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Apoptotic cell death in suspension cultures of *Taxus chinensis* var. *mairei*

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Abstract

Apoptotic cell death was observed in suspension cultures of *Taxus chinensis* var. *mairei* under normal cultivation conditions by using microscopy, total DNA agarose gel electrophoresis and *in situ* end-labeling of fragmented DNA. The morphological and biochemical changes of cells occurred mainly in the non-dividing cell clusters, indicating that the *T. chinensis* cells died mainly by apoptosis. There exists a close relationship between cell apoptosis and Taxol formation. Taxol concentration increased with the increase in content of apoptotic cells and reached a maximum (14.2 mg l^{-1}) after 23 days of culture, corresponding to a maximum ratio of apoptotic to total cells of about 13%.

Introduction

Taxol is an effective anticancer agent and shows significant activity in clinical trials against a wide variety of tumors especially refractory ovarian, non-small cell lung, AIDS-related Kaposi's sarcoma and other cancers (Rowinsky *et al.* 1995). As Taxol is mainly produced by extraction from the bark of *Taxus* species, the very limited resource of *Taxus* species severely limits its production in large amounts. Therefore, the recovery of secondary metabolites from plant cell cultures has been regarded as a promising alternative for production of taxol (Yuan *et al.* 2001).

Suspension cultures of *Taxus* spp. have been extensively investigated due to their high capacity of Taxol production (Ketchum *et al.* 1999, Yuan *et al.* 2001) with Taxol production in suspension cultures of *T. chinensis* var. *mairei* reaching a maximum at the phase of cell death (Yuan *et al.* 2000). As the initiation of secondary metabolism in plant cells is often accompanied by cell differentiation, a phase usually called as 'idiophase' to distinguish it from the tropophase of cell growth, the physiological conditions and multiple genes should affect the growth phase transition in plant cells. Totipotent cells in suspension cultures divide asymmetrically into cell pairs: one cell stops synthesizing DNA and dies, whereas the other continues growing to establish an embryo (Normura & Komamine 1986). During the death process of plant cells, some morphological changes including cell shrinkage, condensation of cytoplasm, nuclear fragmentation and small membrane-sealed packets occur, suggesting that the cells have undergone a form of death similar to the apoptosis in animal cells (Havel *et al.* 1996). Apoptosis is a universal phenomenon and plays an important role in the development of organs and whole organisms, however, the understanding of the apoptosis in suspension cultures of plant cells is limited compared to that in animal cells (Mittler *et al.* 1995).

In this work, the morphological and biochemical features of cell death in suspension cultures of *Taxus chinensis* were investigated by using microscopy, agarose gel electrophoresis and terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (TUNEL).

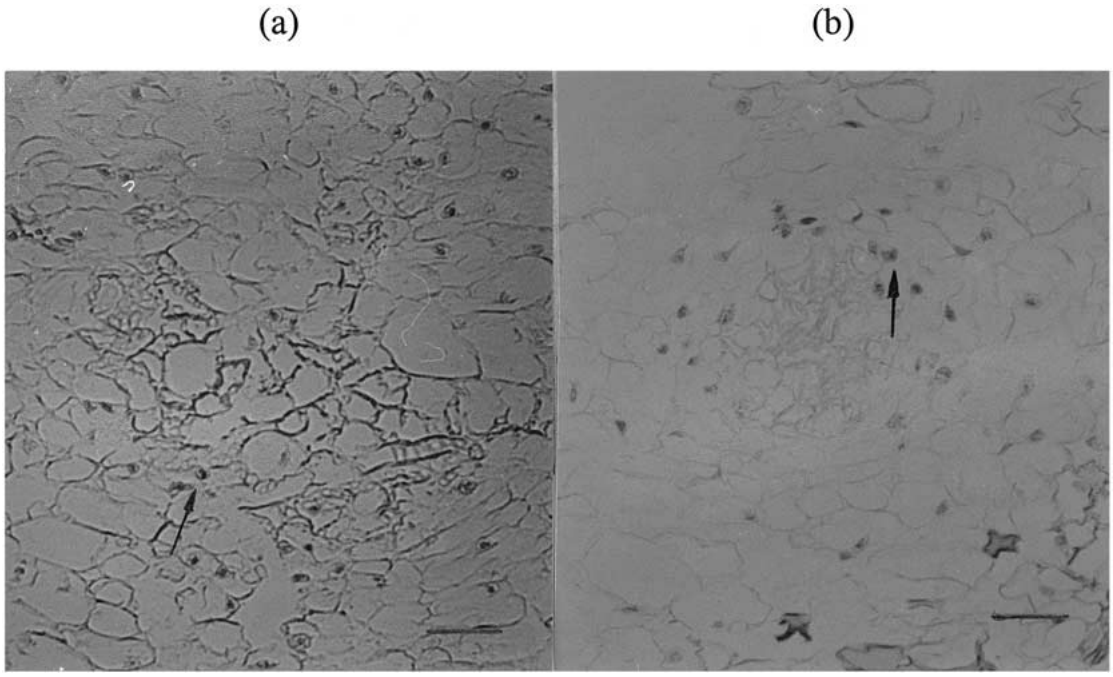

Fig. 1. Structure of cultured *Taxus chinensis* cell clusters under a light microscope. (a) Younger cell clusters with the tracheo-element differentiation at day 5. (b) Cell clusters at day 15. Arrows point the nuclei. The bar is 20 μm.

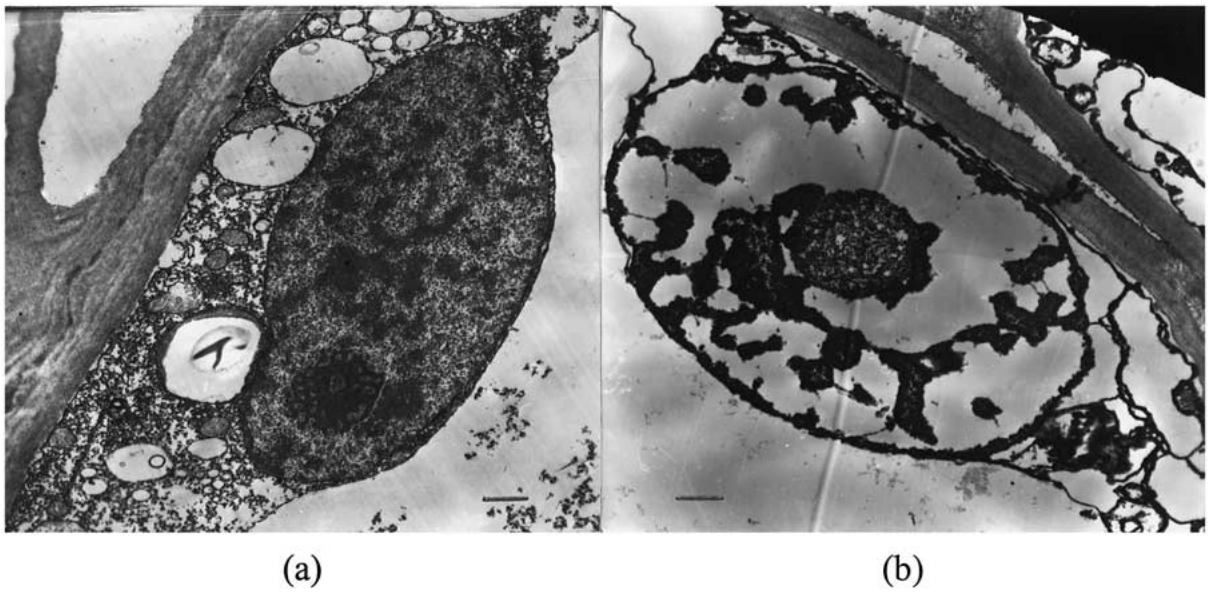

Fig. 2. Electron micrograph of the cultured *Taxus chinensis* cells at different stages of cell growth. (a) The nucleus of a living cell at day 7. (b) The nucleus of a dead cell at day 18. The bar is 1 μm.

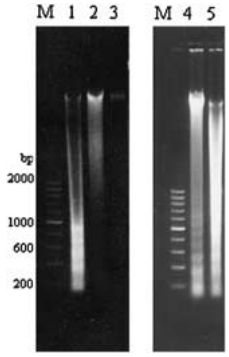

Fig. 3. Agarose gel analysis of total DNA isolated from cultured *Taxus chinensis* cells. Lane M: 200 bp DNA markers, lane 1: 180 bp DNA laddering at day 15, lane 2: DNA fragments at day 9, lane 3: intact DNA at day 7, lane 4: DNA ladders at day 17, lane 5: DNA fragments at day 21.

Materials and methods

Chemicals

Eosin and haemalum were purchased from Sigma. All other chemicals used were of analytical grade and obtained commercially.

Culture conditions

Taxus chinensis var. *mairei* Y-902 cells, kindly provided by the Institute of Botany, Chinese Academy of Sciences, were sub-cultured in Gamborg's B5 medium (Gamborg *et al.* 1968) supplemented with sucrose (30 g l^{-1}), naphthylene acetic acid (5 μM) and 6-benzyladenine (0.01 μM). The medium pH was adjusted to 5.8 with 0.1 M NaOH prior to autoclaving. Culture medium (100 ml) was placed in a 250 ml flask and autoclaved for 20 min at 121 °C. The cells were cultivated for 5 generations at 25 °C in dark in an orbital shaker at 105 rpm. A small part of the suspension cultures of the 5th generation were collected at the mid-growth and filtered through of Microcloth and then inoculated in a B_5 medium at 5% (w/v). Three parallel experiments were conducted and the culture samples were collected at predetermined time intervals for various analyses.

Observation by an optical microscopy

Cell samples (100 mg) were fixed in 5 ml FAA solution [ethanol (70%, v/v)/glacial acetic acid/formalin, 18:1:1, by vol.], dehydrated, embedded in paraffin, sectioned at 10 μm intervals with a microtome and stained with eosin and haemalum following the method of Altamura *et al.* (1991). The images were recorded using an ASA 100 T film (Kodak) under a microscope (Nikon, Japan) and processed with the Adobe Photoshop software (Grand Prairie, USA).

Observation using an electron microscopy

Cell samples (100 mg) were first fixed in 5 ml 0.1 M phosphate buffer (pH 7.2) containing glutaraldehyde (3%, v/v) and paraformaldehyde (4%, v/v) then filtered and post-fixed in 5 ml osmium tetroxide (1%, w/v). Dehydration of the fixed cell samples was carried out by incubation for 10 min in a series of ethanol solutions of increased concentrations before embedding them in spur resin. Sections of 60–90 nm were cut using a diamond knife and stained with uranyl acetate (2%, w/v) in 70% (v/v) ethanol for 1 h and in 0.1 M lead citrate for 1 h, respectively. Electron micrographs were observed using a JEM 100C electron microscope.

Analysis of DNA fragments

Total DNA was extracted following the method of Dellaporta *et al.* (1983) with a slight modification. Fresh cells (0.2 g) were grounded in liquid N_2 with mortar and pestle. The grounded cells were transferred to a sterilized Eppendorf tube and dissolved in 600 μl buffer (pH 8) consisting of hexadecyltrimethyl ammonium bromide (CTAB) (2%, w/v), Tris/HCl (10 mM), EDTA (20 mM), NaCl (1.4 mM) and β-mercaptoethanol (2%, v/v) at 65 °C. The mixture was shaken slightly and incubated at 65 °C for 30 min, then centrifuged at 10 000 *g* for 20 min. The water phase (the upper layer) was extracted with equal volume of chloroform/isoamyl alcohol (24:1, v/v). The supernatant was collected and mixed with equal volume of pellet buffer of pH 8 consisting of CTAB (1%, v/v), Tris/HCl (50 mM) and EDTA (10 mM) at 65 °C for 30 min to precipitate DNA and RNA. The total DNA and RNA were collected by centrifugation at 3000 *g* for 10 min. The pellets were re-suspended in 0.5 ml high-salt buffer (pH 8) consisting of Tris/HCl (10 mM), EDTA (1 mM) and NaCl (1 M). DNA and RNA were precipitated by addition of twice volumes of ethanol at −20 °C for 1 h. The precipitates were centrifuged, washed with 70% (v/v) ethanol and then dried at 37 °C. The dried total DNA and RNA were dissolved in 30 μl buffer (10 mM Tris/HCl, 1 mM EDTA) of pH 8. RNase A (100 μg ml^{-1}) was added to digest RNA at 37 °C for 30 min. The DNA samples were run on 1.2% (w/v) agarose gel stained with

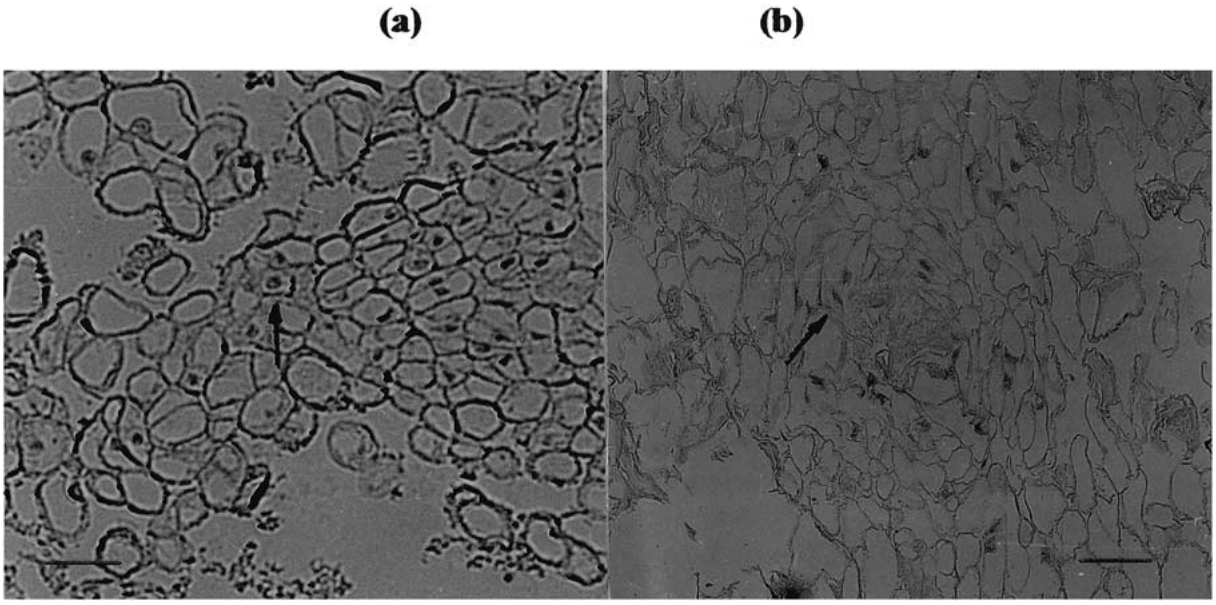

Fig. 4. Normal and apoptotic cells detected by TUNEL in cultured *Taxus chinensis.* (a) Normal cells (pointed by arrow). The bar is 10 μm. (b) Apoptotic cells (pointed by arrow). Scale bar is 20 μm.

ethidium bromide (0.5 μg ml^{-1}) to observe the DNA fragments under UV illumination.

TUNEL assay of apoptotic cells

To detect nuclear DNA cleavage, cell sections were treated by the TUNEL procedure using the *in situ* cell death detection kit, POD (Boehringer, Mannheim), following the manufacturer's instruction. Nuclei were counterstained with hematoxylin. TUNEL-negative nuclei were stained blue, TUNEL-positive nuclei were stained brown. The images were recorded by ASA 100 T film (Kodak) on a microscope (Nikon, Japan) and processed with Adobe Photoshop software (Grand Prairie, TX). Apoptotic cells were counted in three slides containing 30 views with about 200 cells per view.

Extraction of taxol and analysis by HPLC

Extra- and intracellular taxol was extracted and analyzed by HPLC as described in a previous publication (Yuan *et al.* 1998).

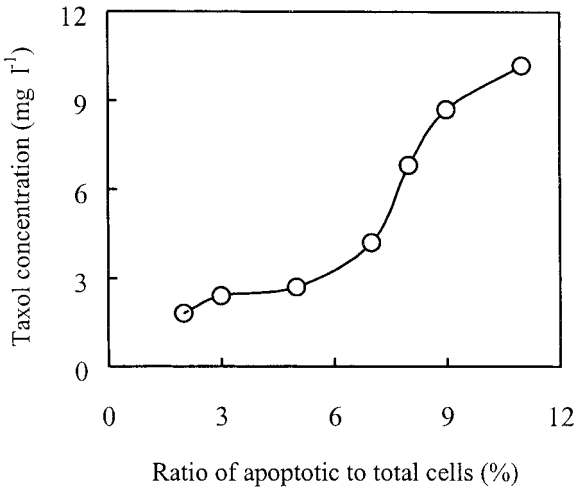

Fig. 5. Taxol production vs. the number ratio of apoptotic to total cells. Apoptotic cells were counted in three slides with 10 views per slide and each view contained ca. 200 cells.

Results and discussion

Morphological changes of T. chinensis *cells*

Cell clusters of different structures were observed during cultivation (Figure 1). The clusters consisted of pale yellow young and mitotically active cells (Figures 1a and 2a) while the darker clusters were composed of old and non-dividing cells (Figure 1b). Young cell clusters are inferior to the old ones in biosyn-

thesis of taxol (Yuan *et al.* 2000). The older cell clusters, which were brown, could be roughly divided into three regions: the inner, middle and outer regions (Figure 1b). In the inner region, the cellular structure disappeared besides the vessels and thickened wall. In the middle region, the cells were still integrated and their cellular structure was clearly visible. In the outer region, the cells were sparsely distributed but not well structured. The cells in the outer region showed some features of apoptotic cell death (Figure 2b), i.e. the cells shrank, the secondary wall thickened and the chromatin condensed to particles. In addition, the cytoplasmic also became compacted. Therefore, it is inferred that the cell death in the outer region might undergo an apoptotic process.

Electrophoresis of the total DNA

To confirm apoptotic cell death, electrophoresis of total DNA was conducted (Figure 3). The progressive delineation of fragmented DNA into distinct 180 bp and oligopolymers was observed at the late time of cultivation (ca. 15th day) as a symbol of DNA ladders. This is a characteristic of the apoptotic cleavage of nuclear DNA, often accompanying with the increases in activities of several deoxyribonucleases (Mittler & Lam 1995).

DNA fragmentation is only one of the biochemical characteristics of apoptosis. The other characteristics of apoptosis, such as the flipping-over of phosphatidylserine, release of cytochrome *c* and activation of certain caspases as observed in animal cells, should also be investigated for a better understanding of the apoptotic cell death of plant cells. The differentiation between the living and apoptotic cells may also help understand the observed low ratio of apoptotic to total cells. The cells stained by TUNEL staining method showed nuclei of brown color (Figure 4b), indicating the DNA fragmentation and apoptosis in the dying cells.

Relation between Taxol production and cell apoptosis

Figure 5 shows that taxol production was closely related with cell apoptosis. The larger the ratio of the apoptotic to total cells was, the higher the taxol concentration in the culture system was. As mentioned earlier, the apoptotic cell death mainly occurred in the cell clusters of brown color. This might show that the cell clusters of brown color had a stronger ability of producing taxol than other ones. However, the detailed relation between cell apoptosis and taxol production is still unclear based on the present observations and needs further investigation.

Acknowledgement

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