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ß-lapachone induced cell death in human hepatoma (HepA2) cells

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Summary. In present study we studied the cytotoxic effects of ß-lapachone, a potent anticancer drug, on the human hepatoma cell line (HepA2) under serum-free condition. Most cells died after 2 μM ß-lapachone addition at 48 hours. No apoptotic characteristics of DNA ladder was documented by agarose DNA electrophoresis. The blockage of cell cycle at S phase and unscheduled DNA synthesis were demonstrated by flow cytometric analysis and anti-bromodeoxyuridine immunocytochemistry. Ultrastructural observation showed that the swollen mitochondria, dilatation and vesiculation of rER and proliferation of peroxisome-like granules appeared within the cytoplasm of HepA2 cells following drug treatment. Using enzyme cytochemistry, both peroxidase and acid phosphatase activities but not catalase activity were localised in these peroxisome-like granules. Therefore, these results suggested that (a) ß-lapachone has a novel cytotoxic effect on human hepatoma cell; (2) ß-lapachone induces the interruption of the cell cycle and unscheduled DNA synthesis in HepA2 cells; and (3) ß-lapachone promotes the proliferation of peroxisome-like granules containing peroxidase and acid phosphatase activities without evidence of catalase activity in hepatoma cell line.

Key words: Hepatoma cells, ß-lapachone, Cell death, Electron microsocpy, Flow cytometry

Introduction

ß-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-*b*]pyran-5,6-dione), a potent anticancer drug, is a natural plant product extracted from various species of tropical trees or directly oxidized from lapachol (Goncalves de Lima et al., 1962; Schaffner-Sabba et al., 1984). ß-Lapachone and its derivatives have been synthesized chemically and have been shown to have a variety of pharmacological properties which elicit anti-viral and anti-parasitic effect (Goncalves et al., 1980; Li et al., 1993b). Marked cytotoxic effects of ß-lapachone on sarcoma cells have been reported (Docampo et al., 1979a). From the view of chemical structure, ß-lapachone is a lipophilic *O*-naphthoquinonic compound. Most naphthoquinonic compounds are shown to possess superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) generating properties in mitochondrial and microsomal suspensions. High production of O_2^- and H_2O_2 within intracellular organelles are toxic to cells or organisms (Docompo et al., 1979b; Forrest et al., 1994; Whittemore et al., 1994). Furthermore, ß-lapachone is assumed to be a DNA topoisomerase I modulator. Unlike camptothecin, ß-lapachone has been shown to inhibit the unwinding activity of Topo I but without producing stabilization of DNA-Topo I complexes (Li et al., 1993a). ß-lapachone also induces the apoptosis of human promyelocytic leukemia (HL-60) or human prostate (DU-145, PC-3 and LNCaP) cancer cells by passing the blockage of cell cycle at G0/G1 (Li et al., 1995; Planchon et al., 1995). However, the cytotoxicty of ß-lapachone on human hepatoma cell line has not been demonstrated. Thus in present study we examined in detail the toxic effect of ß-lapachone in a human hepatoma cell line (HepA2) and reported that ß-lapachone treatment induces the blockage of DNA cycle at S phase and enhances the unscheduled DNA synthesis in HepA2 cells. In addition, ß-lapachone treatment also increases the proliferation of peroxisome-like granules and promotes both activities of endogenous peroxidase and acid phosphatase within peroxisome-like granules in the hepatoma cells.

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Materials and methods

1. Reagents

ß-lapachone was prepared according to the procedures described by Schaffner-Sabba et al. (Schaffner-Sabba et al., 1984). ß-lapachone: mp 158-159 °C (n-hexane/EtOAc) (lit 153-154 °C); NMR; IR; MS. ß-Lapachone was dissolved in ice-cold absolute alcohol as a stock solution at 10 μM concentration and stored in

aliquots at -20 °C.

2. Cell preparation

HepA2 cells used in this study were obtained from the Cell Bank of Taipei Veterans General Hospital. Cells ($2x10^6$) were cultured in 100 μm culture plates in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-activated fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 100 units/ml penicillin and streptomycin at 37 °C in a humidified 5% CO_2 atmosphere for 24 hours. Cells were washed twice with prewarmed PBS and cultured in serum-free DMEM for an additional 1 day.

3. Cytotoxicity of ß-lapachone on HepA2 cells

Following a 24 hr period of serum-free culture, cells were washed twice with prewarmed PBS. and then treated with 0-4 μM ß-lapachone in serum-free medium for 12-48 hrs respectively. After treatment, cells were trypsinized and cell viability was evaluated by trypan blue exclusion under phase contrast microscope.

4. Flow cytometry

Cells treated with or without various concentrations of ß-lapachone under serum-free condition at various time were trypsinized and washed twice with ice-cold 0.05M citrate buffer (pH 7.6) and then stained with a solution containing 0.1% NP40, RNAase A (7,000 units/ml), and 33 mg/ml propidium iodide at 4 °C for 20 min. Cell cycles were analyzed with a FACS can flow cytometry (Becton Dickinson).

5. DNA ladder assay

Treated and control cells were washed with PBS and were lysed in 30 μl lysis buffer (50 mM tris (pH 8.0), 10 μM EDTA, 0.05% SDS and 10 mg/ml proteinase K) for 60 min at 50 °C and treated with 0.5 mg/ml RNase A for an additional 1 hour at 50 °C. Loading buffer (10 mM EDTA, 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue, and 40% (w/v) sucrose) was added (10% final concentration), and heated samples were loaded onto presoldified, 1.8% (w/v) agarose gel containing 0.1 mg/ml ethidium bromide. Agarose gels were run at 100V for 90 min in TBE buffer. Gels were observed and photographed under UV light.

6. DNA synthesis

Cells ($2x10^5$/ml) were cultured on cover slips instead of culture plates in complete DMEM for 24 hours. Following treatment as above description, cells treated with or without various concentrations of ß-lapachone for 6 hours. Final concentration of 10 μM BrdU (5-bromo-2'-deoxy-uridine) was added in the culture medium and incubated for 2 hours. Following two rinses with cold PBS, cells were fixed with ethanol-glycine fixative [70% ethanol in 0.05M glycine buffer (pH 2.0)] for 20 min. Cells were incubated for 30 min with mouse anti-BrdU antibody (1:200) and then with sheep anti-mouse IgG conjugated with alkaline phosphatase. NBT/X-phosphate was used as substrate. The immunostained sections were mounted with glycerol-gelatin medium and were observed and photographed under BH-2 Olympus microscope.

7. Transmittion electron microscopy

After two washes in PBS, treated and control cells were fixed with fixative containing 2% paraform-aldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4, 4 °C) for 30 min. Following osmification and dehydration, cells were embedded in Epoxy-Araldite resin. Sections were cut on an ultramicrotome, mounted on grids and doubly stained with uranyl acetate and lead citrate. Sections were viewed and photographed on a Jeol-2000 electron microscope.

8. Enzyme cytochemistry for catalase, peroxidase and acid phosphatase activities

For catalase

Cells were fixed as above for TEM and rinsed twice with 0.1M glycine-NaOH buffer (pH 10.5) and then incubated for 10 min at 37 °C in a medium containing 20 mg DAB (3-3'-diaminobenzidine), 1.5% H_2O_2 dissolved in 10 ml of 0.1M glycine-NaOH buffer.

For peroxidase

Following fixation, cells were rinsed with 0.1 M phosphate buffer (pH 7.4) and incubated for 10 min at 37 °C in a medium containing 10 mg DAB (3-3'-diamino-benzidine), 0.3% H_2O_2 dissolved in 10 ml of 0.1 M phosphate buffer. For acid phosphatase: After fixation, cells were rinsed twice with 0.1M acetate buffer (pH 5.0) and subsequently incubated for 60 min at 37 °C in a medium containing 12.5 mg sodium-ß-glycero-phosphate as substrate, 2 ml acetate buffer (pH 5.0), 2 ml 0.2% lead nitrate and 1 ml distilled water.

Following incubation, cells were thoroughly rinsed in their respective buffer followed by washes with 0.1M sodium cacodylate buffer (pH 7.4). Cells were postfixed in 1% osmium tetroxide for 1 hour, dehydrated in graded ethanols and embedded in Epoxy-Araldite. Sections were cut as previously described and examined either unstained or doubly stained with uranyl acetate and lead citrate using transmission electron microscopy.

Results

ß-lapachone induces the cell death of HepA2

ß-lapachone was found to be highly toxic to HepA2

cells. Fig. 1 illustrates the cytotoxicity induced by various concentrations of ß-lapachone on HepA2 cells. Approximately 80% cells died following treatment with 2 μM of ß-lapachone for 24 hours. Agarose DNA gel electrophoresis revealed that ß-lapachone induced a typical DNA ladder in human leukemia HL-60 cells (lane 1, Fig. 2) but not produced DNA fragments in HepA2 cells followed 24 h and 48 h treatments (lane 4 and 5, Fig. 2).

ß-lapachone induces S-Phase accumulation and unscheduled DNA synthesis of HepA2

Using flow cytometry, we detected whether ß-lapachone may disrupt the cell cycle of hepatoma (Fig. 3). Compared to serum cultured cells, HepA2 cells were arrested at G1 phase after 24 hour serum-deprived treatment (Fig. 3a,b). Following 12 hours exposure to 2 μM ß-lapachone, the population of cells in G1 decreased by 27%, while the population of S cells increased from 17% to 42%. Similar observation was also observed at 24 hours of treatment accompanied with a noticeable increase in cell debris (Fig. 3c,d). Phase-contrast microscopic observation showed that HepA2 cells appeared as polygonal shape (Fig. 4a). After 24 h ß-lapachone treatment, most cells manifested shrinkage in size, rounded up and then lost attachment to the plate (Fig. 4b). Non-programmed DNA synthesis in ß-lapachone-treated cells was also assessed by BrdU incorporation and anti-BrdU immunostaining. Reaction product of anti-BrdU immunostaining was showed in the nuclei of most ß-lapachone treated-cells but not found within the nuclei of untreated cells (Fig. 4c,d).

Ultrastructural observation and enzyme cytochemistry

Fig. 5a illustrating a normal cell contained numerous mitochondria and an abundance of endoplasmic reticulum. Compared to control cells, ß-lapachone treatment induced the morphological changes including swollen mitochondria, disappearance of mitochondria cristae, aggregation of ribosomes and a conspicious appearance of numerous single membrane-bound organelles arising from rER. These granules are assumed as peroxisome-like granules because they contained a granular matrix with a central nucleoid (Fig. 5b,c). To determine the characteristics of these granules, catalase, peroxidase and acid phosphatase activities by cytochemical methods were carried out. Cytochemical results showed that both activities of catalase, peroxidase and acid phosphatase are weak or not cytochemically detectable in HepA2 cells (data not shown). After ß-lapachone treatment, both of peroxidase and acid phosphatase activities but not of catalase activity were localized within the peroxisome-like granules (Fig. 6). For the peroxidase activity, the reaction product is only

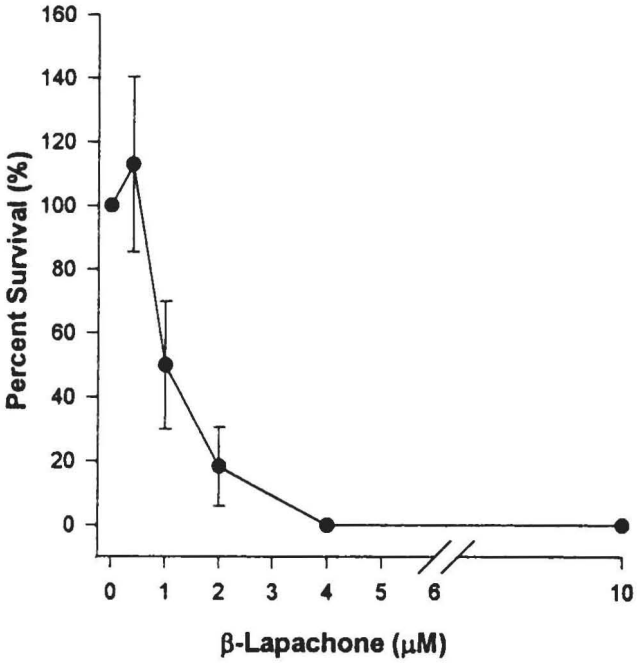

Fig. 1. The percentage survival of human hepatoma (HepA2) cells following a 24 h exposure to various concentration of ß-lapachone. Cells survival was determined by the trypan blue exclusion assay. Points are mean ± SD of three measurements.

Fig. 2. Agarose gel electrophoresis of DNA extracted from HepA2 cells without treated (lane 3) and treated with 2 μm ß-lapachone for 2 h (lane 4) and 4 h (lane 5). Lane 1: DNA fragmentation of human leukemia (HL-60) cells induced by 1 μm ß-lapachone treatment was used as a positive control. Lane 2: DNA marker.

deposited on the membrane of peroxisome-like granules. For the acid phosphatase activity, the distribution of reaction product is not limited on the membrane but also found in the nucleoid matrix of the granules.

Discussion

In this study, we examined the cytotoxicty of ß-lapachone on human hepatoma (HepA2) cells under serum-free conditions. HepA2 cells were sensitive to the effect of ß-lapachone and extensive cell death was observed subsequent to ß-lapachone exposure. Hepatoma cell death induced by ß-lapachone did not involve the production of the DNA ladder and prominent chromosomal condensation (Figs. 1, 2). Similar results have also been reported in ß-lapachone treated 21-MT (a human breast carcinoma cell line), H520 (a human lung carcinoma cell line), SW116 (a human colon adenocarcinoma cell line) and A2780s (a human ovary carcinoma cell line) cells (Li et al., 1995). Following exposure to ß-lapachone, unscheduled DNA synthesis and S phase accumulation in HepA2 cells were also illustrated by flow cytometry and BrdU incorporation (Figs. 3, 4). These findings are consistent with previous experimental results on transformed Chinese hamster embryo fibroblasts (CHEF) after ionizing radiation (Boothman, 1994) or in fibroblasts treated alkylating agent (Boorstein and Pardee, 1984). Camptothecin and ß-lapachone have been suggested to be DNA topoisomerase I modulators which can inhibit the repair of DNA damage caused by alkylating agents and ionizing radiation (Boothman et al., 1987, 1989, 1992; Li et al., 1993a). Though Topo I has been suggested to play a positive role in DNA repair, however, only a slight elevation of Topo I activity has been reported in DNA-damaged cells (Hsiang et al., 1985). By contrast, rapidly down-regulation of Topo I activity in X-ray irradiated neoplastic hamster embryo fibroblasts (CHEF/18) (Boothman, 1994) and radioresistant human (U1-Mel) cell (Hsiang et al., 1985) have been demonstrated (Boothman et al., 1989). Recent studies (Satoh and Lindahl, 1992; Boothman et al., 1994) also

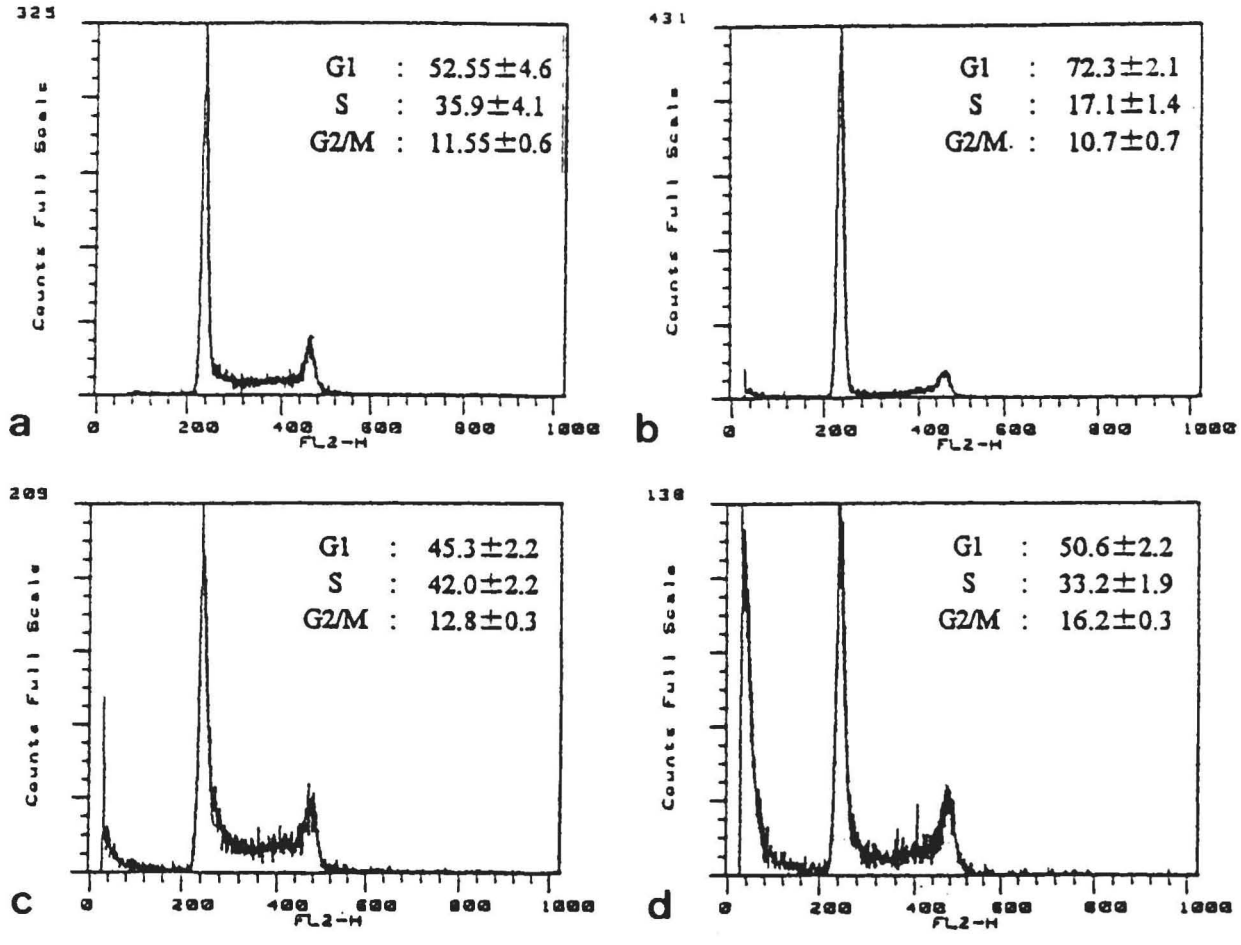

Fig. 3. Cell cycle analysis of HepA2 cells following treatment with ß-lapachone. HepA2 cells were cultured in the presence or absence of calf serum medium for 24 hr **(A and B)**. HepA2 cells were treated with 2 μm ß-lapachone under serum free condition for 12 h **(C)** and 24 h **(D)**. After removal of ß-lapachone, cells were fixed and stained with propidium iodide (PI) as descibed in Materials and methods, and the DNA content was analyzed by flow cytometry.

indicated that Topo I may convert repairable SSBs to unrepairable DSBs and prevent DNA ligation. In response to DNA damage, DNA ligase or a ligase-containing complex may compete with Topo I for SSBs and allow DNA repair. Therefore, down-regulation of Topo I activities following ionizing radiation would allow DNA complex access to SSBs, thus allowing repair. In the presence of Topo I modulator, conversion of SSBs to DSBs may be enhanced by the Topo I-DNA cleavable complexes and thus increases the unscheduled DNA synthesis (Boothman et al., 1994). In present study we agreed the hypothesis of Boothman et al. (1994) and illustrated that the ß-lapachone, a Topo I inhibitor, can enhance the unschedule DNA synthesis, interrupt the cell cycle and then induce the cell death.

In addition to its potential role as a Topo I modulator, ß-lapachone has been suggested to act as a free radical generator by promoting the O_2^- and H_2O_2 production in cellular mitochondrial and microsomal fraction (Goncalves de Lima et al., 1962; Boveris et al., 1978; Docampo et al., 1979b; Goncalves et al., 1980; Boothman et al., 1994; Molina Portela and Stoppani, 1996). The damaging effect of hydrogen peroxide (H_2O_2) and free radicals on cellular DNA has also been

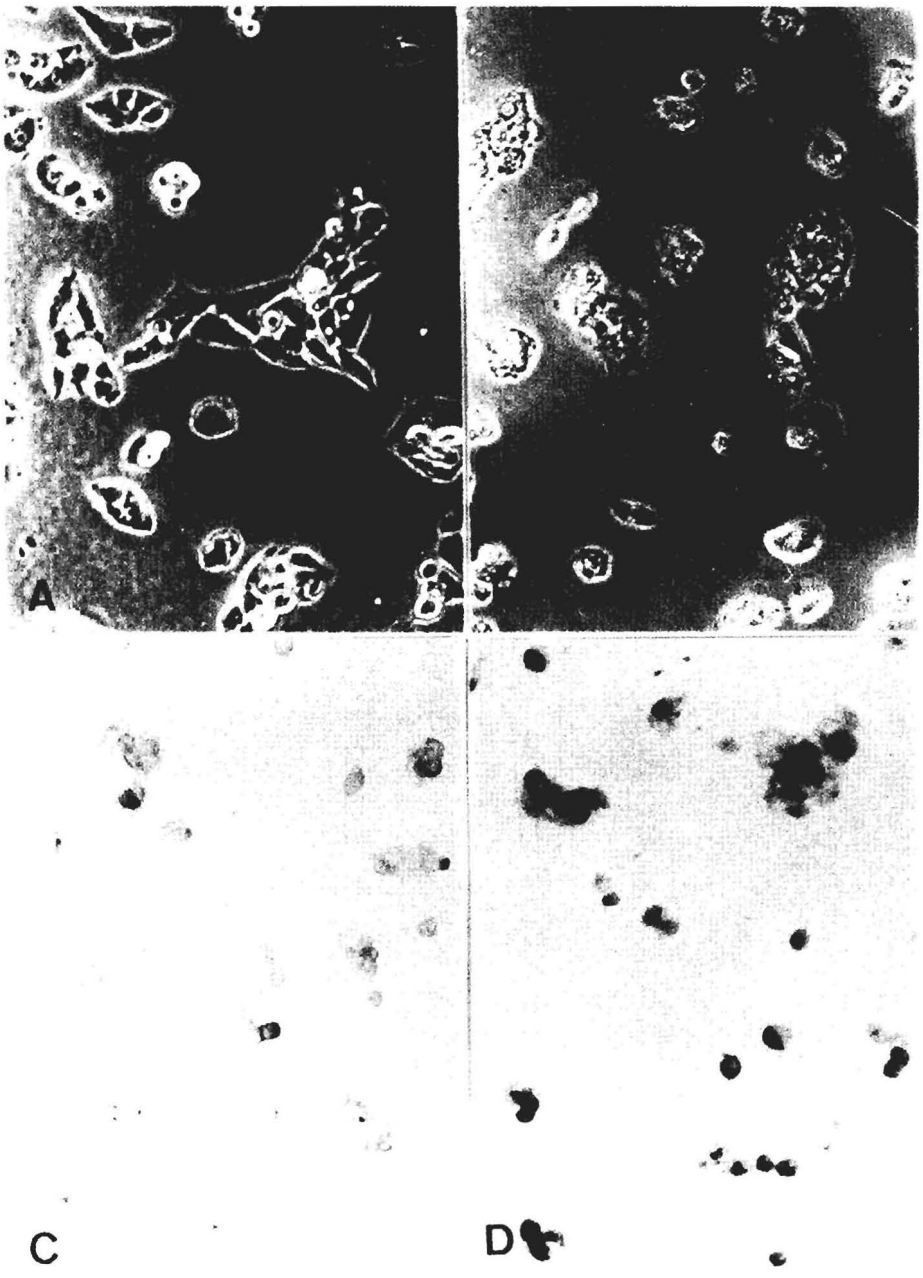

Fig. 4. Light microscopy photographs of Hep A2 cells following ß-lapachone treatment. **A.** untreated cells. **B.** 24 h ß-lapachone treatment. Cells appear shrinkage in size and rounded up (arrow). For the cellular DNA synthesis detection, assay of BrdU incorpulation to untreated (**C**) or ß-lapachone treated cells (**D**) were used. Cells were incubated in medium containing BrdU for 6 h. After removal of medium, cells were fixed, incubated with mouse anti-BrdU antibody and then reacted with secondary antibody (sheep-anti-mouse IgG conjugated with alkaline phosphatase). NBT/X-phosphate was used as coloured substrate for alkaline phosphatase to show the results. x 75

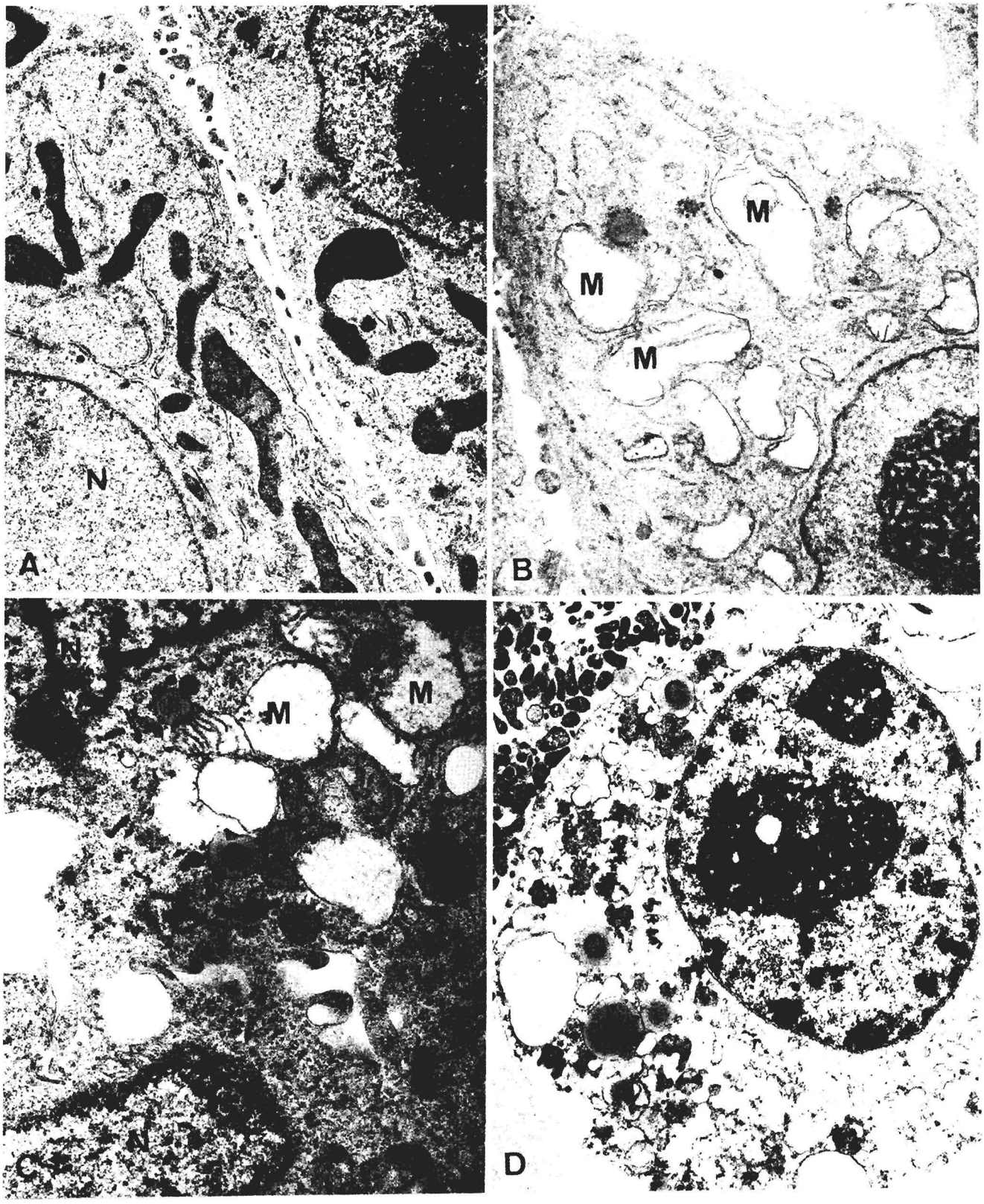

Fig. 5. Ultrastructural alterations of HepA2 cells following ß-lapachone treatment were examined at 0 h **(A)** ; 6 h **(B)** ; 12 h **(C)** and 24 h **(D)** respectively. After drug treatment, morphological changes including swollen mitochondria (M), disappearance of mitochondria cristae, aggregation of ribosomes and proliferation of peroxisome-like granules (G) were seen. N: nucleus. x 10,000

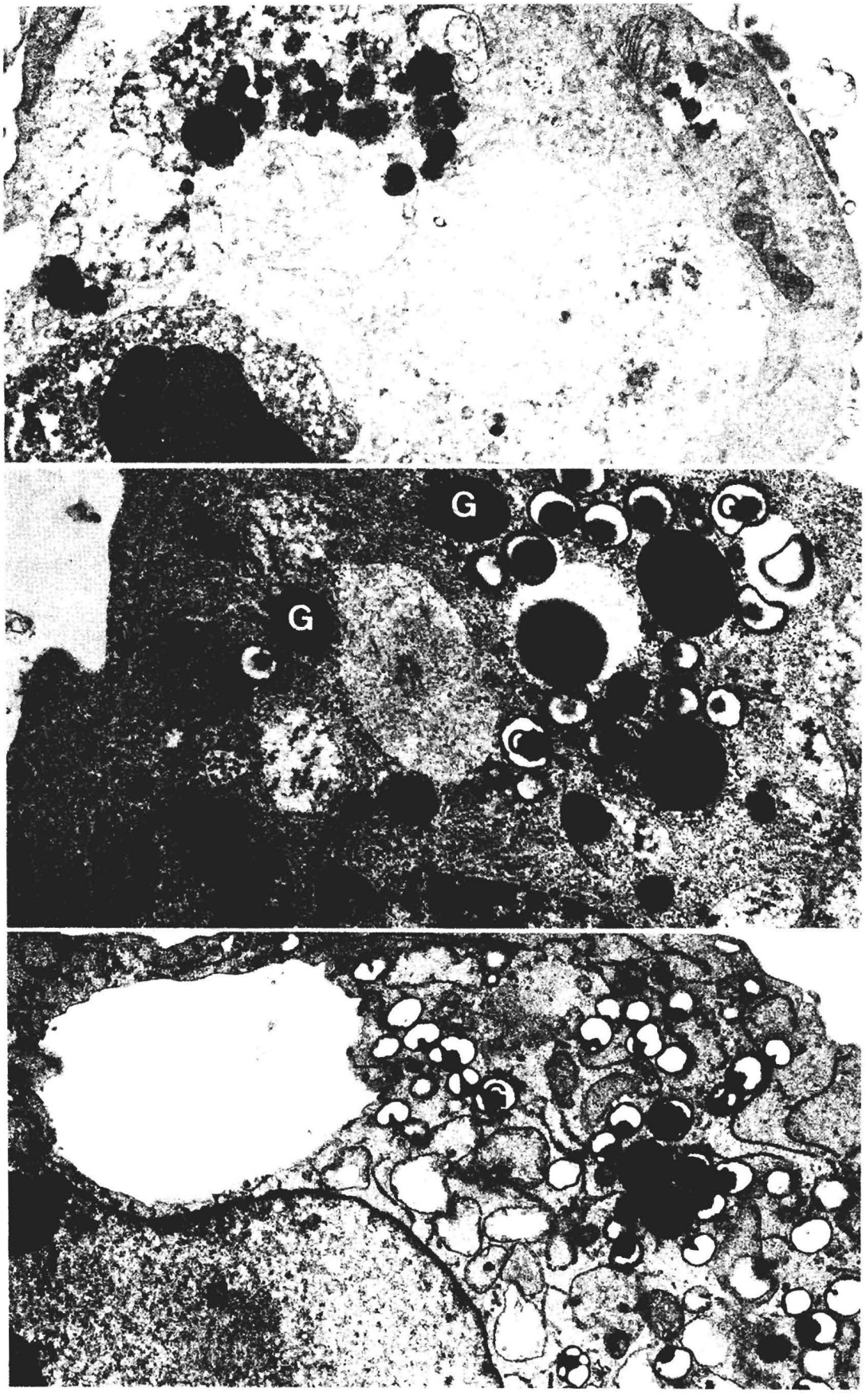

Fig. 6. Electron microscopic localisation of different enzyme activities in 12 h ß-lapachone-treated cells: catalase **(A)**; peroxidase **(B)**; acid phospatase **(C)**. Note the absence of catalase activity in the subcellular components of lapachone-treated cell. Reaction product of peroxidase activity is limited on the membrane of peroxisome-like granules (G) while of acid phosphatase activity is localized both on the membrane and in the matrix of peroxisome-like granules. M: mitochondria; N: nucleus. x 10,000

demonstrated (Imlay and Linn, 1988; Tachon et al., 1994). We supposed that involving of oxidative damage by ß-lapachone treatment was supported by morphological evidence including mitochondrial swelling and proliferation of peroxisome-like granules in cells. Recent studies have confirmed that the oxidative stress is an important component which can induce cell death/apoptosis during development and carcinogenesis (Hockenbery et al., 1993; Whittemore et al., 1994). Moreover, addition of antioxidants (catalase, N-acetylcysteine, ascorbic acid) or elevation of intracellular antioxidant enzyme activities (catalase, peroxidase and acid phosphatase) in cells may defend against free radicals present in the extracellular/intracellular milieu and protect the cells from oxidant-mediate death (Remaley et al., 1984; Jornot and Junod, 1992; Sandstrom and Buttke, 1993; Chiao et al., 1995; Fang et al., 1995). Therefore, we examined whether the expression of catalase, peroxidase and acid phosphatase activities within ß-lapachone treated cells may elevate or not. Results from enzyme cytochemistry revealed that both activities of catalase, peroxidase and acid phosphatase are not detectable in untreated HepA2 cells. Following 12 hr drug treatment, cells displayed a number of peroxisome-like granules which exhibited both of peroxidase and acid phosphatase activities but lacking of catalase activity in the cells (Figs. 5, 6). Recently Oikawa and Novikoff (1995) reported a population of hepatocyte containing no catalase activity in peroxisomes during liver regeneration. Moreover, the reduction of catalase activity and increase of lysosomal enzymes activities in kidney and liver tumour cells have been described by Ghadially and Parry (1965); Goldfischer (1979) and Trew et al. (1979). Since catalase plays an important role involving fatty acid ß-peroxidation and clearance of H_2O_2, loss or lacking of catalase activity may result in the exposure of DNA and other macromolecular to oxidative damage during the time of cell proliferation (Reddy and Rao, 1989; Oikawa and Novikoff, 1995). Hence, we suggested that HepA2 cells may suffer more oxidative damage than in other cells with catalase activity by ß-lapachone treatment.

In summary, we demonstrate that (i) ß-lapachone has a potent cytotoxicity to human hepatoma cell; (ii) ß-lapachone induces the interruption of cell cycle and enhances the unscheduled DNA synthesis on hepatoma cells and (iii) ß-lapachone treatment can damage the mitochondria, increase the proliferation of peroxisome-like granules and elevate the activities of endogenous peroxidase and acid phosphatase in HepA2 cells.

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