EFFECTS OF LOW AND HIGH CONCENTRATIONS OF ANTITUMOUR DRUG TAXOL IN ANAPLASTIC THYROID CANCER CELLS

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Aim: To study the changes of cell cycle, mitochondrial membrane potential and caspase activation in response to an antitumour drug Taxol in ARO and KTC-2 cell lines of anaplastic thyroid carcinoma. Methods: Experiments were done on thyroid anaplastic cancer cell lines ARO and KTC-2 using Western blotting, flow cytometry, light and fluorescent microscopy. Results: Taxol significantly activated caspases in ARO cells starting from drug concentration of 5 nM. Maximum activation was observed at 25 nM and further increase of Taxol concentration to 100 nM resulted in a reduction of caspase activation. Concomitant to caspase activation, a loss of mitochondrial membrane potential was observed. At Taxol concentration of 100 nM, most cells lost their mitochondrial membrane potential. Low Taxol concentrations (10 nM) caused changes in the cell cycle that are typical for apoptosis without cell cycle arrest. Higher drug doses starting from 50 nM arrested cell cycle in G2/M phase. In KTC-2 cell line Taxol concentration as low as 1 nM induced apoptosis. 6–15 nM of the drug caused massive (75–83%) cell death. Upon Taxol action, the increase in the number of cells displaying manifestations of accelerated senescence was insignificant. Conclusion: Taxol induces bona fide apoptosis in thyroid cancer cell cultures at low (1–25 nM) concentrations. Higher drug doses cause the loss of mitochondrial membrane potential and possibly lead to other types of cell death. No accelerated senescence at different Taxol concentrations was observed. The significance of subG1 and G2/M cell populations at low and high doses of Taxol is discussed.

Key Words: taxol, thyroid, anaplastic cancer, cell cycle, apoptosis, mitochondrial membrane potential, accelerated senescence.

MATERIALS AND METHODS

Cell lines and culture conditions. Human ATC cell line ARO was initially provided by J. A. Fagin (University of Cincinnati College of Medicine, Cincinnati, OH). An ATC cell line KTC-2 was kindly donated by Dr. J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan).

Throughout all experiments cancer cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all reagents from Invitrogen Life Technologies, Paisley, UK) in a 5% CO2 humidified atmosphere at 37 °C. After 2 day incubation, when the culture reached about 80% confluence, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) at 37 °C, and a fresh medium was added to each dish. Cells were incubated for additional 24 h, exposed to the drug as described below, and then collected at different time intervals.

Annexin V/propridium iodide staining. Adherent cells were detached by trypsinization and washed once with warm PBS. 1 x 10^6 cells were double stained with fluoresceinisothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) for 15 min at room temperature in a Ca²⁺-enriched binding buffer (apoptosis detection kit, Wako Chemicals), and then analyzed by dual-color cytometry on a FACSCalibur flow cytometer (BDIS, Becton Dickinson, San Jose, CA). For each sample, data from 20,000 cells were acquired in list mode on logarithmic scales. Analysis was performed with the Cell Quest software (BDIS) and WinMDI.

Assessment of mitochondrial membrane potential. Changes of the mitochondrial membrane potential were examined using flow cytometry analysis of cells stained with tetramethylrhodamine ethyl ester (TMRE; Molecular Probes). Cells were incubated with 10 nM TMRE in PBS/5% FBS for 30 min at 37 °C, washed, and stained with propidium iodide for 5 min and analyzed by flow cytometry.

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Abbreviations used: ATC — anaplastic thyroid carcinoma; FITC — fluoresceinisothiocyanate; PI — propidium iodide; SA-β-Gal — senescence-associated β-galactosidase; TMRE — tetramethylrhodamine ethyl ester; X-Gal — 5-bromo-4-chloro-3-indolyl β-D-galactosidase.
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(TMRE, Molecular Probes, Eugene, OR) [9], a cell-permeable dye accumulating in mitochondria with unaltered membrane potential. Cells were harvested by trypsinization at the end of experimental protocol, and 1 x 10^6 cells were incubated with 100 ng/ml TMRE for 15 min at room temperature in HEPES-buffered saline (pH 7.4) followed by the analysis with the FACSкан (20,000 cells/sample). The fluorescence intensity of TMRE was monitored at 582 nm (FL–2).

Staining of activated caspases and intact mitochondria in living cells was performed using microscope slides with 4 chambers. After exposure to Taxol for 24–48 h, cells were washed with warm PBS and incubated for 15 min at 37 °C with respective fluorochrome agents in PBS. The green-fluorescent substrate, rhodamine-110, bis-L-aspartic acid amide (R-22122, Molecular Probes, Eugene, OR), at the concentration of 100 nM for caspase activity, the red-fluorescent agent TMRE (10 nM) for measurement of mitochondrial membrane potential, and the blue-fluorescent Hoechst 33342 dye (Wako, Japan) for nuclei staining (1 mM) were used. The analysis was performed using Nikon fluorescent microscope with digital camera under UV excitation.

**Preparation of cell extracts.** Adherent cells were washed twice with ice-cold PBS supplemented with sodium pyrophosphate and orthovanadate, scraped with a rubber policeman, collected in 1 ml PBS, and centrifuged for 3 min at 1000 rpm at 4 °C. The pellet was then resuspended in 200 μl of lysis buffer (Cell Signaling Technology) containing a cocktail of protease and phosphatase inhibitors. After 15 min on ice, lysates were centrifuged for 15 min at 15,000 g and stored at –80 °C until use. Protein concentration was determined with bicinchoninic acid assay reagent kit (Sigma, St. Louis, MO) according to manufacturer’s protocol.

**Western blotting.** Total cell lysates were boiled in the sample buffer (100 mM Tris–HCl, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 10% dithiothreitol) and separated by SDS-PAGE 7.5–15% gradient gels (Biocraft, Tokyo, Japan). The homogenous 8 and 15% gels were used when better separation of high- and low-molecular-weight proteins, respectively, was needed. Forty micrograms of protein were applied per each lane. Proteins were transferred onto 0.2 μm nitrocellulose membranes (Millipore Corp., Bedford, MA) by semidry blotting. Membranes were blocked with Tris-buffered saline/0.1% Tween 20 containing 5% nonfat dry milk and incubated with primary antibodies to cyclin D1 and p16INK4A (Cell Signaling Technology) at 4 °C overnight. After washing three times with Tris-buffered saline/0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated species-specific secondary antibody (Cell Signaling Technology) for 1 h at room temperature and then washed again three times. Complexes were visualized using ECL reagents (Amersham, Arlington Heights, IL).

**Senescence-associated β-galactosidase (SA-β-Gal) staining.** The β-galactosidase activity assay at pH 6.0 has been considered specific for senescent cells [10]. After experimental treatment, adherent cells were washed with PBS, fixed with 2% formaldehyde at room temperature, washed and incubated at 37 °C with fresh stain solution, consisting of 1 mg X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) per ml of 40 mM citric acid/Na-phosphate, pH 6.0, 5 mM K-ferricyanide, 5 mM K-ferricyanide, 150 mM NaCl, 2 mM MgCl, as described [11]. SA-β-Gal-positive cells were detected by bright-field microscopy. Staining was developed in 4 h and usually reached a peak in 16 h.

**Statistical analysis.** All data were expressed as a mean ± SE. Differences between groups were examined for statistical significance using Student’s t-test. P < 0.05 denoted the presence of a statistically significant difference.

**RESULTS AND DISCUSSION**

It was previously shown that Taxol caused dose-dependent ATC cell death [3]. Exposure of ARO cells to 10 nM of Taxol for 48 h (Fig. 1) caused cell detachment from substrate (round-shaped cell in the picture) and death of most part of cell population. In the presence of 100 nM of Taxol, a 100% cell death was observed. It is believed that apoptosis is the major form of cell death under these conditions. This assumption was confirmed by the results of live cells staining with fluorescent dyes (Fig. 2). There were only rare cells with activated caspases (green spots in the picture) in the control samples. Incubation with Taxol induced caspase activation with the maximum observed at 25 nM of the drug. It should be noted that increase in Taxol concentration to 100 nM caused some decrease in caspase activation (see Fig. 2 [3]).

Using several fluorescent dyes allowed, in parallel with caspase activation, the revelation of changes in mitochondria. The TMRE dye penetrates through the cellular membrane and accumulates in intact organelles. The decrease in the cells stained red in the picture indicates the loss of mitochondrial membrane potential in a part of cell population. Such a decrease became noticeable at 25 nM of Taxol, and at 100 nM of the drug the number of cells with intact mitochondria decreased dramatically (see Fig. 2). These data were confirmed by the results of flow cytometry (Fig. 3). At 100 nM of Taxol, the number of cells with damaged mitochondria (indicated with an oval on the picture) was significantly increased.

Cell staining with PI and further flow cytometry analysis showed that in the control cultures most ARO cells were in the G1 phase of the cell cycle (Fig. 4). Taxol at 2 nM concentration produced no effect on cell distribution. Significant changes were observed at 10 nM of the drug. The quantity of G1 cells decreased and, conversely, the number of subG1 cells significantly increased. The latter suggests an initiation of apoptotic processes in the cells. This was confirmed by the results of Western blotting [3]. It should be noted that there was no G2 arrest at this concentration of the drug. Increase of Taxol concentration to 50 nM, and especially to 100 nM, caused a profound G2/M arrest of ARO cells (see Fig. 4). Almost complete cell accumulation in the G2/M phase was observed only
after 72 h of incubation with 100 nM of Taxol. Thus Taxol allows the progression through the cell cycle for about 3 days. The reason for such protraction is unclear. Perhaps, due to inhomogeneity of long-term cultures, some cell subpopulations may have longer doubling time and/or be a more resistant to the drug.

It should be noted that G2/M-arrested cells do not coincide with G2 peak and are shifted to the right on X axis. ARO cells age possesses genomic instability and aneuploidy can partly account for this shift.

Thus, our results indicate a rather clear difference between the effects of low (10–25 nM) and high (> 50 nM) Taxol concentrations. Low drug concentrations induce bona fide apoptosis, whereas at high concentrations other forms of cell death (necrosis, mitochondrial collapse, etc.) could occur along with apoptosis. It was assumed...
that apoptotic processes may take place in the cells that retain ability to pass cell cycle and, accordingly, to division. Conversely, cell cycle arrest and cell inability to divide due to DNA damage or chemotoxic stress may cause other forms of cell death such as necrosis or result in mitotic catastrophe, accelerated senescence, etc. [11–13].

Fig. 3. Flow cytometry analysis of Taxol effect on mitochondrial membrane potential in ARO cells. a — control, b — 100 nM of Taxol. Incubation time — 24 h. Cells that lost mitochondrial membrane potential are marked with an oval. Data of one typical experiment of 2 are presented.

Fig. 4. Dose dependent changes of ARO cell distributions through cell cycle. Incubation time — 72 h. Arrows indicate the position of cell cycle phases. Data of one typical experiment of 5 are presented.

Cellular senescence has been considered to be an important barrier for cancer development. Since some microtubule-stabilizing agents induce strong cell senescence [14], we examined the extent of senescence in ATC cells after Taxol treatment as potent tumour-suppressing mechanism alternative to apoptosis [15].

There was no significant accumulation of senescent cells both in the control and among the cells exposed to different concentration of the drug (Fig. 5). Initiation of senescence depends, in particular, on the expression of some cell cycle regulatory proteins, first of all, INK-family proteins — cyclin-dependent kinase inhibitors, which affect the G0/G1/S transition [16, 17]. We studied the expression of p16INK4A at increasing Taxol concentrations (Fig. 6, a). The p16INK4A level was very low and did not change at different Taxol concentrations. The level of cyclin D1 which promotes G1/S transition, on the contrary, was very high in both cell lines tested (Fig. 6, a, b). These data are consistent with the results of β-galactosidase activity assay in ATC cells (see Fig. 5), and demonstrate that senescence does not play a significant role in Taxol-dependent cellular effects in contrast to other stress agents such as discodermolide [14].

Fig. 5. Senescence-associated β-galactosidase staining of ARO cells. Arrows show the rare senescent cells. a — 0, b — 5 nM, c — 50 nM, d — 100 nM.
Fig. 6. Expression of p16\textsuperscript{INK4A} and cyclin D1 in ATC cells. a, Western blotting study. b, quantitative estimates of cyclin D1 level. Mean ± SE, n = 3. Taxol concentration 25 nM

One study claimed that the increase in subG1 cell population caused by low doses of Taxol was not associated with apoptosis but rather with di- and tripolar mitosis followed by postmitotic interphase arrest in some lines of human lung and breast cancer cells [13]. According to our data (Fig. 7), low Taxol concentrations induced apoptosis in vast majority of KTC-2 cells. We observed apoptosis in 20% of ATC cells at Taxol concentrations as low as 1 nM after 48 h of incubation. At 6–15 nM of the drug, the proportion of apoptotic cells increased to 75–83%. Thus, an increased level of subG1 cells is paralleled by massive apoptosis suggesting that namely this form of cell death is the predominant mechanism of ATC cell elimination at low Taxol concentrations. In addition, we also did not observe G1 arrest as reported in the above mentioned study [13] at any drug concentration.

It should be noted that 50 nM of Taxol cause apoptotic and, perhaps other forms of death in almost entire cell population exposed to the drug (see Fig. 7). Therefore, very high drug concentrations used in clinic may not necessarily be the best treatment strategy. Drug pharmacokinetics in blood showed that concentration of Docetaxel dropped from 10 µM to 100 nM in several minutes after drug applying with further decrease to 10 nM in 6 h [5].

Based on our observations, one might suggest that optimized treatment would employ a protracted (e. g. 1–2 weeks) regimen of drug administration aimed at attaining low drug concentrations (10–25 nM) in the circulation. Presumably, such modality could be sufficient to induce massive apoptosis of ATC cells and thus provide in a better chance to control the disease.

Fig. 7. Flow cytometry analysis of the apoptotic effects of different Taxol concentrations in KTC-2 cells. PI staining (Yaxis) versus Annexin V (Xaxis). Increase in Annexin V signal indicates apoptotic changes in cell membranes. C-quadrant — living cells, D — apoptotic cells, B — dead, necrotic and late apoptotic cells

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