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CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 11 (2006) pp 384 - 395
http://www.cmbl.org.pl

DOI: 10.2478/s11658-006-0032-4

Received: 21 February 2006
Revised form accepted: 30 May 2006

THE ATYPICAL PATTERN OF CELL DEATH IN B16F10 MELANOMA CELLS TREATED WITH TNP-470.

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Abstract: TNP-470 is an acknowledged anti-angiogenic factor, and was studied clinically as an anti-cancer drug. We previously reported on an additional property of this molecule: the intracellular generation of reactive oxygen species in B16F10 melanoma cells. We showed that a massive generation of ROS occurred in the first few hours after treatment with TNP-470 and that this event was critical to subsequent cell death. In this study, we analyzed the process of cell death and noticed an atypical pattern of death markers. Some of these, such as DNA fragmentation or condensation of chromatin, were characteristic for programmed cell death, while others (the lack of phosphatidylserine flip-flop but permeability to propidium iodide, the maintenance of adhesion to the substratum, no change in mitochondrial transmembrane potential, no effect of the panspecific caspase inhibitor) rather suggested a necrotic outcome. We concluded that TNP-470 induced at least some pathways of programmed cell death. However, increasing damage to critical cell functions appears to cause a rapid switch into the necrotic mode. Our data is similar to that in other reports describing the action of ROS-generating agents. We hypothesize that this rapid programmed cell death/necrosis switch is a common scenario following free radical stress.

Key words: Reactive oxygen species, Angiogenesis, Melanoma, Apoptosis

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Abbreviations used: FITC – fluorescein isothiocyanate; PCD – programmed cell death; ROS – reactive oxygen species; TMRM – tetramethylrhodamine methyl ester; z-VAD-fmk – N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone

INTRODUCTION

TNP-470 is a semisynthetic derivative of the fungal alkaloid fumagillin. Both substances possess anti-angiogenic properties and inhibit the proliferation of endothelial cells via the p53/p21 pathway [1, 2]. Having a relatively low toxicity compared to its parental molecule, TNP-470 became apparent as a promising compound for anticancer therapy. To date, over 200 papers have been published describing the TNP-470-induced inhibition of tumor growth. In an experimental model of cancer, TNP-470 was able to yield improvements up to and including complete remission of primary tumors and metastases [3]. Several biological effects of TNP-470 such as growth inhibition or changes in protein expression were reported for non-endothelial cells; however, their mechanisms were not investigated [4-7]. We previously described a novel activity of TNP-470, namely the rapid induction of B16F10 melanoma cell death, and found that reactive oxygen species (ROS) generation was responsible for this cytotoxic effect of the drug [8]. Fatal damage such as membrane permeabilisation was detected within the first few hours of TNP-470 treatment, depending on the culture medium or drug concentration. Investigating the mechanism of action of TNP-470, we tried to identify if the induced cell death had a programmed or spontaneous character. To answer this question, we analyzed some markers which can be used to differentiate between programmed cell death (PCD) and necrosis, for example intranucleosomal DNA degradation [9], the presence of phosphatidylserine in the cell membrane outer leaflet [10], chromatin condensation [11], cell adhesion [12], mitochondrial transmembrane potential [13], and changes in the volume and granulation of the cells [14]. Moreover, we monitored intracellular ATP concentration and the influence of a panspecific caspase inhibitor [15] on the survival of cells treated with TNP-470.

METHODS

Cell culture

B16F10 (murine melanoma) cells were provided by Dr. Ewa Augustin from the Gdansk University of Technology, Poland. Cells were cultured in F10 HAM medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco), 100 μg/ml of streptomycin and 100 μg/ml of penicillin (both from Polfa, Warszawa, Poland). The cultures were kept in a humidified atmosphere of 5% CO_2 at 37ºC and passaged with 0.25% trypsin (Biomed, Lublin, Poland).

Chemicals

TNP-470 [O-(chloroacetyl-carbamoyl) fumagillol] was provided by Dr. H. Toguchi from Takeda Industries (Osaka, Japan). The panspecific caspase inhibitor N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was purchased from Calbiochem (San Diego, CA).

Cytotoxic assay

Cells were plated onto 96-well plates (2×10^4 cells per well) and cultured overnight. On the next day, the medium was removed and the cells were preincubated for 2 h with 25 μM of the caspase inhibitor z-VAD-fmk before suspension in fresh medium. Afterwards, the medium was changed for one containing TNP-470 or TNP-470 plus 25 μM z-VAD-fmk. After 24 h, the medium was removed and a solution containing neutral red (Sigma-Aldrich, Munich, Germany) (1:50 v/v) was added. The cells were incubated for 30 min, washed with Phosphate Buffered Saline (PBS) and lysed with Sorensen buffer (8.04 g of sodium citrate, 20 mM HCl in 50% methanol). Absorbance at 540 nm was measured with a Multiscan 340 microplate reader (Labsystems, Helsinki, Finland).

DNA fragmentation assay

Low molecular weight DNA was eluted via a modified version of a method described elsewhere [16]. For each experimental point, 2×10^6 cells were collected, washed with PBS and fixed with 80% ethanol overnight at -20ºC. Afterwards, cells were centrifuged and suspended in 100 μl of hypotonic lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% [v/v] Triton X-100, pH 8.0). After 1 h incubation at 4ºC, the lysates were centrifuged at 20,000 x g for 30 min. The collected supernatants were treated with RNA-se A (Sigma, St. Louis, USA) for 30 min, followed by similar treatment with proteinase K (Boeringher-Mainheim, Mannheim, Germany). DNA was precipitated with 50% isopropanol, air-dried, and resuspended in an electrophoresis loading buffer. The samples were run in 1% agarose gel with 0.5 μg/ml ethidium bromide.

Flow cytometry analyses

Cells were harvested with trypsin and incubated with TNP-470 in sterile polypropylene tubes (Sarstedt, Numbrecht, Germany) at 5×10^5 cells/tube. The cells had to be harvested first and the experiments performed in the tubes because the trypsinisation of cells previously treated with TNP-470 resulted in total disruption of the cells. For annexinV-FITC/propidium iodide (PI) staining, an Annexin V KIT (Caltag Laboratories, Burlingame, USA) was used according to the manufacturer's guidelines. For the comparison of the mitochondrial transmembrane potential ($\Delta\psi$), tetramethylrhodamine methyl ester (TMRM) staining was used. The cells were suspended in a total volume of 0.5 ml of 200 nM TMRM (Molecular Probes, Carlsbad, USA) in PBS buffer, and incubated in the dark for 10 min. Samples were analyzed in a Coulter Epics XL cytometer (Beckman Coulter Inc., Buckinghamshire, UK), and the obtained data was analyzed with WinMDI 2.8 software (©Joseph Trotter).

Light and fluorescent microscopy

Cells were seeded onto sterile coverslips one day before the experiment. The next day, the medium was changed for fresh medium containing TNP-470. For

positive control PI staining, the cells were permeabilised with cold acetone/methanol 7:3 at -20ºC for 10 min. Coverslips were mounted onto microscope slides containing drops of 50 μg/ml PI solution, incubated for 5 min in the dark, and analyzed using a Nikon Eclipse 800 E microscope (Nikon, Kanagawa, Japan). Photographs were recorded on a Hamamastu ORCA digital camera (Hamamatsu, Hamamatsu City, Japan) and analyzed with Lucia 4.5 software (Laboratory Imaging Ltd., Praha, Czech Republic). The same light intensity and time of exposure were applied for every sample.

Intracellular ATP measurement

Measurements of the intracellular ATP concentration were performed using a method described by Smolenski [17]. Cells treated with TNP-470 (1×10^6 cells per point) were harvested with trypsin, centrifuged, and suspended in 50 μl of 13% $HClO_4$ (Buch Chemika, Buch, Switzerland). The samples were incubated on ice for 15 min and centrifuged at 13000 × g for 5 min, and the collected supernatant was neutralized with 3 M K_3PO_4 (POCH, Gliwice, Poland) up to pH 5-6. The cell extracts were stored at -20ºC until analysis, which was no longer than two weeks later.

RESULTS

Knowing from previous experiments that TNP-470 is able to destroy B16F10 cells in a dose-dependent manner within a short period of time, we analyzed the effects of TNP-470 during the first 3 h of incubation. Propidium iodide staining indicated strong chromatin condensation typical for programmed cell death. TNP-470 was able to permeabilise cells by itself, so no additional permeabilisation was necessary to stain the DNA (Fig. 1). However, the cells did not lose their adhesion to the substratum. Instead, a shrinkage of stress fibers and an increase in granulation were observed. The intranucleosomal DNA degradation pattern, another marker of programmed cell death, also known as the "DNA ladder", was visible at 50 μM and higher concentrations of TNP-470 (Fig 2).

Exposure of phosphatidylserine in the outer leaflet of the cell membrane is associated with apoptosis. Surprisingly, the content of phosphatidylserine detectable on the surface of B16F10 cells decreased with increasing doses of TNP-470. Furthermore, a population of the cells characterized by a high signal for PI and a very low signal for annexin V appeared. This phenotype was not due to cytosol shedding, because the forward scatter (FS) parameter of the cells was not affected by TNP-470 (Fig. 3). On the other hand, the side scatter (SS) was increased, confirming our previous observation of a higher granularity of TNP-470-treated cells (Fig. 3)

In terms of changes in transmembrane mitochondrial potential, we did not observe significant differences between particular concentrations of TNP-470 after 2 h of incubation, while we found a decrease in the TMRM signal when the

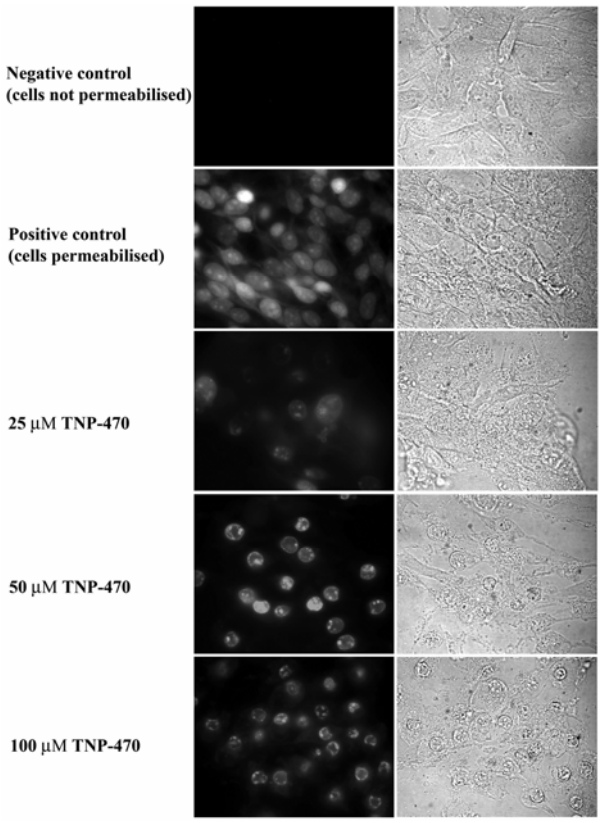

Fig. 1. Left column: photographs of PI-stained cells. Right column: photographs taken under light microscopy; magnification 1000x.

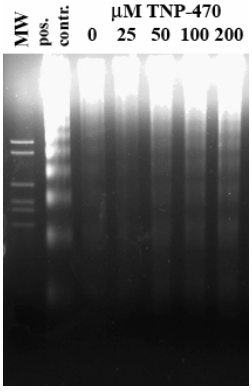

Fig. 2. Lanes contain (from left): DNA marker (MW); an extract of low molecular weight DNA from U937 cells stimulated with 100 U/ml of TNFα for 3 h (positive control); extracts from B16F10 cells stimulated with TNP-470 at various conc.: 0 μM, 25 μM, 50 μM, 100 μM and 200 μM for 3 h.

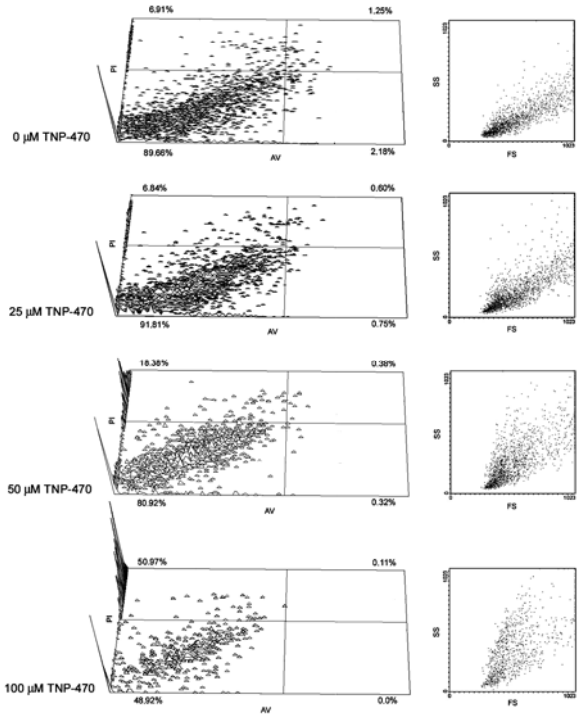

Fig. 3. Left column: density plots of annexin V-FITC signal (AV) vs. PI signal (PI). The density plots are divided into quadrants, and the % of total cell number within each quadrant is indicated. Right column: dot plots of light scatter parameters: forward scatter (FS) vs. side scatter (SS). All values are given in arbitrary units.

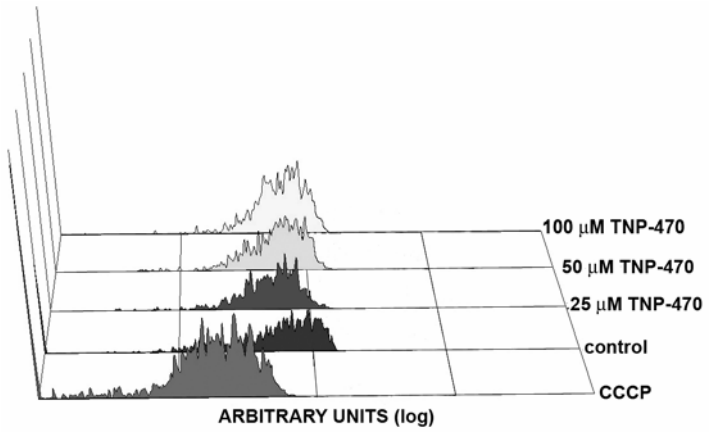

Fig. 4. Mitochondrial transmembrane potential was examined in B16F10 cells treated with 25 μM, 50 μM and 100 μM TNP-470 for 2 h. The chemical uncoupler CCCP (100 μM) was used as a positive control.

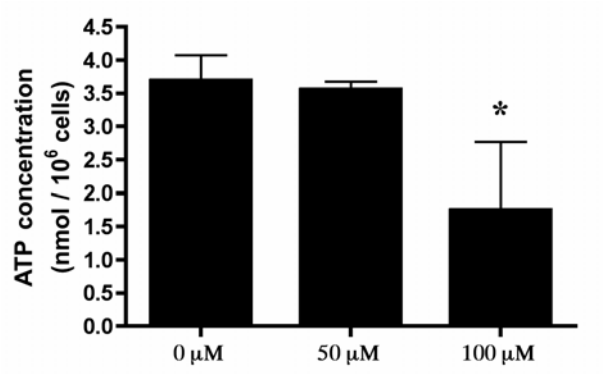

Fig. 5. The intracellular concentration of ATP was estimated via HPLC analysis. Cells treated with TNP-470 for 2 h were lysed, and the ATP content was measured by comparison with the standard. *indicates $P < 0.05$, according to Student's T test for unpaired data.

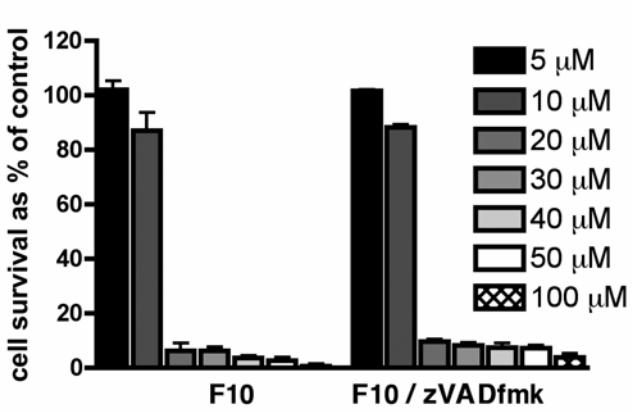

Fig. 6. Cells were pretreated with 25 μM of the panspecific caspase inhibitor zVAD-fmk and subsequently exposed to TNP-470 in the continued presence of zVAD-fmk for 24 h. Control cells were not treated with the inhibitor.

chemical uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used (Fig. 4).

The intracellular ATP concentration measured after 2 h was at control levels at 50 μM TNP-470, although a significant drop was noticed at 100 μM TNP-470 (Fig. 5). Finally, we performed a 24-h cytotoxic assay, during which cells were first preincubated with the panspecific caspase inhibitor z-VADfmk, and afterwards treated with TNP-470 in the continued presence of the inhibitor. However, we saw no protective effect of z-VADfmk, even at low concentrations of TNP-470 (Fig. 6).

DISCUSSION

Programmed cell death acts as a "security gateway" for multicellular organisms. It protects the local environment from inflammatory processes which could occur in the case of uncontrolled release of cellular contents. There are some physiological situations when programmed cell death is necessary (e.g. in the development of the nervous system, in the functioning of the immune system, in spermatogenesis), and these events proceed via a pathway of apoptosis. This kind of cell decay can also be induced by some physical stimuli such as irradiation, hypoxia, cell-cell contact, soluble biomolecules and xenobiotics. The existence of death receptors, regulatory proteins, and specific cascades of proteases dedicated to apoptosis suggests that, in spite of the ATP consumption involved, programmed cell death is beneficial for living organisms [reviewed in 18-20]. Later researchers distinguished several patterns of programmed cell death (PCD) other than classical apoptosis, e.g. autophagy, paraptosis, dark cell death, apoptosis-like PCD, necrosis-like PCD and caspase-independent apoptosis [21]. These PCD modes can share some of the markers of classical apoptosis.

We previously showed that TNP-470 could induce the rapid cell death of B16F10 melanoma cells *in vitro*. Looking at the cell death pattern, we found two evident markers of PCD, namely chromatin condensation and intranucleosomal DNA degradation. However, while the loss of adhesion is a characteristic feature of apoptosis *in vitro* [12, 22] and was shown for apoptotic B16F10 cells [23], we found that the dead cells already permeabilised by TNP-470 were still attached to the substratum. Intranucleosomal degradation of DNA during apoptosis is performed by a set of controllable endonucleases [24]. These enzymes cleave dsDNA at accessible sites between nucleosomes, resulting in the generation of DNA fragments of c. 180 bp in length. Multiple-length repeats of such fragments give a characteristic "DNA ladder" pattern. Since we detected this pattern at 50 μM and higher concentrations of TNP-470, we hypothesized that an apoptotic pathway might have been initiated. Treatment of B16F10 cells with 100 μM of TNP-470 for 2 h resulted in a 2-fold drop in intracellular ATP levels, whereas at the same point in time, 50 μM of TNP-470 had not significantly affected ATP concentration. While apoptosis and other PCD are endergonic processes, a lack of ATP can disrupt the programmed cell death scenario and cause a switch to necrosis [25]. Also, under our experimental conditions, we were not able to detect elevated phosphatidylserine content with annexin V staining, but rather noticed the reverse effect. In attempting to explain this, we could exclude the possibility of cytosolic shedding from our light scattering data. Two alternative explanations can be proposed: massive disintegration of the membrane, which generally lowers the content of phosphatidylserine, or lipid peroxidation induced by ROS, which affects annexin V binding. With regard to the second explanation, some reports claim that phosphatidylserine is more vulnerable to peroxidation than other phospholipids [26-27]. In addition, there

were no significant changes in the transmembrane mitochondrial potential in the cells treated with TNP-470. Similarly, we could not detect any protective effect of the panspecific caspase inhibitor. These results suggest that TNP-470-induced cell death does not occur through classical apoptosis or other PCD alone.

The occurrence of both necrosis and apoptosis after TNP-470 application was already shown using the rat liver metastasis model by Ahmed *et al.* [28]. However, this data was collected from *in vivo* experiments, and the systemic but not local action of TNP-470 on tumor liver metastases was being studied. Apoptosis and necrosis are common features of many tumor nodules. In fast-growing solid tumors, there is a certain ratio of proliferation to cell death, which can be modulated by external factors like angiogenesis and inflammation [29]. TNP-470 applied systemically exerts an anti-angiogenic effect and can contribute to hypoxia in fast-growing tumors, which cause programmed or spontaneous cell death. The authors postulated exactly such a mechanism as an explanation of their findings. So, the effect of TNP-470 presented there was not direct, but mediated by the systemic response. In our study, we analyzed the direct effect of TNP-470 on melanoma cells and thus excluded any additional influence of the tumor microenvironment.

Taken together, our results are similar to other data concerning the influence of ROS generators on cell death. Menadione, which generates superoxide radicals, can induce both apoptosis and necrosis. Shortly after menadione is added, the apoptotic phenotype prevails over the necrotic phenotype, but at longer incubation times this tendency reverses [30]. At a certain point, the same cell exhibits both apoptotic and necrotic markers [31]. As in our study, application of z-VADfmk did not protect from death even cells in a very early apoptotic state, whereas N-acetylcysteine did. Moreover, a peroxynitrite-generating agent was described to cause apoptosis or necrosis, depending on the dose [32]. Again, in accordance with our TNP-470 experiments described elsewhere [8], cell death was still induced when the ROS generator had been eliminated long prior to the cell membrane becoming permeable.

In conclusion, substances that have the ability to generate ROS are able to induce cell death with mixed features of PCD and necrosis. However, necrosis seems to be the ultimate mode of death. Therefore, we postulate that shortly after the first appearance of ROS, some PCD pathways are activated but then, due to the constantly induced and increasing damage, it is not possible for the cells to control these processes to their conclusion. Thus, the inhibition of apoptotic executive pathways is not enough to protect these cells from death. Effective protection can be achieved only via the application of antioxidants acting on the primary cause of damage, which is ROS generation.

Acknowledgments. This project was supported by the Polish State Committee for Scientific Research grant No. 6 P04A 017 21 (to J.B.).

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