J Cancer Res Clin Oncol (1996) 122:214–218

ORIGINAL PAPER

Jaakko O. Pulkkinen · Liisa Elomaa · Heikki Joensuu
Paula Martikainen · Kristina Servomaa
Reidar Grénman

Paclitaxel-induced apoptotic changes followed by time-lapse video microscopy in cell lines established from head and neck cancer

Received: 15 August 1995/Accepted: 16 September 1995

Abstract Paclitaxel (Taxol) is a potent chemotherapeutic drug for squamous-cell carcinoma (SCC) of the head and neck *in vitro* with microtubule-stabilizing activity that arrests cells in G2-M. To study the mechanism of its cytotoxic effect on SCC *in vitro*, we exposed five laryngeal SCC cell lines to 10 nM paclitaxel. The cell lines were studied by time-lapse video microscopy for 96 h, and by agarose gel electrophoresis. Paclitaxel blocked the cells in the premitotic phase for 6–24 h, after which the cells died morphologically by apoptosis. Mitotically arrested cells were seen within a few minutes after exposure to paclitaxel. No mitoses were seen in the paclitaxel-treated cells. A few apoptoses were also seen in the control cultures grown without paclitaxel, but they represented only 6%–20% of the frequency of apoptoses seen in the paclitaxel-treated group. In some paclitaxel-treated cultures the cells escaped the mitotic arrest without cytokinesis and formed multinucleated cells that eventually died. Agarose gel electrophoresis showed oligonucleosomal DNA fragmentation characteristic of apoptosis. We conclude that time-lapse video microscopy is an efficient method of observing drug-induced morphological changes in cell culture. Paclitaxel at a 10 nM concentration rapidly induces a premitotic block, which usually leads to apoptotic cell death. In some cases multinucleated cells are formed that morphologically also eventually die by apoptosis.

Key words Apoptosis · Paclitaxel · Squamous-cell carcinoma cell line · Time-lapse video microscopy · Head and neck cancer

Abbreviation *SCC* squamous-cell carcinoma

J. Pulkkinen · R. Grénman (✉)
Department of Otorhinolaryngology, Turku University Central Hospital, FIN-20520 Turku, Finland.
Fax: +358 21 613 550

L. Elomaa
Department of Oncology, Turku University, Turku, Finland

P. Martikainen
Department of Pathology, Turku University, Turku, Finland

R. Grénman
Department of Medical Biochemistry, Turku University, Turku, Finland

H. Joensuu
Department of Oncology and Radiotherapy, Helsinki University Central Hospital, Helsinki, Finland

K. Servomaa
Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland

Introduction

Paclitaxel (Taxol) is a structurally complex natural plant product with a novel mechanism of action. It has a range of activity that includes ovarian cancer, melanoma, non-small-cell lung carcinoma, breast carcinoma and leukemia (Runowicz et al. 1993).

Paclitaxel is a potent inhibitor of eukaryotic cell replication, blocking cells in the late G2-M phase of the cell cycle (Schiff and Horwitz 1980). It is an unusual mitotic inhibitor because it promotes the formation of discrete bundles of stable microtubules that result from the reorganization of the microtubule cytoskeleton (Schiff et al. 1979).

Wyllie et al. (1980) characterized two mechanisms of cell death: necrosis and apoptosis, also called programmed cell death. These two processes differ both morphologically and biochemically. The morphological changes associated with necrosis are swelling, followed by rupture of the cell membranes and dissolution of the organized cell structure. In contrast, apoptosis is characterized by chromatin condensation, cellular shrinkage, cytoplasmic blebbing and fragmentation of genomic DNA as a consequence of the activation of

a specific endogenous endonuclease. DNA is broken into fragments of 180–200 base pairs (bp), i.e. oligonucleosome length.

Many chemotherapeutic agents have been shown to induce apoptosis in sensitive cells. It has been reported that paclitaxel causes apoptotic changes in human small-cell lung cancer (SCC) cells (at the mean inhibition concentration of 22.6 nM) (Ohmori et at. 1993), in a human ovarian tumor cell line (Liu et al. 1994), in two human carcinoma cell lines (Willingham and Bhalla 1994) and in human leukemic cells (Tang et al. 1994). The tendency of a cancer cell to undergo apoptosis may be especially important for the chemotherapy of malignant tumors with a low growth rate, which are typically resistant to cytostatic agents (Carson and Ribeiro 1993).

Our group has previously shown that paclitaxel induces growth inhibition in laryngeal SCC cell lines at nanomolar concentrations and DNA flow cytometry showed a G2-M block (Elomaa et al. 1995). In this study we demonstrate that paclitaxel in nanomolar concentrations induces apoptosis in human laryngeal SCC cell lines, and study the associated morphological changes by time-lapse video microscopy.

Materials and methods

Materials

Paclitaxel was kindly provided by Bristol-Mayers Squibb (Princeton, Pa., USA). A 0.1 mM stock solution was prepared by diluting the original solution containing 6 mg paclitaxel with isotonic saline, and the final dilutions were made immediately before each experiment in our laboratory.

Cell culture

Five laryngeal SCC cell lines established in our laboratory were investigated. Information about the origin of the cell lines [primary tumour location, TNM staging, specimen site, type of lesion, histological grade (1–3) and the passage numbers used] are listed in Table 1. The cells were maintained in complete Eagle's minimal essential medium with 2 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. All cells were cultured as monolayers in plastic tissue-culture dishes (Nunc, Roskilde, Denmark).

Time-lapse video microscopy

Between 1 and 4 days after plating a medium containing 10 nM paclitaxel was applied. The medium was equilibrated with 5% CO_2 at 37 °C in an incubator for 10 min. The culture flask was then capped and transferred to a 37 °C heated stage of an inverted microscope (Nikon Diaphot, Nikon Corp., Tokyo, Japan). Cells were viewed using phase-contrast optics at 20 × objective magnification coupled to a JVC 3CCD KY-F30 video camera (Victor Company, Tokyo, Japan). The field to be analysed was selected so that at the beginning of filming it contained approximately 20–50 cells. The time-lapse video recording was performed so that two successive pictures were taken at 1-min intervals (Panasonic AG-6720A). The video recorder and the microscope were coupled to a timer (LIBT2, Red Lion, USA), which lit the microscope lamp for 5 s in every minute syncronously with the recorder. Filming was continued for 96 h. Subsequently the film was viewed frame by frame on a video monitor. The cumulative number of cells rounding up, completing mitosis or apoptosis and multinucleated cells per field were counted at 3-h intervals. The pH of the medium remained stable during the experiment. One control culture was filmed for each cell line, and at least two paclitaxel-treated cultures were filmed. The time (h) during which the cells stayed mitotically arrested was calculated by a time-shift of the two curves (imaging premitotic or apoptotic cells) until they overlapped each other.

Analysis of DNA fragmentation

Cells (10^7) were harvested after treatment with 10 nM paclitaxel for 24 h and 48 h. The cells were incubated in a cell lysis buffer (50 mM TRIS-HCl, pH 8, 2 mM EDTA, 100 µg/ml proteinase K and 0.5% sodium dodecyl sulphate) at 37 °C overnight, and the DNA was extracted twice with phenol/chloroform and once with chloroform. DNA was precipitated with ethanol, rinsed with 70% ethanol, and dried. The extract was dissolved in TRIS/EDTA buffer (10 mM TRIS/HCl, pH 8, containing 1 mM EDTA) and then treated with RNase (50 µg/ml) for 1 h at 37 °C. Extraction and precipitation of DNA were repeated as described above. Cellular DNA (0.5–1.0 µg) was treated with 5 U Klenow polymerase using 0.5 µCi ^{32}P-labelled dCTP in the presence of 10 mM TRIS/HCl (pH 7.5)/5 mM $MgCl_2$. The reaction was incubated for 10 min at room temperature and terminated after addition of 10 mM EDTA. The unincorporated nucleotides were removed by three consecutive precipitation cycles of isopropanol with 2.5 M ammonium acetate. The labelled DNA was resuspended in TRIS/EDTA (pH 7.5). 0.5 µg DNA was applied on a 1.8% agarose gel and electrophoresed for 2 h at 100 V. After the gel had been dried, the filter was exposed for autoradiography.

Table 1 Characteristics of the cell lines

Cell line	Tumour site	TNM stage	Specimen site	Histological grade	Passages used
UT-SCC-8	Supraglottic larynx	T2N0M0	Larynx	G1	4, 49, 53
UT-SCC-9	Glottic larynx	T2N0M0	Neck metastasis	G1	24, 27, 38
UT-SCC-19A	Glottic larynx	T4N0M0	Larynx	G2	24, 25, 43
UT-SCC-19B	Glottic larynx	T4N0M0	Larynx[a]	G2	32, 33, 45
UT-SCC-29	Glottic larynx	T2N0M0	Larynx	G1	9, 10, 15

[a] Persistent disease at the primary site after irradiation

Table 2 The cumulative number of cells completing mitoses or undergoing apoptoses during 96 h in a medium containing 10 nM paclitaxel. The figures are percentages of the initial cell number and *n* is the initial cell number in parenthesis. The time in premitotic phase was estimated by a time-shift of the curves so that they overlapped each other

Cell line	Control culture			10 nM paclitaxel			
	Mitoses (%)	Apoptoses (%)	*n*	Mitoses (%)	Apoptoses (%)	Time (h) in premitotic phase	*n*
UT-SCC-8	142	13	31	0	84	24	25
UT-SCC-9	146	12	55	0	57	14	45
UT-SCC-19A	240	25	20	0	51	16	43
UT-SCC-19B	150	17	36	0	46	24	50
UT-SCC-29	65	12	40	0	57	6	45

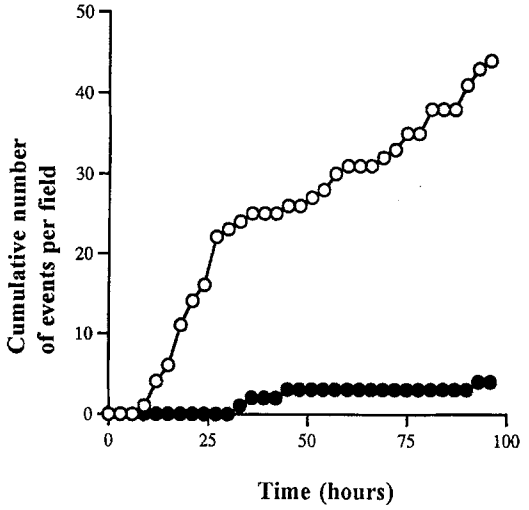

Fig. 1 UT-SCC-8 cells grown in Eagle's minimal essential medium (control) and followed by time-lapse video microscopy. The filming began 48 h after plating. The initial cell number in the viewing screen at the beginning of the filming was 31. The cumulative number of cells undergoing mitoses (○) or apoptoses (●)

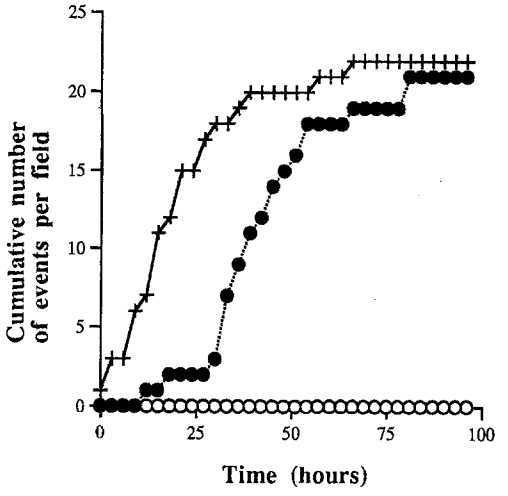

Fig. 2 Effects of 10 nM paclitaxel on UT-SCC-8 cells followed by time-lapse video microscopy. The initial number of cells in the viewing screen at the beginning of the filming was 25. The cumulative number of cells rounding up (+) or undergoing mitoses (○) or apoptoses (●)

Data analysis

The apoptotic frequency of control cells and paclitaxel-treated cells was calculated as the ratio (apoptoses)/(initial cell number + mitoses), referred to as the apoptotic ratio.

Results

Time-lapse video microscopy of SCC cells in culture

Time-lapse video microscopy was used to analyse the morphological changes induced by 10 nM paclitaxel in the cultured laryngeal SCC cells. The results of the time-lapse video microscopy are shown in Table 2 and in Figs. 1 and 2. As expected on the basis of our previous experiments, the control cultures of all cell lines showed frequent mitoses (Elomaa et al. 1995). Apoptoses were also seen in the control cultures; they represented 12%–25% of the initial cell number, depending on the cell line (Table 2), and the apoptotic ratio varied from 0.052 to 0.075 (mean 0.064). In the cultures treated with 10 nM paclitaxel the cells rounded up at the same pace as the control cells underwent mitoses. They stayed mitotically arrested for 6–24 h, after which the cells died morphologically by apoptosis i.e. by cellular shrinkage, violent pulsation and blebbing of the plasma membrane during the subsequent hour. No mitoses or necroses were observed in the paclitaxel-treated cells. The apoptotic ratio varied from 0.35 to 0.92 (mean 0.61). In some paclitaxel-treated cultures some of the cells escaped the mitotic arrest without cytokinesis and formed multinucleated cells. These multinucleated cells eventually died very rapidly (in a few minutes) showing cell shrinkage, i.e. one of the typical signs for apoptotic cell death (Fig. 3).

DNA electrophoresis

DNA ladder fragmentation was detected by agarose gel electrophoresis, which confirmed the apoptotic type of

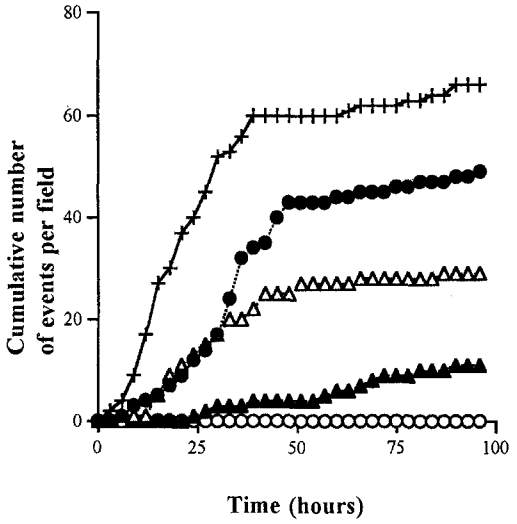

Fig. 3 Effects of 10 nM paclitaxel on UT-SCC-29 cells followed by time-lapse video microscopy. The initial cell number in the viewing screen at the beginning of the filming was 53. The cumulative number of cells rounding up (+) or undergoing mitoses (○) or apoptoses (●), multinucleated cells (△) or dying multinucleated cells (▲)

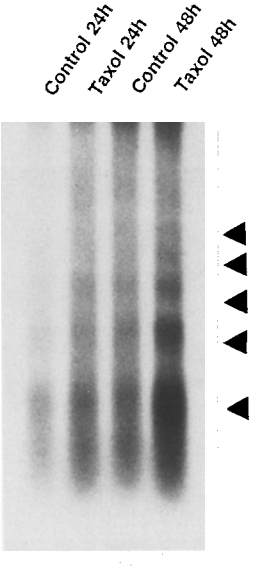

Fig. 4 Effects of 10 nM paclitaxel on DNA fragmentation. UT-SCC-19A cells were cultured with no additives (*Control*) or in 10 nM paclitaxel for 24 h or 48 h as indicated. Subsequently DNA was isolated from the cultures and analysed for DNA laddering. Comparable results were obtained in all cell lines and in two independent experiments. *Arrowheads* from *bottom* to *top* indicate mono- or oligonucleosomal DNA fragments with the nominal length of 200*n* base pairs ($n = 1, 2, 3, 4, 5$)

cell death. The DNA agarose gel electrophoresis "ladder" pattern was observed on DNA extracted from cells treated with paclitaxel.

Figure 4 shows the appearance of DNA fragments detected by agarose gel electrophoresis. The DNA fragmentation patterns are similar for all samples in which the cells were exposed to paclitaxel for periods of 24 h or 48 h.

The hallmark of the apoptotic process, nucleosomal fragmentation, was detectable even in untreated control cells. Oligonucleosomal DNA fragmentation was visible in all cultures at 24 h and it was of increased intensity at 48 h.

Discussion

The spontaneous occurrence of apoptosis has been described in many different types of cancer (Wyllie et al. 1980). Video microscopy revealed spontaneous apoptoses in all of our cell lines established from head and neck cancer, and they represented 6%–20% of the frequency of apoptoses seen in the paclitaxel-treated cultures. Similarly agarose gel electrophoresis showed DNA laddering as a sign of spontaneous apoptoses in all cell lines (example in Fig. 4).

A 10 nM dose of paclitaxel induced full growth inhibition in our laryngeal SCC cell lines. In flow-cytometric analysis a G2-M block was demonstrated in all our five cell lines after exposure to 10 nM paclitaxel for 24 h (Elomaa et al. 1995). Mitotically arrested cells were observed with time-lapse video microscopy after addition of paclitaxel, and they were copious after 24 h. This timing corresponds to the developement of the premitotic block demonstrated in DNA flow-cytometry studies (Elomaa et al. 1995).

Detection of apoptosis by DNA gel electrophoresis depends on the number of apoptotic cells in the sample. Extensive apoptosis can easily be visualised by electrophoresis after staining with ethidium bromide, whereas the measurement of a low level of nucleosomal autolysis is more difficult. To increase the sensitivity for detection of fragmented DNA, we carried out a simple and rapid method using [^{32}P]dCTP end-labelling of DNA fragments with Klenow polymerase (Rösi 1992).

Although apoptotic cell death was detected both by time-lapse video microscopy and agarose gel electrophoresis, we found video microscopy to be a specific and sensitive method to detect apoptotic cell death, and to give information about the time course of these events.

The multinucleated cells eventually died morphologically by quick cellular shrinkage, and a change to a normal Dulbecco's modified Eagle medium after they had been kept for 96 h in a medium containing 10 nM paclitaxel even facilitated their tendency to die (data not shown).

These effects were seen with a paclitaxel concentration of 10 nM, which is only 1/10–1/100 of the concentration achieved in the patient serum after a single dose

of about 200 mg/m^2. However, the exposure time of 96 h to paclitaxel may be longer than what is achieved in head and neck cancer tissue *in vivo* after a single infusion of paclitaxel.

The tendency for spontaneous apoptosis of our cell lines might have some correlation to their sensitivity to paclitaxel. This, however, requires further study in a larger series of cell lines. The paclitaxel-induced G2-M block might act as a radiosensitizer as this phase is known to be a radiosensitive phase of the cycle (Sinclair 1968). It has been suggested that paclitaxel potentiates tumour response to radiation by other mechanisms in addition to blocking the cell cycle in mitosis (Milas et al. 1994).

Acknowledgements We wish to thank Mrs. Marita Potila for her excellent technical assistance and Dr. Valtteri Niemi for his constructive comments made on an earlier draft of this paper.

References

Carson DA, Ribeiro JM (1993) Apoptosis and disease. Lancet 341:1251–1254

Elomaa L, Joensuu H, Kulmala J, Klemi P, Grénman R (1995) Squamous cell carcinoma of the larynx is highly sensitive to paclitaxel *in vitro*. Acta Otolaryngol (Stockh) 115:340–344

Liu Y, Bhalla K, Hill C, Priest DG (1994) Evidence for involvement of tyrosine phosphorylation in taxol-induced apoptosis in a human ovarian tumor cell line. Biochem Pharmacol 48:1265–1272

Milas L, Hunter NR, Mason KA, Kurdogly B, Peters LJ (1994) Enhancement of tumor radioresponse of a murine mammary carcinoma by paclitaxel. Cancer Res 54:3506–3510

Ohmori T, Podack ER, Nishio K, Takahashi M, Miyahara Y, Tadeka Y, Kubota N, Funayama Y, Ogasawara H, Ohira T, Ohta S, Saijo N (1993) Apoptosis of lung cancer cells caused by some anti-cancer agents (MMC, CPT-11, ADM) is inhibited by bcl-2. Biochem Biophys Res Commun 192:30–36

Rösi F (1992) A simple and rapid method for detection of apoptosis in human cells. Nucleic Acids Res 20:5243

Runowicz CD, Wiernik PH, Einzig AI, Goldberg GL, Horwitz SB (1993) Taxol in ovarian cancer. Cancer 71:1591–1596

Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroplast cells. Proc Natl Acad Sci USA 77:1561–1565

Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly *in vitro* by taxol. Nature 277:665–667

Sinclair WK (1968) Cyclic X-ray responses in mammalian cells *in vitro*. Radiat Res 33:620–643

Tang C, Willingham MC, Reed JC, Miyashita T, Ray S, Ponnathpur V, Huang Y, Mahoney ME, Bullock G, Bhalla K (1994) High levels of p26BCL-2 oncoprotein retard taxol-induced apoptosis in human pre-B leukemia cells. Leukemia 8:1960–1969

Willingham MC, Bhalla K (1994) Transient mitotic phase localization of *bcl*-2 oncoprotein in human carcinoma cells and its possible role in prevention of apoptosis. J Histochem Cytochem 42:441–450

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306