad Prism. We used one-way Anova test with posttests Dunnet's or Bonferroni. Results were compared with control samples, and means were considered significant if $P < 0.01$.

Results

Cell proliferation and time-lapse videomicroscopy. The growth of Bowes human melanoma cell line as assessed by colorimetric WST-1-based assay after treatment with 10 µg/ml etoposide and 150 µg/ml potassium chromate is shown at Fig. 1 and Fig. 2, respectively. When compared with control cultures, etoposide slightly slowed proliferation of Bowes cells in the interval of 24 hours of treatment, with longer treatment intervals (up to 96 hours) being no different from controls. Potassium chromate provoked a very quick decrease in Bowes cell viability as soon as 4 hours after the beginning of treatment but this trend didn't continue in the following 8 hours. The next significant reduction in cell viability occurred in the time interval 12–24 hours after the beginning of treatment.

Digitalized time-lapse videosequences of Bowes cells treated with 10 µg/ml etoposide revealed that with exception of a short period (about 8 hours of the treatment), where blebbing developed in some cells (approximately 5–8 % of cells in the visual field), the morphological appearance and behavior of Bowes cells did not undergo major alterations; i.e. cells showed migratory and mitotic activity and no features of cell damage (vacuolization, shrinkage or ballooning) were observable (Fig. 3 and 4). In the chromate treated cells, there appeared the first significant changes such as cell shrinkage and blebbing approximately 6 hours after the beginning of the treatment (Fig. 5) followed in the later time periods (18 hours of the treatment) by cell fragmentation yielding numerous spherical bodies (Fig. 6). After 24 hours, all treated cells were dead, with many of them remaining clumped together.

Labelling of externalized phosphatidylserine with Annexin V-FITC. Annexin V-FITC labeling of the cells reveals very early apoptotic changes. Furthermore, in combination with PI labeling it serves to distinguish the early apoptotic phases from the late apoptotic phases based on differing penetration rate of individual dyes. In our experiments, the number of A positive cells along with the number of both A and PI positive cells were followed during selected time intervals in treated and control cultures.

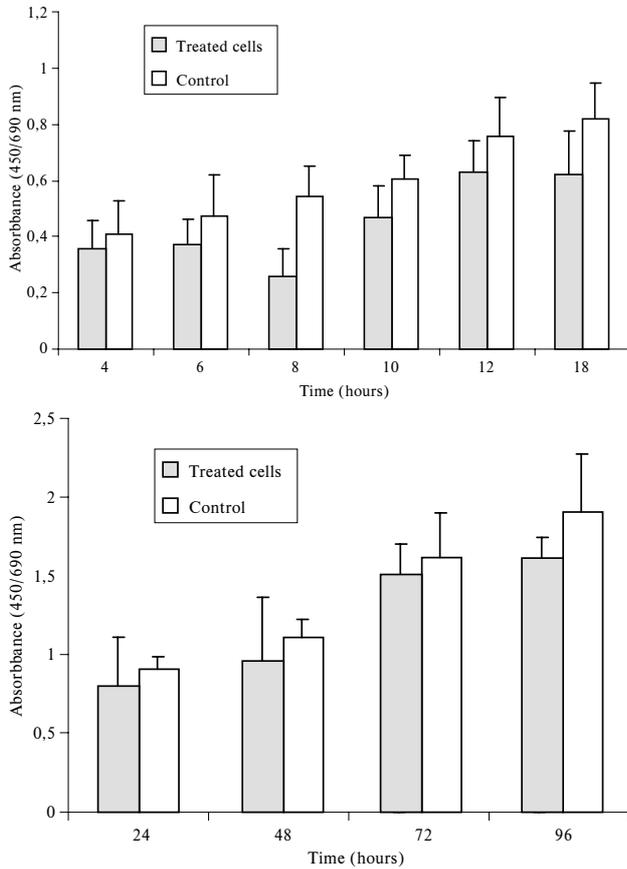


Fig. 1: Viability and proliferation of Bowes human melanoma cells exposed to 10 µg/ml etoposide during 96 hours as measured by colorimetric WST-1 assay. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

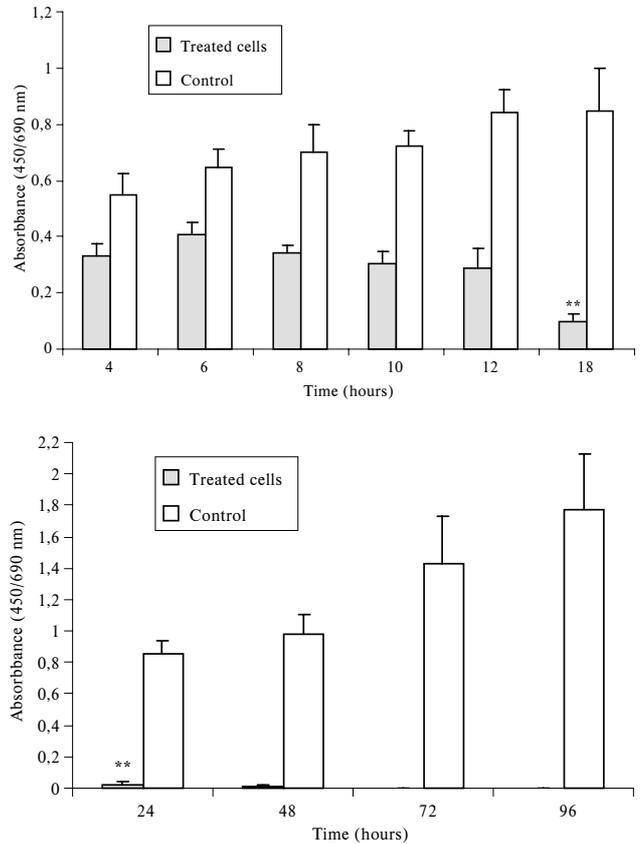


Fig. 2: Viability and proliferation of Bowes human melanoma cells exposed to 150 µg/ml potassium chromate during 96 hours as measured by colorimetric WST-1 assay. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

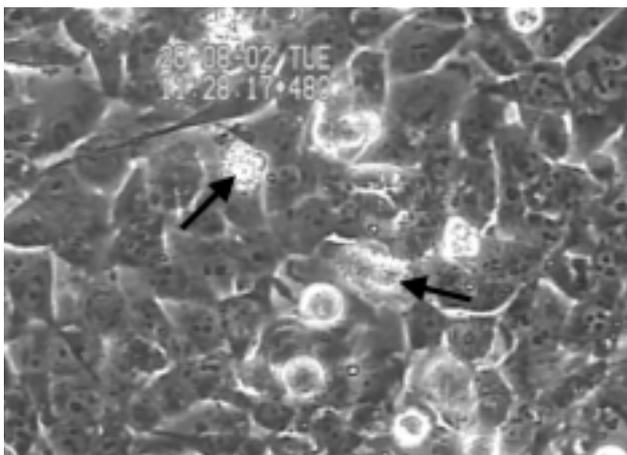


Fig. 3: Morphological appearance of Bowes human melanoma cells after treatment with 10 µg/ml etoposide during 8 hours. The arrows denote blebbing cells. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

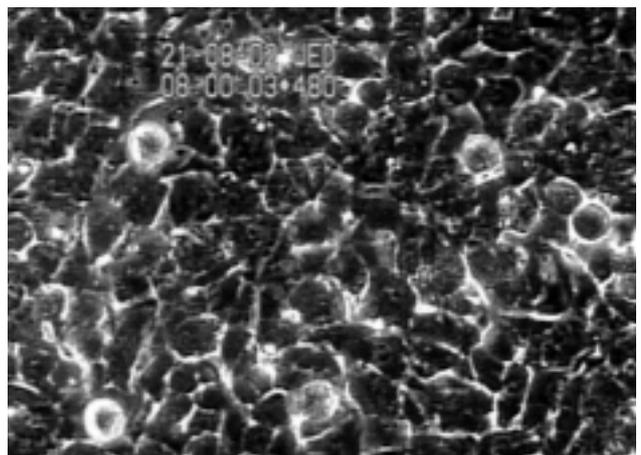


Fig. 4: Morphological appearance of Bowes human melanoma cells after treatment with 10 µg/ml etoposide during 24 hours. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

In etoposide-treated cells, the maximum of A positive cells (15%) was observed at 96 hours of exposure. Between these intervals, a proportion of A-positivity oscillated, generally being no significant from control cultures (Fig. 7). A and PI positive cells were almost not present.

In chromate-treated cells, a significant proportion of A positive cells was detected at 8 hours of treatment (8%), and the maximum (99%) was reached at 24 hours of treatment as compared with control cells (Fig. 8). A and PI positive cells were found only at the very end of the treatment (data not shown).

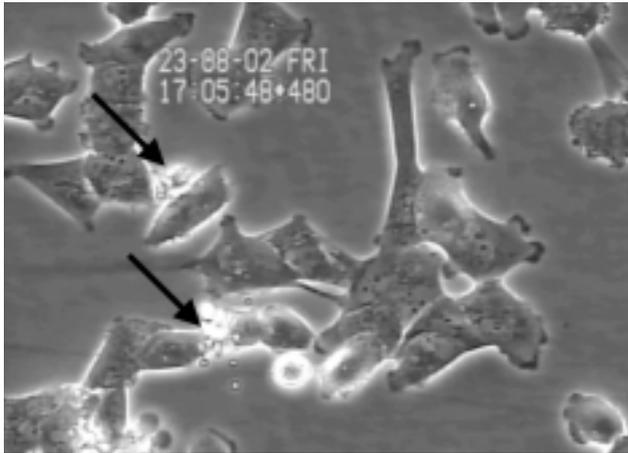


Fig. 5: Morphological appearance of Bowes human melanoma cells after treatment with 150 µg/ml potassium chromate during 6 hours. The arrows denote blebbing cells. The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

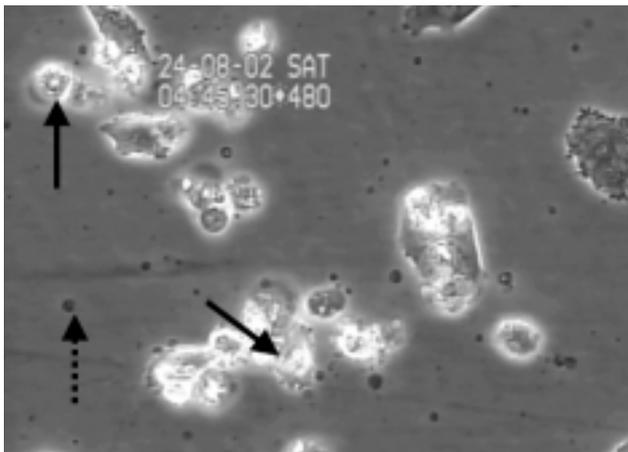


Fig. 6: Morphological appearance of Bowes human melanoma cells after treatment with 150 µg/ml potassium chromate during 18 hours. The arrows denote fragmenting cells. Visible are spherical structures originating from the fragmented cells (dashed arrow). The image represents a printout of the digitalized sequence. Olympus IX 70, phase contrast 200x.

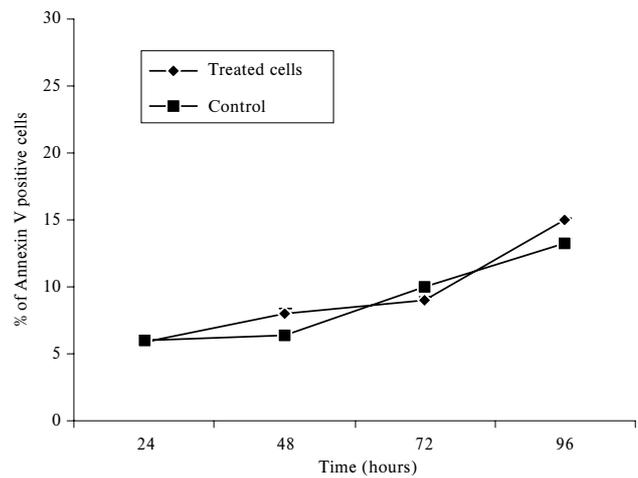
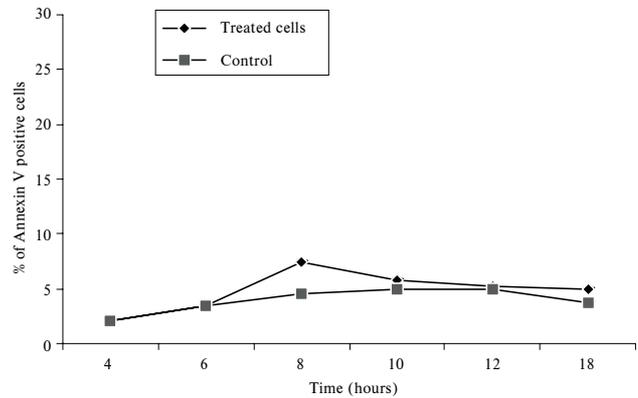


Fig. 7: Annexin V-FITC positivity of Bowes human melanoma cells exposed to 10 µg/ml etoposide during 96 hours. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

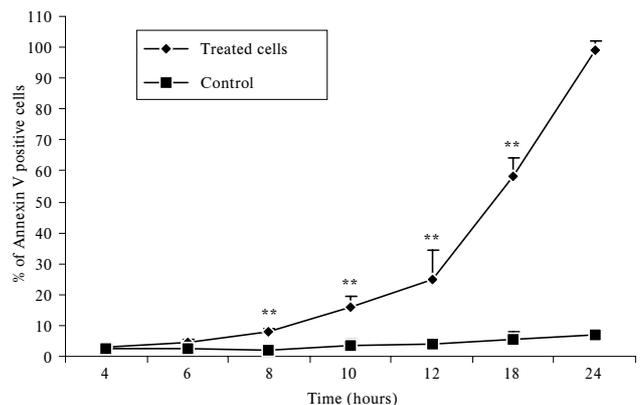


Fig. 8: Annexin V-FITC positivity of Bowes human melanoma cells exposed to 150 µg/ml potassium chromate during 24 hours. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test.

Nuclear fragmentation staining with DAPI. The distribution of the cells with fragmented nuclei during treatment with etoposide and potassium chromate is shown at Fig. 9 and 10. In etoposide treated cultures, very weak fragmentation was observed at 8 hours of treatment and next at 96 hours of treatment. In chromate treated cultures, the proportion of cells with marginalized and fragmented chromatin steadily raised to reach maximum at 24 hours.

Visualization of DNA fragmentation. The appearance of DNA of etoposide and chromate treated cells is shown at Fig. 11 and 12. At no probed treatment intervals specific internucleosomal DNA fragments were detectable.

Immunocytochemical detection of activated caspase-3. The conversion of inactive procaspase-3 into its active form (caspase-3) marks the beginning of the executory stage of apoptosis. The presence of caspase-3 in the cell is thus regarded as a point of no return in death cascade. We have detected caspase-3 positive cells by means of immunofluorescence staining.

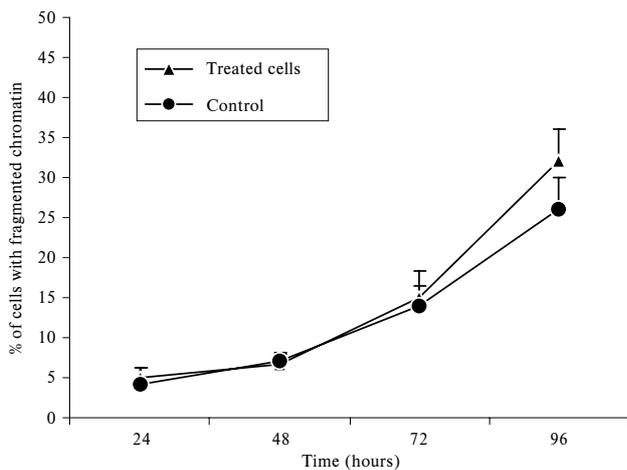
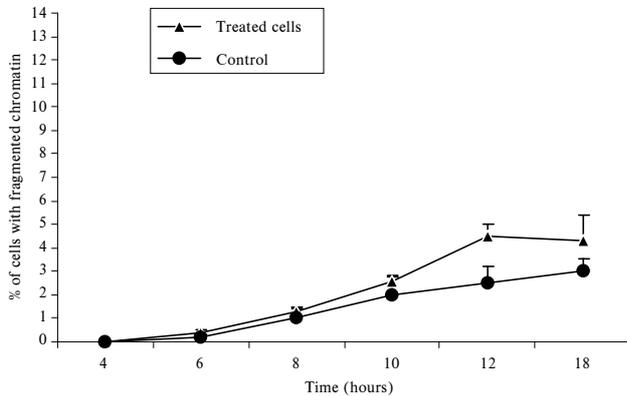


Fig. 9: Fragmentation of Bowes human melanoma cells nuclei exposed to 10 $\mu\text{g}/\text{ml}$ etoposide during 96 hours assessed by nuclear specific dye DAPI. Values represent the mean \pm SD of three different experiments. $**P < 0.01$ with one way-Anova test and Dunnet's post test.

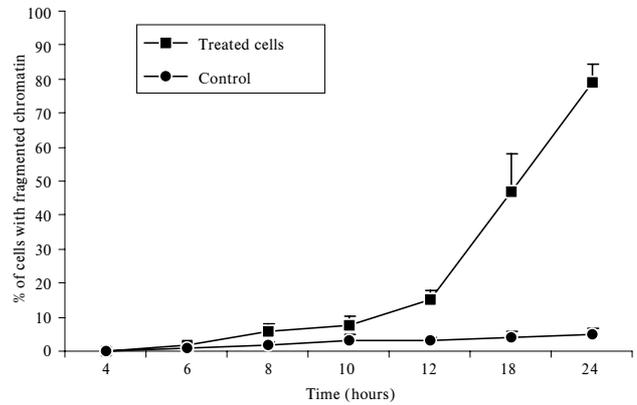


Fig. 10: Fragmentation of Bowes human melanoma cells nuclei exposed to 150 $\mu\text{g}/\text{ml}$ potassium chromate during 24 hours assessed by nuclear specific dye DAPI. Values represent the mean \pm SD of three different experiments. $**P < 0.01$ with one way-Anova test and Dunnet's post test.

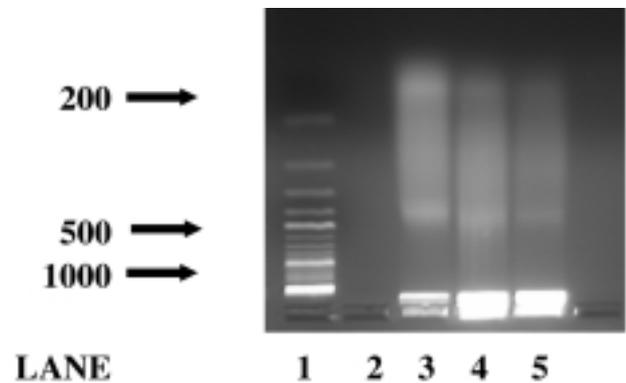


Fig. 11: The appearance of DNA from Bowes human melanoma cells treated with 10 $\mu\text{g}/\text{ml}$ etoposide at time intervals 8 (lane 3), 18 (lane 4), and 24 hours (lane 5). Size marker is shown in lane 1, negative control in lane 2.

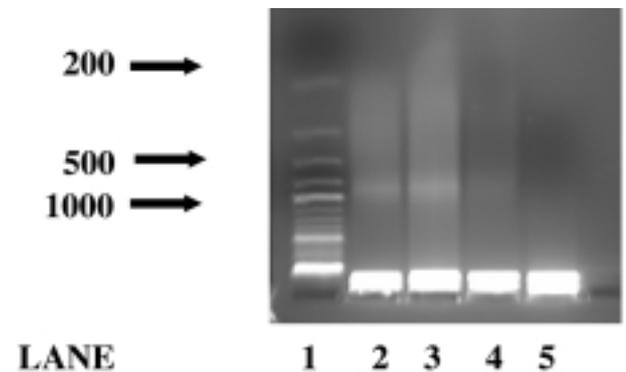


Fig. 12: The appearance of DNA from Bowes human melanoma cells treated with 150 $\mu\text{g}/\text{ml}$ potassium chromate at time intervals 8 (lane 2), 24 (lane 3), and 96 hours (lane 4). Size marker is shown in lane 1, negative control in lane 5.

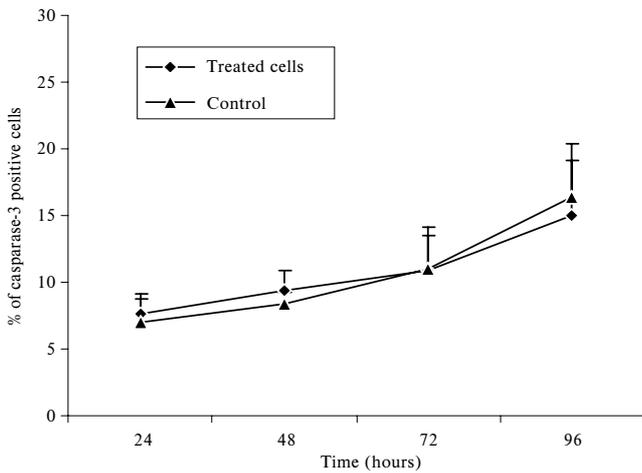
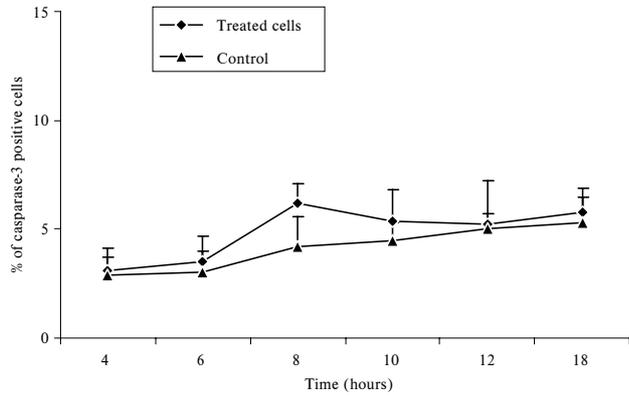


Fig. 13: Activation of caspase-3 in Bowes human melanoma cells exposed to 10 $\mu\text{g/ml}$ etoposide during 96 hours as detected by immunofluorescence staining. Values represent the mean \pm SD of three different experiments. $**P < 0.01$ with one way-Anova test and Dunnet's post test.

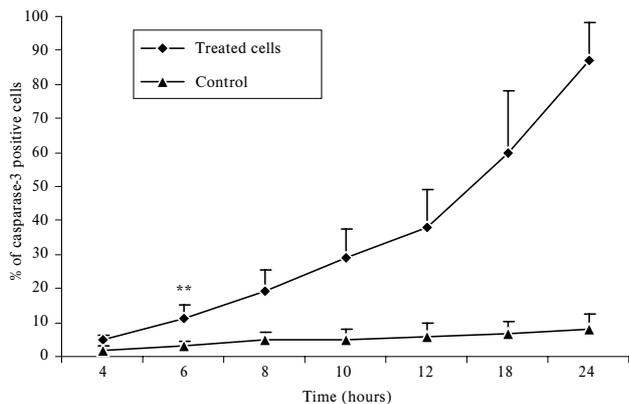


Fig. 14: Activation of caspase-3 in Bowes human melanoma cells exposed to 150 $\mu\text{g/ml}$ potassium chromate during 24 hours as detected by immunofluorescence staining. Values represent the mean \pm SD of three different experiments. $**P < 0.01$ with one way-Anova test and Dunnet's post test.

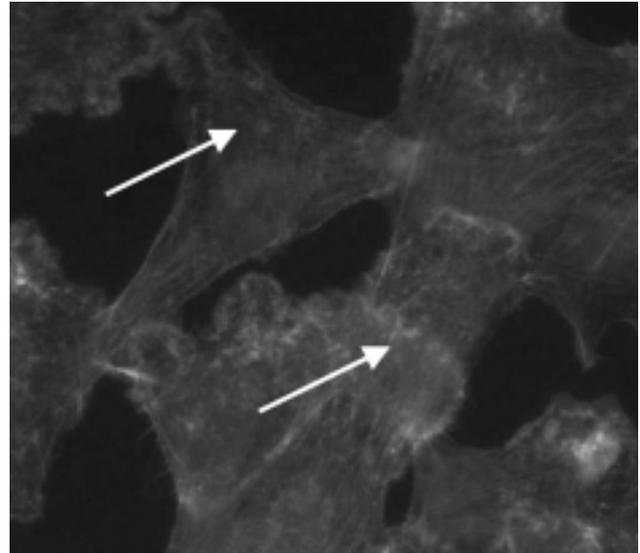


Fig. 15: Localization and arrangement of F-actin cables in adherent Bowes human melanoma cells. F-actin forms a continuous net spreading throughout the body of the cell (arrow), with its especial prominence in the ruffling edges (arrow). Immunofluorescence staining FITC-conjugated phalloidin. Nikon E 400, 400x.

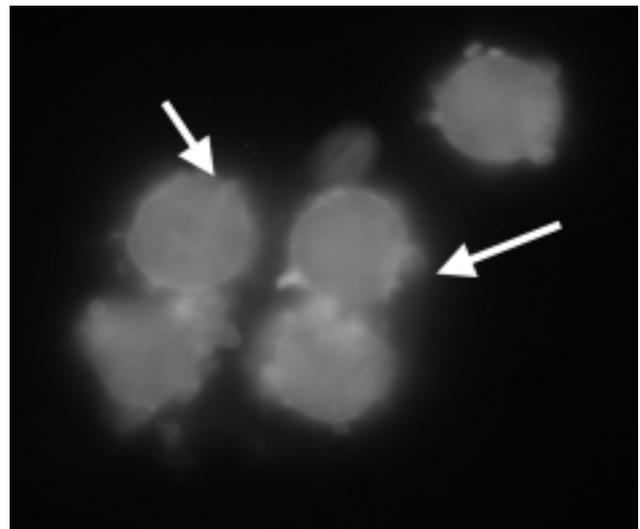


Fig. 16: Localization and arrangement of F-actin cables in apoptotic Bowes human melanoma cells after the treatment with 150 $\mu\text{g/ml}$ potassium chromate for 8 hours. F-actin architecture shows different arrangement, with major localization points being blebs (arrow). Immunofluorescence staining FITC-conjugated phalloidin. Nikon E 400, 400x.

In etoposide-treated cells, the proportion of caspase-3 positive cells transiently increased at 8 hours of treatment but after this time interval again decreased to the level observed in control cells. Positively staining Bowes cells were again detectable in significant numbers at 96 hours of treatment (Fig. 13).

In case of chromate treatment, cells started to stain positively for caspase-3 as soon as 4 hours after the beginning of treatment, with significant increase after 6 hours of treatment. The proportion of positive cells steadily grew and reached its maximum at 24 hours of treatment (Fig. 14).

Immunocytochemical staining of F-actin. In adherent Bowes cells F-actin molecules localize to the cytoplasm and ruffling edges of the plasma membrane (Fig. 15). Treated cells regrouped F-actin cables and accumulated them in the blebbing plasma membrane (Fig. 16). There was no qualitative difference in distribution pattern of F-actin cables after treatment with both chemicals.

Discussion

The studies into appearance, dynamics and regulation of apoptosis induced *in vitro* have revealed many useful facts as well as peculiarities about this type of cell death and together with *in vivo* studies contributed to the better understanding of this phenomenon and its role in many pathological situations. Over past decades many research teams have been trying to find certain universal hallmarks of apoptosis which would be easily obtainable, reliable and unequivocally interpretable. Nowadays, after long and strenuous work, it is generally known that there are very few features that may be deemed universal to all apoptotic models (2). Thus in practice, it seems necessary to employ several methodological approaches to verify whether a particular study model we employ does not produce erroneous results in turn incorrectly interpreted. Several reviews and studies focusing on most commonly used techniques in detection of apoptosis came to the same conclusion (13,26). This conclusion appears to be of particular importance, especially when an *in vitro* model (cell line) which is resistant to standard apoptosis inducers is investigated.

In our experiment, we wanted to find out about dynamics of selected apoptotic markers and usefulness of their detection in Bowes human melanoma cells treated with established proapoptogenic compounds - etoposide and potassium chromate. The examined markers and intervals of their analyses were chosen to embrace the entire spectrum of possible apoptotic features in time with respect to the standardly used techniques.

Etoposide is a derivative of podophyllotoxin and topoisomerase II inhibitor (3,13). It introduces DNA strand breaks, and by interfering with expression of several apoptosis regulating genes triggers apoptotic pathway (7). Its efficiency has been documented in various cell lines and currently it is being used in treatment of hematological malignancies (13,21). The standard etoposide concentration

of 10 $\mu\text{g/ml}$ temporarily decreased Bowes cell viability and induced limited apoptosis during 8 hours of treatment (Fig. 1). These effects were of a transient nature as in the longer time intervals exposed cells grew and behaved normally (Fig. 1 and 4) as compared with control cultures. The individual assays revealed that blebbing and caspase-3 activation were detectable concurrently while there was no detectable chromatin and DNA fragmentation. These results support previous findings which proved that melanoma cells are often resistant to chemotherapy. This resistance has been attributed either to direct loss of the p53 protein, an important cell cycle regulator and apoptosis promoter, or to a mutation of the p53 effector Apaf-1 (24). Inhibition of topoisomerase II might therefore operate via this pathway and that would explain only a weak apoptotic response in our model. Moreover, when melanoma cells are treated with factors activating different signaling pathways such as interferon β or prostaglandin A1, growth inhibition and apoptosis are observed (9,18).

Considering the fact that etoposide functions only in S phase of the cell cycle, our observation of its maximal effect at 8 hours of treatment coincides with proliferative profile of Bowes melanoma cells (Fig. 1). On the other hand, this cytostatic effect did not continue in the later time intervals (24-96 hours) which would be partially explicable with the already mentioned p53-based resistance and cell line-specific growth properties and metabolism. The detected apoptotic markers at 96 hours of treatment (phosphatidylserine externalization) should be in this case attributed to natural apoptosis occurring in a culture after reaching confluency and depleting nutrition sources. Here it is interesting to note that changes in the plasma membrane composition were not detected prior to or simultaneously with other apoptotic markers at 8 hours of treatment where a number of cells started blebbing and expressed activated caspase-3 (Fig. 13). In this case it would actually mean that phosphatidylserine externalization was somehow suppressed or did not accompany the process of apoptosis which casts a suspicion on the universality of this hallmark.

Potassium chromate at concentration of 150 $\mu\text{g/ml}$ is a representative of hexavalent chromium compound that has been identified as a potent apoptosis instigator activating several different signaling apoptosis pathways (22). In melanoma Bowes cells, it produced a set of morphological, biochemical and molecular changes which were detectable as soon as 4 hours after the beginning of treatment (Fig. 5), and culminating in time interval between 12-18 hours (Fig. 6). Morphologically, cells displayed all the known characteristics (blebbing, shrinkage, fragmentation) with exception of preceding nucleolar brightening suggesting that transcription process was not probably altered prior to other changes. Curiously, phosphatidylserine externalization was not significantly increased in treated cells until at approximately 8 hours of treatment when marked caspase-3 activation had already been present. This finding seemingly contradicts other authors who claim that changes in the

plasma membrane structure and molecular composition as revealed by Annexin V based staining belong to the first harbingers of programmed cell death (2,10,16). Still, the whole thing is probably more complex since there are reports evidencing phosphatidylserine externalization as a change accompanying other processes than apoptosis (17).

Despite the clear presence of activated caspase-3 and fragmented nucleus along with F-actin filaments rearrangement (Fig. 10 and 16) in dying cells we were not able to detect the specific DNA laddering after treatment with both etoposide as well as potassium chromate (Fig. 11 and 12). The absence of DNA fragments may be explained by several factors such as inactivity or absence of DNAses, high intracellular zinc content preventing fragmentation or transient nature of the process itself (5). Furthermore, some cell lines have been documented as being devoid of DNA fragmentation as well as other characteristic features upon undergoing apoptosis (4,11,14) and this may well be the case of Bowes human melanoma cells.

Conclusions

Bowes human melanoma cells represent a useful model for studying programmed cell death and its modulation *in vitro* with respect to clinical importance of malignant melanoma.

Etoposide at concentration of 10 µg/ml slowed proliferation and induced only transient and limited changes in this cell line. These changes appeared at 8 hours of treatment and may be characterized by simultaneous plasma membrane blebbing and caspase-3 activation, with longer treatment intervals (up to 96 hours) producing no observable or detectable changes. The observed resistance of Bowes human melanoma cell line to etoposide may be due to an alteration of the p53-based apoptosis pathway in these cells.

Potassium chromate at concentration of 150 µg/ml induced apoptosis in Bowes human melanoma cells. Analyses of the appearance of individual apoptotic markers proved that Annexin V based detection of phosphatidylserine externalization was delayed in comparison with caspase-3 activation or other morphological changes affecting the plasma membrane, cytoplasm and cytoskeleton. Despite the fact that apoptotic nuclei underwent characteristic degradation, no DNA specific fragmentation was evidenced at any examined treatment intervals.

Our results suggest that in a given model as well as in any other studies where apoptosis is to be detected, a combination of tests must be applied to rule out the possibility of misleading results. Out of utilized markers, the least reliable proved to be detection of externalized phosphatidylserine and DNA fragmentation which are, in our opinion, very liable to deviations. On the other hand, caspase-3 activation and nuclear degradation tested very consistently in this as well as other experimental models. Also, we recommend that whenever it is possible one should use conventional

morphological methods based on microscopy (for instance time-lapse videomicroscopy) to have a feedback visual control of the studied model.

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