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Effect of Lidamycin on Telomerase Activity in Human Hepatoma BEL-7402 Cells[1]

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Objective To investigate the effect of lidamycin (LDM) on telomerase activity in human hepatoma BEL-7402 cells under the condition of LDM inducing mitotic cell death and senescence. **Methods** Chromatin condensation was detected by co-staining with Hoechst 33342 and PI. Cell multinucleation was observed by Giemsa staining and genomic DNA was separated by agarose gel electrophoresis. Fluorescent intensity of Rho123 was determined for mitochondrial membrane potential. MTT assay and SA-β-gal staining were employed to analyze the senescence-like phenotype. The expression of proteins was analyzed by Western blot. Telomerase activity was assayed by telomerase PCR-ELISA. **Results** Mitotic cell death occurred in LDM-treated cells characterized by unique and atypical chromatin condensation, multinucleation and increased mitochondrial membrane potential. However, no apoptotic bodies or DNA ladders were found. In addition, apoptosis-related proteins remained nearly unaltered. Senescence-like phenotype was identified by increased and elongated size of cells, growth retardation, enhanced SA-β-gal activity and the changes of senescence-related protein expression. Telomerase activity markedly decreased ($P<0.01$) in LDM-treated hepatoma BEL-7402 cells. **Conclusion** Mitotic cell death and senescence could be triggered simultaneously or sequentially after exposure of hepatoma BEL-7402 cells to LDM. The decrease in telomerase activity may play a key role in the defective mitosis and aging morphology. Further investigation of detailed mechanism is needed.

Key words: LDM; Mitotic cell death; Cellular senescence; Telomerase activity

INTRODUCTION

Lidamycin (LDM), also called C-1027, is a member of the enediyne antibiotic family which has been focused on its potent antitumor activity due to its unique ability to damage the DNA of tumor cells by directly inducing single-strand or sequence-specific double-strand breaks[1-2]. LDM is produced by a *Streptomyces globisporus* C-1027 strain isolated from a soil sample collected in Qian-jiang county, Hubei province, China[3-4]. LDM consists of an enediyne chromophore of 843 Daltons and an acidic apoprotein of 10 500 Daltons from 110 amino acid residues[5]. The former is the active moiety attacking DNA and is protected by the latter from loss of its DNA cleavage activity and can be dissociated and reestablished[6]. Recent reports have shown that LDM is highly cytotoxic toward tumor cells, can trigger apoptosis and mitotic cell death in human hepatoma BEL-7402 cells other than inhibition of DNA and RNA synthesis and displays a remarkable therapeutic efficacy against murine and human hepatomas *in vivo*[7-9].

Mitotic cell death, also called mitotic catastrophe or delayed reproductive death which differs from apoptosis, is characterized by an aberrant form of mitosis associated with formation of multinucleated giant cells that are temporarily viable but reproductively dead[10]. Besides, mitotic cell death also possesses other features including enlargement of cell volume and retardation at G_2/M phase of cell cycle[8]. Though the mechanism of mitotic cell death has not been clearly elucidated, it appears to involve the defects in mitotic machinery like multiple rounds of DNA synthesis without intervening cytokinesis, chromosome missegregation, dysfunction of cell checkpoints, centrosome overduplication, *etc.*[11-13]. Telomere erosion can explain the occurrence of mitotic cell death in continuously grown Chinese hamster don cells[14].

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Senescence is defined as a state in which a cell no longer has the ability to proliferate but continues cell metabolism[15]. So, cellular senescence is characterized by a ubiquitous and progressive decline in the functional capacity of various physiological systems and loss of capability of an individual to maintain homeostasis. There exist two kinds of senescent form: premature senescence and replicative senescence. Previous studies indicate that premature senescence can be provoked by overstimulation of the Ras/Raf/MEK/mitogen-activated protein kinase (MAPK) pathway involved in p53-p21 and p16 whereas replicative senescence represents a DNA damage-like response, but both of them share some common signaling pathways and morphological features such as increased size, delayed growth, enlarged nuclei, flattened and elongated appearance and enforced SA-β-gal activity[16-19]. Cellular stresses are also likely to participate in the activation of senescence[20]. Additionally, some researchers have linked senescence with the abnormality of telomere and telomerase[21-22].

Telomerase is a specialized RNA-dependent DNA polymerase located within nuclei, whose function is to synthesize and add the repetitive nucleotide sequence to the ends of chromosome[23]. There are two essential components in human telomerase ribonucleoprotein complex. One is human telomerase reverse transcriptase (hTERT), a catalytic protein subunit mainly localized in the nuclei of cancer cells and proliferating normal cells, while the other is human template RNA (hTR) which is constitutively expressed[24-25]. It was reported that telomerase is active in 70%-90% of malignant tissues and many immortal cell lines, but most normal human somatic cells have no detectable active manifestation of telomerase[26]. Deregulation of telomerase is thought to facilitate cellular immortality and tumorigenesis by providing cancer cells with infinite proliferation capacity. For example, reconstitution of telomerase activity by retroviral transduction of hTERT has been used to extend the lifespan of umbilical cord blood-derived cells[27]. Some researchers also revealed that increased telomerase activity correlates with increased malignant potential and stage[28], therefore, inhibition of telomerase could limit the growth of human cancer cells and further lead to progressive telomere shortening and cell death[29-30]. Recent reports have shown that cellular senescence is also critically influenced by telomerase and telomere[21-22]. Thus, telomerase is considered a new tumor marker and expected to function in tumor diagnostics and therapeutics.

This study investigated the impact of telomerase activity on mitotic cell death and cellular senescence induced by low concentration of LDM in human hepatoma BEL-7402 cells. Results suggest that decreased telomerase activity with increased LDM may play a key role in the defective mitosis and senescence-like phenotype.

MATERIALS AND METHODS

Materials

Lidamycin was generously provided by Professor Lian-Fang JIN from our institute and stored at -20℃ as an 100 μmol/L stock solution in 9 g/L NaCl solution. MTT, Hoechst33342, propidium iodide (PI) and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Telomerase PCR ELISA kit was purchased from Roche and ECL Western blot kit was commercially available from Amersham Pharmacia. All other chemicals were of analytical grade.

Cell Culture

Human hepatoma BEL-7402 cells (the Key Laboratory of Cell Proliferation and Regulation Biology of the Ministry of Education, Beijing Normal University) were planted in DMEM (Gibcol BRL) supplemented with 100 mL/L fetal bovine serum (HyClone), 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37℃ in a humidified incubator of 5% CO_2.

Chromatin Condensation Shown by Co-staining With Hoechst 33342 and PI

Cells were continuously incubated for 72 h after exposure to LDM for 2 h and then harvested by trypsinization, washed once in PBS (pH 7.4) and co-stained by DNA-specific fluorescent dye Hoechst 33342 (2 μg/mL) and PI (40 μg/mL) for 15 min at 37℃. After that, cells were washed once, resuspended in PBS (pH 7.4) and observed under a fluorescence microscope (BH2 system, Olympus) equipped with a $\lambda_{455\ nm}$ filter.

Successive Changes of Cell Nuclei Monitored by Giemsa Staining

Cells were seeded in 6-well plates with a coverslip each well. After 2 h of treatment with LDM, cells were continuously cultured in LDM-free DMEM medium. Cells on the coverslips were taken out respectively at 0, 24, 48, and 72 h, immediately fixed in methanol: glacial acetic acid (3:1) at 4 ℃ and then stained with Giemsa solution for 15 min. After washed completely in distilled water, cells on the coverslips were observed and photographed.

Genomic DNA Separation and Agarose Gel Electrophoresis

Genomic DNA separation and agarose gel electrophoresis were performed as described previously[31]. Briefly, 2×10^6 cells after LDM treatment were scraped with a cell scraper. Washed once in PBS, the cells were lysed in 100 μL lysis buffer (10 mmol/L EDTA, 0.5% sodium lauryl sarcosine, 50 mmol/L Tris-HCl, pH 8.0) containing 100 μg/mL RNase at 37℃ for 0.5 h. Then 100 μL of lysis buffer containing 100 μg/mL proteinase K was added and kept at 50℃ for 3 h. The genomic DNA samples were extracted three times in the same volume of Tris-phenol: chloroform: iso-amyl alcohol (25:24:1). The DNA samples (15 μg/lane) were resolved on 1.5% agarose gel and detected using ChemiImager 5500 gel imaging system.

Determination of Mitochondrial Membrane Potential ($\Delta\Psi m$)

Rhodamine 123 is an indicator dye which can often be utilized as a fluorescent probe to estimate the membrane potential of mitochondria ($\Delta\Psi m$) by changes of fluorescent intensity from rhodamine 123 because of its accumulation by mitochondria in proportion to $\Delta\Psi m$[32]. The cells were incubated for 72 h after exposure to 0.1 nmol/L or 0.5 nmol/L LDM for 2 h and then harvested and washed twice in PBS. Then rhodamine 123 was added to the final concentration of 0.5 μmol/L. The mixture was placed in dark at 37℃ for 30 min. Then, the cells were washed twice and resuspended in PBS, adjusted to 1×10^6/sample and filtered with nylon membrane. $\Delta\Psi m$ was assessed by the fluorescent uptake detected by flow cytometry with excitation at 505 nm and emission at 534 nm.

Observation of Cell Morphology

The cells were incubated in LDM-free DMEM for 48 h after exposure to different concentrations of LDM for 2 h, observed and photographed under an inverted microscope.

Growth Curve Assay

MTT assay was used to indirectly describe the survival curve of human hepatoma BEL-7402 cells after treatment with LDM. The establishments of growth curves were performed at a 5-d interval, 2.0×10^3 cells were seeded in each well of 96-well plates and treated with LDM for 2 h. A 50 μL MTT (2 mg/mL) was added to each well before assay and incubated for an additional 4 h at 37℃, followed by addition of 150 μL/well DMSO. After the complete solution of the pellet, the value at each time point was read on a microplate reader (Model 550, Bio-Rad) at λ_{570nm}.

Senescence-assocaiated β-galactosidase (SA-β-gal) Staining

Cells were assayed for senescence-associated β-galactosidase activity at pH 6.0 as previously described[33]. In brief, 4×10^4 cells/well were seeded in 24-well plate and incubated for 48 h. After 2 h treatment of LDM and 72 h of continuous incubation, the attached cells were first rinsed twice in PBS, then fixed in 5 ml/L glutaraldehyde and finally stained with 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution for 16 h at 37℃. Cells expressing β-galactosidase activity (stained blue) were counted under an inverted microscope. At least three fields of 300 cells were scored for each assayed sample.

Western Blot Analysis

Cells treated with LDM for 2 h and incubated for 72 h were harvested and washed in PBS, lysed on ice in lysis buffer (100 mmol/L Tris-HCl, pH 6.8, 25 g/L SDS, 10% β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride and 10% glycerol) for 10 min, followed by ultrasonication. The supernatant was obtained by centrifugation at 4℃, 12000×g for 10 min. Bradford method was used to determine the protein concentration with bovine serum albumin as a standard. Western blot analysis was performed as previously described with some modifications[34]. In short, an equal loading of protein (routinely 30 μg/lane) was electrophoresed on a 10% or 12% SDS-polyacrylamide gel (Fluka), semi-dried electrotransfered onto nitrocellulose membrane (Hybond-P, Amersham Pharmacia) and blocked overnight at 4℃ with 5% nonfat milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween-20, pH 7.6). Then the membranes were washed five times in TBST and blotted with TBST dilution (from 1:300 to 1:1000) of primary antibodies directed respectively against Bax (Santa Cruz), Bcl-2 (Santa Cruz), Caspase-3 (Santa Cruz), Caspase-9 (a kind gift from Dr. Xiao-Dong WANG, University of Texas Southwestern Medical Center, Dallas, USA), p16 (NeoMarkers), pRb (Santa Cruz), p53 (Santa Cruz), pp53 (Ser 15) (Santa Cruz), p21 (Santa Cruz), cyclin D1 (NeoMarkers), and actin (Santa Cruz) for a further 1.5-3 h at room temperature, washed five times in TBST, and then incubated with a 1:2000 dilution of the horseradish peroxidase-conjugated second antibody (Amersham Pharmacia) in TBST for 2 h. Blots were developed using ECL Western blot kit

(Amersham Pharmacia).

Telomerase Activity Assayed by Telomerase PCR-ELISA

Telomerase PCR-ELISA was conducted using telomerase PCR ELISA kit (Roche) following the manufacturer's protocols. Telomerase activity was measured after 2 h of exposure to different concentrations of LDM (0.1-1 nmol/L) and followed by 72 h successive culture. After rinsed twice in ice-cold PBS, cells were scraped and harvested. Next, 10^5 cells were homogenized in 200 μL of pre-cooled lysis buffer and then kept on ice for 30 min. The supernatant was collected *via* centrifugation at 16000×g for 20 min at 4℃ and stored at -80℃. Protein concentrations of the extracts were measured using ultra-spectrophometer before use. Subsequently, 10 μg of protein extract was assayed in 50 μL reaction mixture containing 25 μL of reaction mixtures and some sterile DEPC-H_2O. After incubated at 25℃ for 30 min to elongate the primer and then heated at 94℃ for 5 min to inactivate the telomerase, the reaction mixture was subjected to PCR amplification in a thermal cycler: denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s, and extension at 72℃ for 90 s. Finally, the reaction mixture was incubated at 72℃ for another 10 min to fully extend the amplified fragments. Next, ELISA assay was carried out following the manufacturer's instructions. Telomerase activity was represented by the difference of the absorbance (△A) according to the following formula:

$$\triangle A = A_{450} - A_{690}$$

RESULTS

Unique and Atypical Chromatin Condensation Shown by Co-staining With Hoechst 33342 and PI

To ascertain the occurrence of mitotic cell death, co-staining assay with Hoechst 33342 and PI was performed. The results indicated that human hepatoma BEL-7402 cells treated with 0.1 and 0.5 nmol/L LDM for 2 h and followed by a 72-h incubation in drug-free medium displayed a unique and atypical chromatin condensation characterized by a series of features different from typical apoptosis such as integrated karyotheca, adherence on the bottom, patchy chromatin condensation and no formation of apoptotic bodies in the late phase of cell death process (Fig. 1).

Cell Multinucleation Displayed by Giemsa Staining

Human hepatoma BEL-7402 cells treated with 0.1 and 0.5 nmol/L LDM for 2 h were continuously incubated in drug-free medium until the specific time dots and then stained with Giemsa solution. The results showed that cell multinucleation occurred at 24 h in human hepatoma BEL-7402 cells after LDM treatment. Giant mononuclear cells began to appear at 48 h. Vacuolization of cell plasma became more significant at 72 h (Fig. 2). The percentages of multinucleation were 12.3% and 35.7% after 72 h incubation with 0.1 nmol/L and 0.5 nmol/L LDM, respectively, of which the number of micronuclei reached about 12-15 per cell.

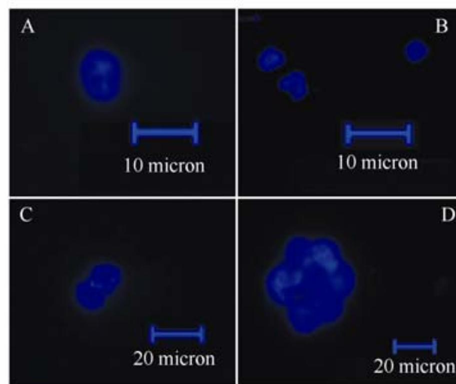

FIG. 1. Atypical chromatin condensation induced by LDM in human hepatoma BEL-7402 cells determined by co-staining of Hoechst 33342 and PI. (The cells were continuously incubated for 72 h after 2 h LDM treatment. A, Untreated cells; B, 0.1 nmol/L LDM treated cells; C and D, 0.5 nmol/L LDM treated cells.)

Exclusion of Apoptosis From Mitotic Cell Death

In order to further distinguish mitotic catastrophe induced by LDM from typical apoptosis, features of cell apoptosis were assayed. Figure 3 shows that the mitochondrial membrane potential of LDM-treated cells was relatively elevated compared to the control. Analysis of genomic DNA (Fig. 4) made it clear that there were no DNA ladders of apoptotic cells emerged in the late phase of cell death in human hepatoma BEL-7402 cells after 2 h treatment with LDM. Additionally, the protein levels of pre-apototic Bax and anti-apoptotic Bcl-2 decreased. No proteolytical activation of caspase-3 and caspase-9 was detected in LDM-treated BEL-7402 cells and no significant changes were found in their corresponding precursors (Fig. 5).

Senescence-like Phenotype Triggered by Low Concentration of LDM

As shown in Fig. 6, human hepatoma BEL-7402 cells became flattened and enlarged, and the granules

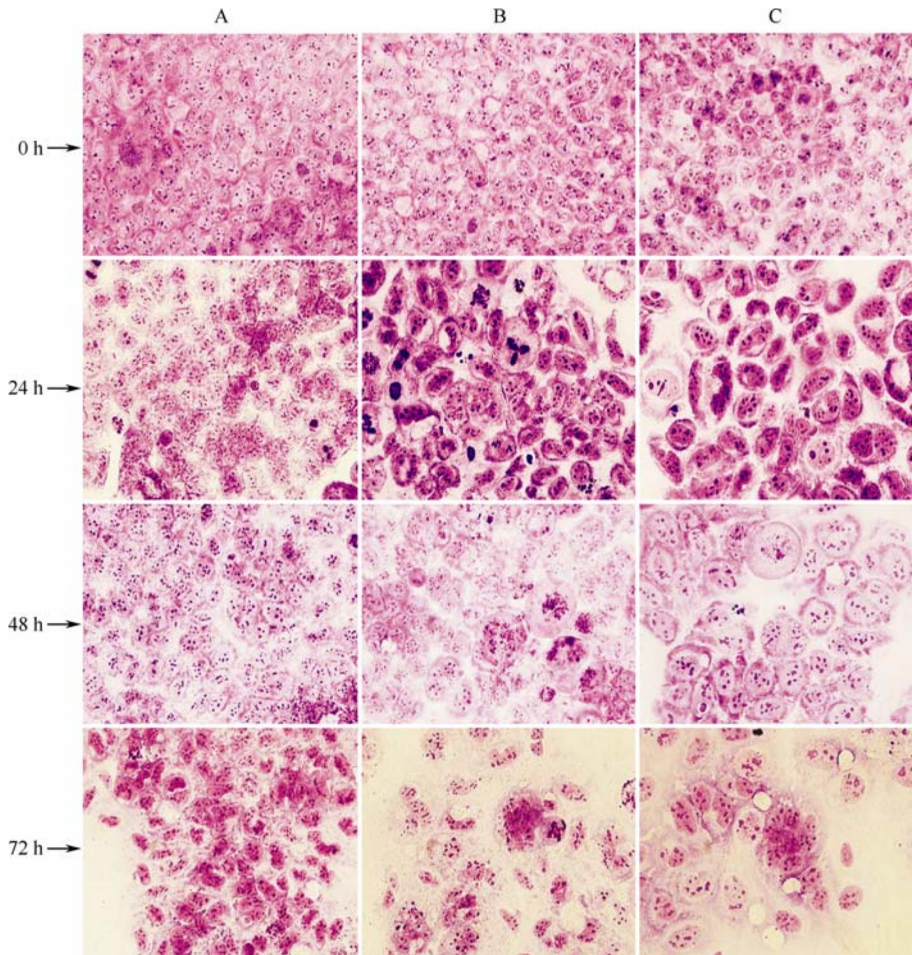

FIG. 2. Giemsa staining of human hepatoma BEL-7402 cells treated with different concentrations of LDM. A, Untreated cells; B, 0.1 nmol/L LDM treated cells; C, 0.5 nmol/L LDM treated cells. The magnification was 400×.

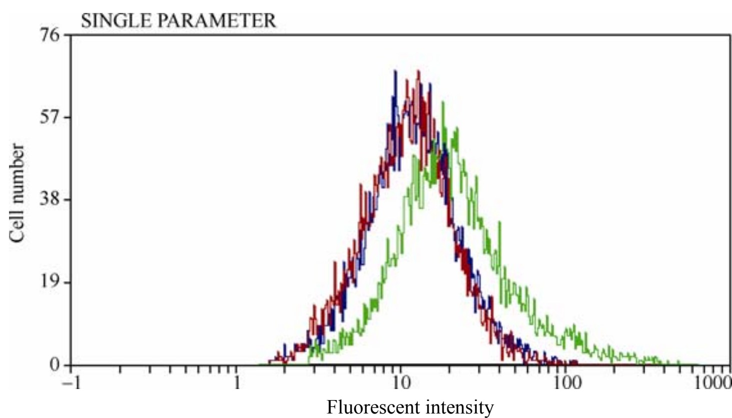

FIG. 3. Time-lapsed changes of mitochondrial membrane potential (ΔΨm) in human hepatoma BEL-7402 cells. Blue line reprents the control; red line represents the sample treated with 0.1 nmol/L LDM; green line represents the sample treated with 0.5 nmol/L LDM.

in cell plasma manifolded although BEL-7402 cells still grew adhering on the bottom of the well similar to those of senescent cells. However, BEL-7402 cells treated with 1 μmol/L LDM became round and smaller, a lot of which detached from the bottom and floated into the medium.

FIG. 4. Electrophoretic analysis of genomic DNA in human hepatoma BEL-7402 cells. M represents the standard DNA marker (from up to down: 2000 bp, 1600 bp, 1200 bp, 800 bp, 600 bp, 400 bp, and 200 bp); lane A, untreated cells; lane B, 0.1 nmol/L LDM treated cells; lane C, 0.5 nmol/L LDM treated cells. This is a representative result from three separate experiments.

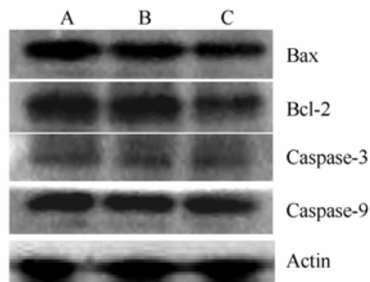

FIG. 5. Western blot analysis of apoptosis-related proteins in human hepatoma BEL-7402 cells. A, Untreated cells; B, 0.1 nmol/L LDM treated cells; C, 0.5 nmol/L LDM treated cells.

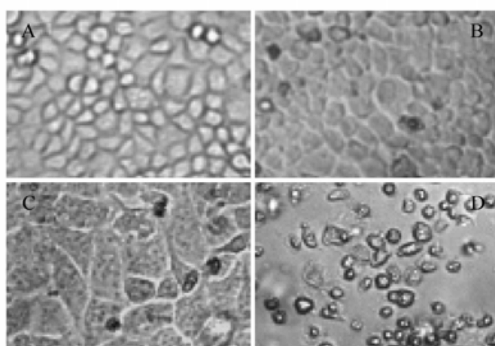

FIG. 6. Cellular morphology induced by LDM in human hepatoma BEL-7402 cells. A, Untreated cells; B, 0.1 nmol/L LDM treated cells; C, 0.5 nmol/L LDM treated cells; D, 1 μmol/L LDM treated cells. The photographs were taken at the magnification of 200×.

Senescence-like Growth Retardation Induced by Low Concentration of LDM

To further investigate the effect of LDM on cellular senescence, senescence-like growth arrest was detected by MTT assay. As shown in Fig. 7, LDM-treated human hepatoma BEL-7402 cells exhibited a significant decrease in growth rate and delay in double time of proliferation in a dose-dependent manner compared with the control. At the time of 120 h after incubation, the difference in cell number between the control and the LDM-treated group was further enlarged and cell number of the groups treated with 0.1 nmol/L and 0.5 nmol/L LDM was decreased by 51.8% and 70.5% respectively, compared with the control.

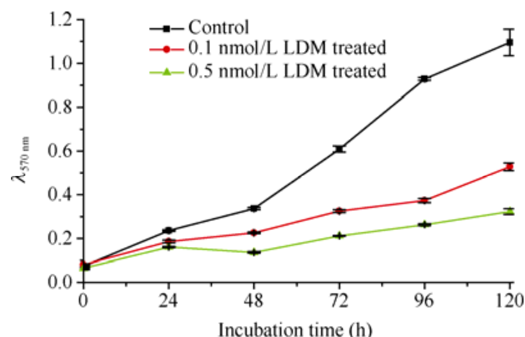

FIG. 7. Growth inhibition by LDM in human hepatoma BEL-7402 cells.

Increased SA-β-gal Expression Induced by Low Concentration of LDM

SA-β-gal expression is a recognized, senescence-associated and biological index and has been shown to closely correlate with senescence in aging cell cultures *in vitro* and *in vivo*[33,35]. We observed SA-β-gal expression at 72 h after LDM treatment in human hepatoma BEL-7402 cells. The treated cells displayed enhanced activity of SA-β-gal. Moreover, BEL-7402 cells treated with 0.1 and 0.5 nmol/L LDM increased the SA-β-gal expression by 17.2-fold and 29.3-fold, respectively (Fig. 8).

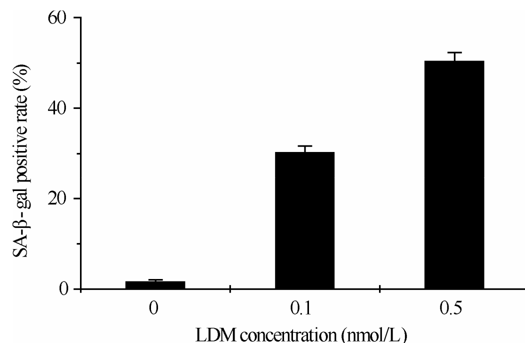

FIG. 8. Increased percentages of SA-β-gal stained human hepatoma BEL-7402 cells.

Alterations of Cell Cycle Related Proteins at the Expression Level After LDM Treatment

To understand the changes of protein expression leading to mitotic catastrophe and cellular senescence, cell cycle related proteins were analyzed by Western Blot in human hepatoma BEL-7402 cells treated with LDM. As shown in Fig. 9, LDM increased the protein levels of p16 and pRb. Conversely, p53, pp53 (Ser 15), p21, and cyclin D1 declined at the protein levels.

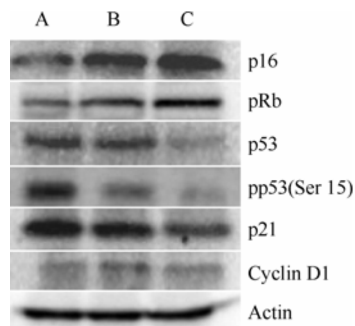

FIG. 9. Western blot analysis of cell cycle related proteins in human hepatoma BEL-7402 cells. Lane A, Untreated cells; Lane B, 0.1 nmol/L LDM treated cells; Lane C, 0.5 nmol/L LDM treated cells.

Decreased Telomerase Activity Induced by Low Concentration of LDM

Telomerase activity was considered to be closely correlated with proliferation and malignancy of tumor cells and cellular senescence. To investigate the impacts of telomerase activity on mitotic catastrophe and cellular senescence, PCR-ELISA was used to quantify the telomerase activity in human hepatoma BEL-7402 cells. The data showed a remarkable dose-dependent decrease in telomerase activity ($P<0.01$) within the concentration range of 0.1-1 nmol/L LDM, which could be thought enough to induce mitotic cell death and cellular senescence (Fig. 10).

DISCUSSIONS

A unique and atypical chromatin condensation was found in LDM-treated human hepatoma BEL-7402 cells. The cells with this kind of condensation are characterized by integrated karyotheca, small light “dot” representing segregated condensed chromatin and absence of formation of apoptotic bodies which are identical to those of previous report[8]. Furthermore, no PI-positive cells were found in this process, suggesting that all cells were alive instead of being necrosed because of their integrated cell membrane and karyotheca.

Cell multinucleation is one of the remarkable features in mitotic cell death. Significant multinucleation by Giemsa staining in human hepatoma BEL-7402 cells appeared after treatment with low concentration of LDM. In fact, primal multinucleation started at 12 h after LDM treatment, indicating that multinucleation occurred very quickly in LDM-treated BEL-7402 cells.

The mitochondria are highly sensitive to their microenvironment and decreased mitochondrial membrane potential is an event occurred at the early phase of typical apoptosis[36]. In the present study, a relative increase of mitochondrial membrane potential has suggested that human hepatoma BEL-7402 cells, rather than cell apoptosis, are in a state of mitotic catastrophe. In addition, Bcl-2 and Bax are the two most important apoptosis regulating proteins of *bcl-2* gene family. The former is a member of the anti-apoptotic family, while the latter is a member of the pro-apoptotic family and the ratio of Bcl-2/Bax determines the occurrence of apoptosis[37]. As shown in Fig. 5, although both Bcl-2 and Bax decrease with LDM treatment, their ratio has no significant alteration. So does the activity of caspase-3 and caspase-9, which may be explained by the inactivation of caspase-dependent mitochondrial apoptosis pathway.

Taking together, these data indicate that low-concentration LDM is committed to mitotic cell death rather than apoptosis in human hepatoma BEL-7402 cells.

During the investigation of mitotic cell death, we have found that LDM-treated BEL-7402 cells display some morphological characteristics (Fig. 6) identical to senescent cells such as increased cell volume, enhanced granules in cell plasma, and flattened and prolonged cell morphology[16-18]. This may possibly be the primary signs of cellular senescence, indicating the occurrence of aging. The increased SA-β-gal activity further confirms the fact that LDM-treated BEL-7402 cells are in a state of aging. As reported, induction of SA-β-gal activity and mitotic catastrophe are mutually independent events[35]. Then, are mitotic catastrophe and senescence independent in human hepatoma BEL-7402 cells induced by low concentration of LDM? This may be explained at the level of protein expression.

As shown in Fig. 9, the levels of p53, pp53 (Ser 15), p21 and cyclin D1 are declined and the levels of p16 and pRb are increased in LDM-treated BEL-7402 cells. p53 is a tumor suppressor. The inhibition of p53 can accelerate mitotic cell death. However, excess activity of p53 could induce premature aging of mice in multiple tissue types[18,38-39]. p21 is a p53-regulated protein and is stimulated by p53. p21 is a cyclin-dependent kinase

inhibitor (CDKI) which blocks cell cycle progress by inhibiting the binding of cyclin D1 and cyclin-dependent kinases Cdk4/6 that phosphorylates and inactivates Rb and related proteins p107 and p130. p16 is another CDKI, which is possibly necessary for the maintenance of senescent phenotype, which increases markedly in senescent cells and correlates with increasing Rb hypophosphorylation[20]. Some researchers also have found that increased levels in either p53-p21 or p16 alone are able to induce Rb hypophosphorylation and initiate senescence program[22], suggesting that inhibition of p53 may primarily lead to the occurrence of mitotic cell death whereas overexpression of p16 is probably responsible for senescence-like phenotype in human hepatoma BEL-7402 cells after treatment with LDM. It is reported that enhanced intensity of staining for SA-β-gal is due to the up-regulation of p16[22,38].

In conclusion, these data suggest that mitotic cell death and senescence coexist in LDM-treated human hepatoma BEL-7402 cells and they seem not to be independent events. To some extent, both of them may share common cross talk in the signal transduction pathway. Studying senescence and cell death may help us to understand the similarities and differences.

Telomerase activity has been shown to be a new reliable pathological marker of tumor malignancy. Previous reports indicate that telomere and telomerase are intimately related to tumorigenesis and cellular senescence[21,28]. The tumor-promoting effects of telomerase activity are very complex. Tumor cells must resolve the telomere length problem in order to realize immortalization, while reactivation of telomerase is a prerequisite of malignant growth in telomerase-positive cells. So, it is not surprising that telomerase is highly expressed in most human tumors[26]. On the contrary, inhibition of telomerase activity does not benefit tumorigenesis, but contributes to the formation of tumor suppressor mechanism, including apoptosis, mitotic cell death and senescence *etc*. Our studies have revealed that telomerase activity is decreased with increased LDM concentration in human hepatoma BEL-7402 cells (Fig. 10), suggesting that mitotic catastrophe and senescence are associated with the reduced telomerase activity in LDM-treated human hepatoma BEL-7402 cells. Whether telomere loss or attrition affects mitotic catastrophe and senescence induced by LDM remains to be investigated.

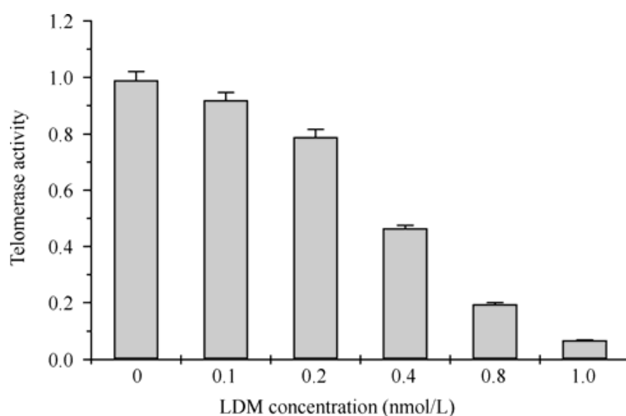

FIG. 10. Decreased telomerase activity by LDM in human hepatoma BEL-7402 cells.

REFERENCES

1. Xu Y J, Zhen Y S, Goldberg I H (1994). C1027 chromophore, a potent new enediyne antitumor antibiotic, induces sequence-specific double-strand DNA cleavage. *Biochemistry* **33**, 5947-5954.
2. Dziegielewski J, Beerman T A (2002). Cellular responses to the DNA strand-scission enediyne C-1027 can be independent of ATM, ATR, and DNA-PK kinases. *J Biol Chem* **277**, 20549-20554.
3. Hu J L, Xue Y C, Xie M Y, *et al.* (1988). A new macromolecular antitumor antibiotic, C-1027. I. Discovery, taxonomy of producing organism, fermentation and biological activity. *J Antibiot* **41**, 1575-1579.
4. Zhen Y S, Min X Y, Yu B, *et al*. (1989). A new macromolecular antitumor antibiotic, C-1027 III. Antitumor activity. *J Antibiot* **42**, 1294-1298.
5. Otani T, Yasuhara T, Minami Y, *et al*. (1991). Purification and primary structure of antitumor antibiotic C-1027, from *Streptomyces globisporus*. *Biol Chem* **55**, 407-417.
6. Shao R G and Zhen Y S (1995). Relationship between the molecular composition of C1027, a new macromolecular antibiotic with enediyne chromophore, and its antitumor activity. *Acta Pharm Sin* **30**, 336-342. (In Chinese)
7. Wang Z, He Q Y, Liang Y Y, *et al.* (2003). Non-caspase-mediated apoptosis contributes to the potent cytotoxicity of the enediyne antibiotic lidamycin toward human tumor cells. *Biochem Pharmacol* **65**, 1767-1775.
8. He Q Y, Liang Y Y, Wang D S *et al*. (2002). Characteristics of mitotic cell death induced by enediyne antibiotic lidamycin in human epithelial tumor cells. *Int J Oncol* **20**, 261-266.

9. Huang Y H, Shang B Y and Zhen Y S (2005). Antitumor efficacy of lidamycin on hepatoma and active moiety of its molecule. *World J Gastroenterol* **11**, 3980-3984.
10. Liang Y X and Li D D (2004). Mitotic cell death. *Chin J Cell Biol* **26**, 26-28.
11. Allen C E and Wu L C (2000). Downregulation of KRC induces proliferation, anchorage independence, and mitotic cell death in HeLa cells. *Exp Cell Res* **260**, 346-356.
12. Grafi G (1998). Cell cycle regulation of DNA replication: the endoreduplication perspective. *Exp Cell Res* **244**, 372-378.
13. Liang Y X, Zhang W, Li D D, *et al*. (2004). Mitotic cell death in BEL-7402 cells induced by enediyne antibiotic lidamycin is associated with centrosome overduplication. *World J Gastroenterol* **10**, 2632-2636
14. Sohn S H, Multani A S, Gugnani P K, *et al*. (2002). Telomere erosion-induced mitotic catastrophe in continuously grown Chinese hamster don cells. *Exp Cell Res* **279**, 271-276.
15. Park I K, Morrison S J and Clarke M F (2004). Bmi 1, stem cells, and senescence regulation. *J Clin Invest* **113**, 175-179.
16. Lin A W, Barradas M, Stone J C, *et al*. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev* **12**, 3008-3019.
17. Serrano M, Lin A W, McCurrach M E, *et al*. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602.
18. Cao L, Li W M, Kim S, *et al*. (2003). Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes Dev* **17**, 201-213.
19. Park S Y, Choi B, Cheon H, *et al*. (2004). Cellular aging of mitochondrial DNA-depleted cells. *Biochem Biophys Res Commun* **325**, 1399-1405.
20. Satyanarayana A, Greenberg R A, Schaetzlein S, *et al*. (2004). Mitogen stimulation cooperates with telomere shortening to activate DNA damage responses and senescence signaling. *Mol Cell Biol* **24**, 5459-5474.
21. Yang F, Li M Y (2005). Effects of telomere and telomerase on cellular senescence. *Med Recapit* **11**, 197-199. (In Chinese)
22. Sharpless N E, DePinho R A (2004). Telomeres, stem cells, senescence, and cancer. *J Clin Invest* **113**, 160-168.
23. Mokbel K (2000). The role of telomerase in breast cancer. *Eur Surg Oncol* 26, 509-514.
24. Yan P, Benhattar J, Seelentag W, *et al*. (2004). Immunolistochemical localization of hTERT protein in human tissues. *Histochem Cell Biol* **121**, 391-397.
25. Yi X M, Shay J W, Wright W E (2001). Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. *Nucleic Acids Res* **29**, 4818-4825.
26. Shay J W, Bacchetti S (1997). A survey of telomerase activity in human cancer. *Eur J Cancer* **5**, 787-791.
27. Terai M, Uyama T, Sugiki T, *et al*. (2005). Immortalization of human fetal cells: The life span of umbilical cord blood-derived cells can be prolonged without manipulating p16^{INK4a}/RB braking pathway. *Mol Biol Cell* **16**, 1491-1499.
28. Hahn W C (2003). Role of telomeres and telomerase in the pathogenesis of human cancer. *J Clin Oncol* **21**, 2034-2043.
29. Hahn W C, Stewart S A, Brooks, M W, *et al.* (1999). Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* **5**, 1164-1170.
30. Herbert B, Pitts A E, Baker S I, *et al*. (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci USA* **96**, 14276-14281.
31. He Q Y, Zhang H Q, Pang D B, *et al*. (1996). Resistance to apoptosis of harringtonine-resistant HL-60 cells induced by tetrandrine. *Acta Pharmacol Sin* **17**, 545-549.
32. Russell C, Scaduto J, Grotyohann L W (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys J* **76**, 469-477.
33. Dimri G P, Lee X, Basile G, *et al*. (1995). A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* **92**, 9363-9367.
34. Liang Y, Yan C, Schor N F (2001). Apoptosis in the absence of caspase 3. *Oncogene* **20**, 6570-6578.
35. Chang B D, Broude E V, Dokmanovic M, *et al*. (1999). A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* **59**, 3761-3767.
36. Duchen M R (2004). Roles of mitochondria in health and disease. *Diabetes* **53** (Suppl 1), S96-102.
37. Wiebren A T, Joost J W, Johannes J B, *et al*. (2001). The importance of biological factors (bcl-2, bax, p53, PCNA, MI, HPV and angiogenesis) in invasive cervical cancer. *Eur J Obstet & Gynecol Reprod Biol* **97**, 223-230.
38. Chang B D, Xuan Y Z, Broude E V, *et al*. (1999). Role of p53 and p21vaf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. *Oncogene* **18**, 4808-4818.
39. Krishnamurthy J, Torrice C, Ramsey M R, *et al.* (2004). *Ink4a/Arf* expression is a biomarker of aging. *J Clin Invest* **114**, 1299-1307.

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