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APOPTOSIS AND APPEARANCE OF MULTINUCLEAR C6 GLIOMA CELLS AFTER TREATMENT BY MICROTUBULE POISONS

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Microtubules play a crucial role in a large number of cellular functions, including chromosome separation during cell division. Microtubule poisons, drugs that perturb microtubule function, are used for the treatment of a number of malignant tumors, although the precise mechanisms of their cytotoxic effect are still not well understood.

In this study, we have investigated the effects of two microtubule poisons, colchicine and paclitaxel, on rat astrocytoma C6 cell line in vitro. Cells were incubated 24-72 hours with, or without poisons, fixed and processed for analysis by light and electron microscopy.

Both type of drugs displayed effects on the microtubule network of C6 glioma cells observed by electron microscopy. Furthermore, microtubule poisons triggered apoptotic cell death, although the extent of apoptosis was rather low, while the number of cells arrested in mitosis was significant (33% after 24h treatment with paclitaxel). The most striking effect of paclitaxel was obesreved after 72h, when over 66% of cells displayed multiple nuclei, implying that glioma cells escape mitotic arrest, that results in formation of multinucleated cells.

The results showed that C6 glioma cells are largely resistant to induction of apoptosis by microtubule poisons, so further studies are needed to examine the possibilities to overcome resistance.

Key words: apoptosis, colchicine, microtubules, multinucleate cells, paclitaxel

INTRODUCTION

Microtubules, stiff hollow cylindrical structures made of heterodimeric tubulin subunits, are important part of the cytoskeleton. They play crucial roles in a large number of cellular functions – control the position of organelles, intracellular trafficking of vesicles, mitochondria and other components throughout the cell, and have a specific role in mitotic spindle formation and chromosome separation during cell division (Mollinedo and Gajate, 2003).

Microtubules are highly dynamic polymers and their polymerization dynamics is tightly regulated by proteins that bind along them, and it can be

influenced by a number of drugs (Andersen, 2000; Jordan and Wilson, 2004). Microtubule poisons, drugs that perturb microtubule function, are used for the treatment of certain solid tumors and hematologic malignancies, although precise mechanisms of their cytotoxic effect are still under study (Jordan and Wilson, 2004; Brito and Rieder, 2009).

A number of poisons are able to interact with microtubules promoting either their disassembly (microtubule disruption) or assembly (microtubule stabilization), and thereby blocking microtubule dynamics, leading to disruption of the mitotic spindle in dividing cells (Jordan and Wilson, 1998). The dominant effect of these poisons is the arrest of cells in metaphase and blockade of mitosis, but it has also been shown that they induce apoptosis in normal and malignant cells (Bumbasirevic *et al.*, 1995; Bumbasirevic *et al.*, 1997, Mircic *et al.*, 2005).

Apoptosis is a type of programmed cell death, characterized by specific morphological changes (Kerr *et al*., 1972). It occurs during various physiological and pathological conditions, when cells die in a controlled manner, following activation of caspase cascades (Galluzzi *et al*., 2007). This process can also be induced by a number of chemotherapeutic agents in normal and malignant cells, including microtubule poisons (Mollinedo and Gajate, 2003; Jordan and Wilson, 2004).

Glioblastoma are the most common brain tumors, characterized by uncontrolled growth, diffuse infiltration, lack of differentiation and are highly resistant to current therapeutic approaches including radiation and chemotherapy (Galanis and Buckner, 2000). This is largely a consequence of a highly deregulated tumor genome leading to augmented survival pathways and defects in the apoptosis signaling machinery (Furnari *et al*., 2007). Thus, developing of innovative therapeutic strategies is needed for a successful treatment of glioblastoma.

In this study, we have investigated the potential of two microtubule poisons, colchicine and paclitaxel, to induce apoptosis in rat astrocytoma C6 cell line *in vitro*, as this cell line shows similarities to human glioblastoma (Grobben *et al*., 2002).

MATERIAL AND METHODS

Cell culture

The rat glioma cell line C6 was a kind gift from Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, in 25-cm^2 tissue culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 0.01% sodium pyruvate and antibiotics (1% penicillin/streptomycin solution), all purchased from Invitrogen (Carlsbad, CA, USA). After the conventional trypsinization procedure, the cells were seeded at 2 x 10^7 cells/well in 6-well plates or 3x10^7 cells/well in 4-well chamber slides, cultivated overnight and then exposed to drugs.

Media and reagents

DMEM, l-glutamine, phosphate-buffered saline (PBS), fetal calf serum (FCS), fungizone, gentamycin sulfate and paclitaxel were purchased from Sigma (St. Louis, Missouri, USA) and Invitrogen (Carlsbad, CA, USA), while colchicine, propidium iodide, glutaraldehyde, cacodylate buffer, propylene oxide and Epon from Merck (Damstadt, Germany). Uranyl acetate was from SERVA Electrophoresis (Heidelberg, Germany). Antibiotics, sterile conical flasks, eight or four-chamber culture slides and sterile disposable pipettes were purchased from Galenika a.d. (Belgrade, Serbia).

Determination of apoptotic, mitotic and multinuclearity index

On toluidine blue stained 1 μm thick sections, apoptotic, mitotic and multinuclear cells were counted on 5 randomly chosen high power fields (100 x oil immersion objective, Olympus CH microscope, Japan). We used one section per specimen and 3-5 specimens per individual test, by the aid of a square eye piece graticule. The identification of cells was based on previously defined morphological criteria (Walker, 1988). The percentage of these cells was determined on 1000 cells per section and expressed as apoptotic (AI), mitotic (MI) or multinuclearity (MN) index.

Transmission Electron Microscopic Examination

C6 glioma cells were incubated in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 0.01% sodium pyruvate and antibiotics at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Cells were incubated 24-72 hours with, or without microtubule poisons, 2 μM colchicine or 2 μM and 10 μM paclitaxel. After treatments, cells were washed twice in PBS (pH 7.4), using low speed centrifugation (2000 RPI, 5 minutes) and pellets were fixed in 3% solution of glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) over night at +4^{o}C. Following osmification, pellets were dehydrated in an ethanol gradient, cleared in propylene oxide, and then embedded in Epon. Semithin sections were cut with a diamond knife on a Leica Ultracut UCT EM FCS ultramicrotome (Leica Microsystems, Austria), stained with Toluidine blue and analyzed by an Olympus BX41 light microscope (Olympus, Tokyo, Japan). All the slides were photo-documented with an Olympus C-5060 ADU wide zoom camera and the Olympus DP-soft Image Analyzer programme (Olympus GmbH, Hamburg, Germany). The ultrathin sections from chosen representative areas were cut with the same ultramicrotome, collected on copper grids, and stained with uranyl acetate and lead citrate. The sections were examined by FEI Morgagni 268D transmission electron microscopy (FEI Europe, Eindhoven, Netherlands) and photographed with a Mega ViewIII Soft Imaging System digital camera (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Quntitative data were expressed as the mean value with standard error (SE). We used the One-way ANOVA test with the Bonferroni as the Multiple Comparasion test. Differences were considered significant at $p<0.05$. The units

for statistical description and analysis were mean values of parameters obtained per specimen on 5 randomly chosen fields.

RESULTS

The untreated C6 glioma cells displayed oval and slightly indented euchromatic nuclei with clearly visible nucleoli. On electron micrographs, their cytoplasm showed numerous free ribosomes, solitary cisterns of rough endoplasmic reticulum and elongated mitochondria (Fig. 1).

The application of either colchicines, or paclitaxel induced substantial alterations of the microtubule network. While colchicine brought to the disappearance of microtubules, C6 glioma cells incubated with paclitaxel

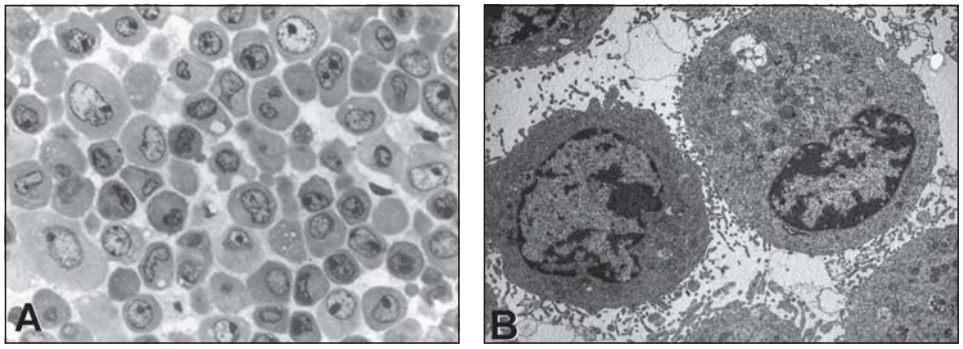

Figure 1. Morphology of C6 glioma cells in control cultures observed by light microscopy on 1 μm thick sections stained by Toluidine blue (A), and by transmission electron microscopy (B). Magnification: A – 1000x; B – 7000x

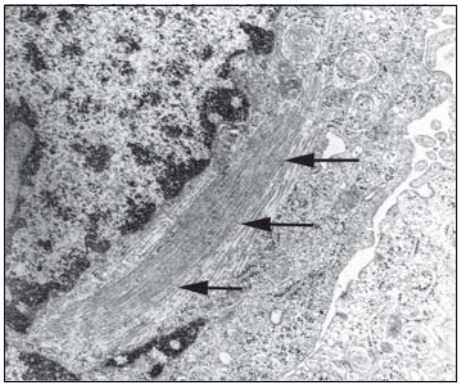

Figure 2. Ultrastructural characteristics of a C6 glioma cells incubated 24 hours with 2 μm of paclitaxel showing prominent microtubule bundles (arrows). 28000x

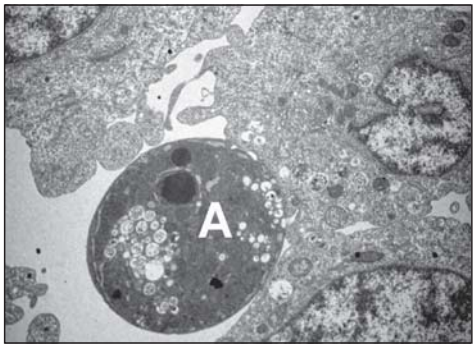

Figure 3. Ultrastructural characteristics of C6 glioma cells incubated 24 hours with 2 μm of paclitaxel. Typical morphological features of apoptotic cell (A) are seen. 14000x

displayed prominent bundles of microtubules (Fig. 2). In addition, application of these agents resulted in diminished cell growth, mitotic blockade and cell death. A number of cells displayed characteristics of apoptotic cell death (Fig. 3), including typical nuclear chromatin condensation, dispersion of nucleoli, condensation of the cytoplasm and subplasmalemmal dilatation of the endoplasmic reticulum. The earliest ultrastructural alterations were noticed in mitochondria, which showed a condensed appearance with multiple intracristal dilatations (Fig. 4).

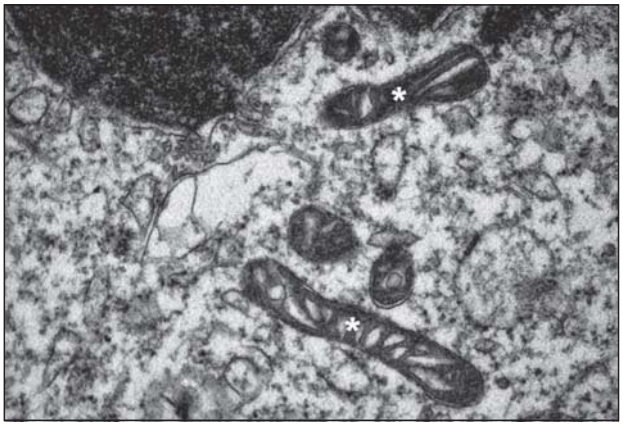

Figure 4. Ultrastructural characteristics of a C6 glioma cell incubated 24 hours with 2 μm of colchicine, showing condensed mitochondria (asteriks) with intracristal dilatations. 36000 x

The apoptotic indices (AI) as determined on 1 μm thick sections stained by Toluidine blue were moderately increased after treatment with microtubule poisons in comparison to control cultures (Fig. 5). In addition, the application of microtubule poisons displayed considerable effect on mitotic indices (MI) of C6 glioma cells, which was significantly increased after 24 hours of incubation with colchicine, or paclitaxel, indicating mitotic arrest (Fig. 5).

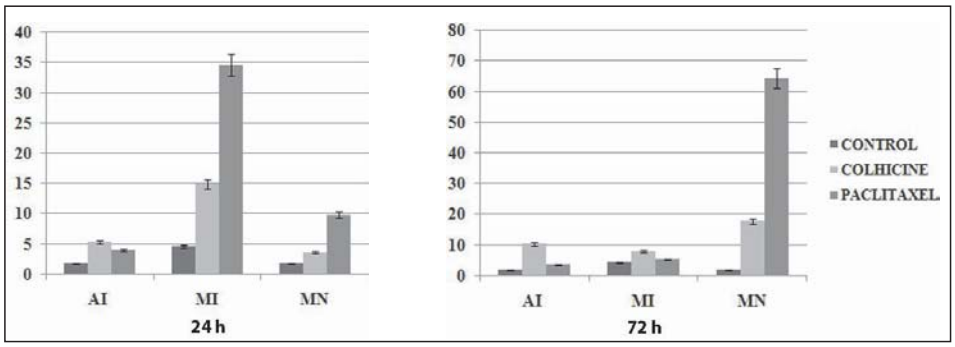

Figure 5. Graphic representation of apoptotic (AI), mitotic (MI) and multinuclearity (MN) indices (mean values with standard deviation) of C6 glioma cells incubated for 24 and 72 hours without drugs (control), with 2 μm colchicine and 2 μm paclitaxel

The most striking effect of these drugs on C6 glioma cells was the appearance of multinucleated cells (Fig. 6), whose number was significantly increased (3.57% and 17.61% by colchicine, or 9.86% and 64.33% by paclitaxel, after 24 and 72 hours respectively; Fig. 5).

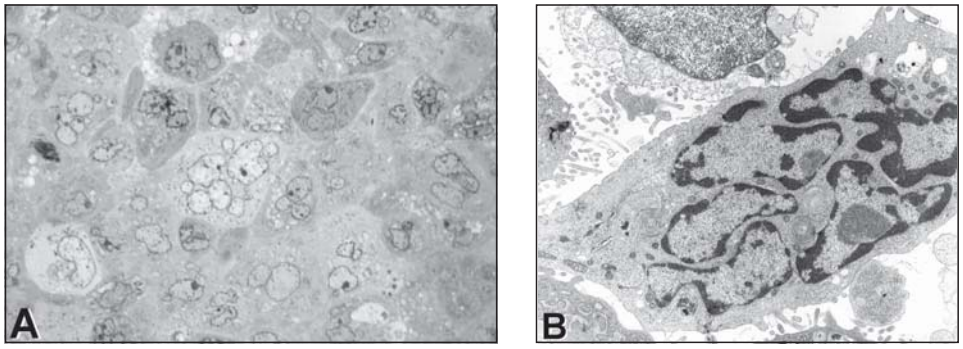

Figure 6. C6 glioma cells incubated 72 hours with 2 μm of paclitaxel. A. Light microscopic appearance of a number of multinuclear cells (1 μm thick sections stained by Toluidine blue). B. Electron microscopic appearance of multinucleated C6 glioma cell. 10000x

DISCUSSION

In this study we demonstrated that both types of microtubule poisons, colchicine and paclitaxel, trigger apoptotic cell death in C6 glioma cells, although the extent of apoptosis was not significant. We confirmed by TEM analysis that colchicine produced the disappearance of microtubules in both interphase and mitotic C6 glioma cells, as a consequence of disassembly of microtubules by binding to tubulin dimers (Mandelkow and Mandelkow, 1995). It was previously shown that colchicine inhibits microtubule polymerization by binding at the α-β dimerization interface of tubulin with the B-ring on the α-subunit and the A and the C-rings on the β-subunit (Chaudhuri *et al*., 2000). Paclitaxel also binds to tubulin molecules at N-terminal 31 amino acids of β-tubulin (Rao *et al*., 1999), but it acts by inhibiting microtubule disassembly, and thus promoting the formation of unusually stable cytoplasmic microtubule bundles, and disrupting normal dynamic reorganization of the microtubular network, required for mitosis and cell proliferation (Mollinedo and Gajate, 2003). Prolonged drug exposure induces protracted arrest of the cell cycle at G2/M phase, that may result in apoptosis.

In this study, the effect of microtubule poisons in the induction of apoptosis was seen already after only 4 hours of incubation (data not shown), without a significant increase after 24 and 72 hours. On the other hand, exposure of C6 glioma cells to both types of microtubule poisons, caused an increase in mitotic index, which was a consequence of disturbed normal function of microtubules, and cells were in the mitotic blockade due to the absence of functional mitotic spindle (Gajate and Mollinedo, 2005).

The induction of apoptosis by microtubule poisons is by the activation of apparently different signal pathways, depending on drug concentration (Wang *et al*., 2000). Protracted arrest of the cells in G2/M phase of the cell cycle because of incompetent mitotic spindle, triggers apoptosis in various cell types, and this is believed to be the primary mechanisms of cytotoxic effect of microtubule poisons (Harmon *et al*., 1992; Mollinedo and Gajate, 2003; Jordan and Wilson, 2004). We have previously shown that in thymocytes colchicines, nocodazole and paclitaxel induce apoptosis in both interphase and dividing cells (Bumbaširević *et al*., 1995; Bumbasirevic *et al*., 1997). The possible link between microtubules and apoptosis could be the number of Bcl-2 family members that interact with microtubules. One of the BH-3 proteins, Bim binds to dynein light chains which are associated with microtubules (Puthalakath *et al*., 2001). Binding of microtubule poisons to microtubules results in release of Bim, which then translocates to mitochondria, where it binds to anti-apoptotic Bcl-2 family members and initiates apoptosis. Additionally, release of Bim from microtubules can be achieved by JNK that phosphorylates Bim (Lei *et al*., 2003). Furthermore, phosphorylation of Bcl-2, which occurs during prolonged G2/M arrest, leads to inactivation of its binding to proapoptotic Bax, and induction of apoptosis (Mollinedo and Gajate, 2003). It seems that many other proteins are also involved in microtubule poison induction of apoptosis, as for example survivin, which binds and stabilizes microtubules, and further suppress apoptotic activity of caspase-3, -7 or -9 (Deveraux and Reed, 1999). Disruption of this interaction results in loss of survivin's antiapoptotic function, increased caspase-3 activity and induction of apoptosis (Giodini *et al*., 2002).

Contrary to low effectiveness in apoptosis induction in C6 glioma cells, microtubule poisons in this study were effective in generating mitotic blockade. The consequence of protracted arrest of cells, instead of apoptosis, was aberrant mitotic exit and formation of multinucleated cells, especially after paclitaxel treatment.

Our study is similar to studies which suggested that the nuclei of C6 glioma cells exposed to a cisplatin, are morphologically the most affected organelle (Biggiogera *et al*., 1997) and showed that number of cells present hyperlobulated, or multiple nuclei (Krajči *et al*., 2000). In addition, evidence of apoptosis was not observed in noscapine-treated C6 glioma cells, but they undergo excessive DNA synthesis and atypical nuclear divisions, resulting in multinucleated cells (Landen *et al*., 2004). Previous experiments reported that prolonged exposure to microtubule poisons results in abnormal mitotic exit, that occurs without complete chromosome segregation known as "mitotic slippage" (El Hajouji *et al*., 1998). Features of cell death induced by microtubule poisons, and death after aberrant mitotic exit are called mitotic catastrophe (Okada and Mak, 2004; Galluzzi *et al*., 2007). It seems that suppression of microtubule dynamics by paclitaxel leads to the triggering of the mitotic spindle assembly checkpoint that results in an arrest at the G2/M phase of the cell cycle and subsequent apoptosis, or cells undergo aberrant mitotic exit and formation of multinucleated cells (Jordan and Wilson, 2004).

This study showed that C6 glioma cells are largely resistant to induction of apoptosis by microtubule poisons, so further studies are needed to examine possibilities for overcoming resistance. Targeting apoptosis resistance has become the main stream research for designing new combination therapy for glioblastoma (Krakstad and Chekenya, 2010). Recently, it has been shown that treatment of rat glioblastoma cell lines by N-(4-Hydroxyphenyl) retinamide sensitizes cells to paclitaxel (Janardhanan *et al*., 2009).

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APOPTOZA I POJAVA MULTINUKLEARNOSTI C6 GLIOMSKIH ĆELIJA TRETIRANIH ANTIMIKROTUBULARNIM OTROVIMA

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SADRŽAJ

Mikrotubuli imaju važnu ulogu u brojnim ćelijskim funkcijama, uključujući ulogu u razdvajanju hromatida tokom ćelijske deobe. Antimikrotubularni otrovi,

lekovi koji ometaju funkciju mikrotubula, koriste se u tretmanu mnogih malignih tumora, mada precizan mehanizam njihovog citotoksičnog dejstva nije u potpunosti rasvetljen.

U ovom radu, je ispitivan potencijal dva mikrotubularna otrova, koji razgrađuju (kolhicin) ili stabilizuju mikrotubule (paklitaksel), u indukciji apoptoze kod C6 ćelijske linije astrocitoma pacova *in vitro*. Ćelije su inkubirane 24-72 sata sa ili bez otrova, fiksirane i obradjene za analizu pomoću svetlosne i elektronske mikroskopije.

Obe primenjene substance ispoljile su efekte na mikrotubule ćelija C6 glioma, što smo uočili pomoću elektronske mikroskopije. Pored toga, antimikrotubularni otrovi indukovali su apoptozu ograničenog broja ćelija, dok je njihov efekat na blok mitoze bio izražen (33% posle 24 sata inkubacije sa paklitakselom). Međutim, najznačajniji efekat ovih supstanci ogledao se u pojavi multinukleusnih ćelija (66% posle 72 sata inkubacije sa paklitakselom), ukazujući da ćelije glioma uspevaju da napuste mitotsku blokadu i da aberantno završe ćelijsku deobu stvaranje multinuklearnih ćelija.

Naši rezultati ukazuju da ćelije C6 glioma ispoljavaju rezistenciju na indukciju apoptoze pomoću antimikrotubulskih otrova, te su dalja istraživanja neophodna za iznalaženje načina za prevazilaženje rezistencije.