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Characteristics of Mitochondrial Events in Synthetic Bile Acids-induced Apoptosis of Human Osteosarcoma Cells

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Abstract : Apoptosis of osteosarcoma cells induced by bile duct derivates, HS-1200 was investigated with relation to mitochodria. HS-1200 induced cytochrome c and Smac/DIABLO release from mitochondria which are major factors related to apoptosis. In these apoptosis processes, release of cytochrome c was not blocked by caspase inhibitor, but release of Smac/DIABLO was blocked. BKA, a kind of PTP (permeablity transition pore) inhibitor, did not block both of them. Interestingly, the alteration of MMP was not observed by means of using JC-1 dye. Although MitoTracker, DiOC-6 and Rhodamine123 were used to confirm previous results, the decrease of MMP was not observed. In order to investigate whether this phenomenon is apoptosis-specific or cell-specific process, genistein was added to cells which usually decreased MMP. After adding genistein, MMP was not decreased, suggesting this phenomenon is cell-specific process.

Conclusionally, HS-1200 induced apoptosis of osteosarcoma cells via mitochondria, cytochrome c and Smac/DIABLO were released from mitochondria without decrease of MMP. The release of Smac/DIABLO was dependent of caspase.

Key words : Bile duct derivates, Apoptosis, MMP, Osteosarcoma cells

INTRODUCTION

Bile acids are polar derivatives of cholesterol essential for the absorption of dietary lipids and regulate the transcription of genes that control cholesterol homeostasis. Different bile acids exhibit distinct biological effects. Importantly, natural bile salts were reported to inhibit cell proliferation and induce apoptosis in various cancer cells (Blake *et al*. 1998, Martinez *et al*. 1998).

Recently, it has been reported that several synthetic bile acids induced apoptosis in human hepatocellular carcinoma, human breast carcinoma, human leukemic T and mastocytoma cells (Baek *et al*. 1997, Park *et al*. 1997, Im *et al*. 1999, Choi *et al*. 2001, Im *et al*. 2001, Seo *et al*. 2003) and inhibited angiogenesis in human hepatocellular carcinoma cells (Suh *et al*. 1997). Moreover, ursodexocycholic acid (UDCA) and its

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synthetic derivatives, HS-1030 and HS-1183, inhibited SV40 DNA replication, and predominantly inhibited the initiation stage of DNA replication (Kim *et al.* 2003). Especially HS-1200 was known to have the most efficient antitumor effect.

Apoptosis is an evolutionarily conserved, innate process by which cells systemically inactivate, dissemble, and degrade their own structural and functional components to complete their own demise. Cells undergoing apoptosis usually develop characteristic morphological changes, including nuclear condensation and pyknosis, and degradation of DNA into oligonucleosomal fragments (Wyllie *et al.* 1980, Williams 1991). It can be activated intracellularly through a genetically defined developmental program or extracellularly by endogenous proteins, cytokines and hormones, as well as drugs, xenobiotic compounds, radiation, oxidative stress, and hypoxia (Wyllie *et al.* 1980, Williams 1991).

It was demonstrated that alterations in mitochondrial function in general and induction of the mitochondrial permeability transition play a key part in the regulation of apoptosis (Kroemer *et al.* 1997, Green & Reed 1998). Their intermembrane space was proposed to contain several potentially apoptogenic factors, including cytochrome c, procaspases 2, 3 and 9, and apoptosis inducing factor (AIF), which were liberated through the outer membrane in order to participate in the degradation phase of apoptosis (Susin *et al.* 1999a).

Osteosarcoma is one of the most common primary malignant tumors of bone. Numerous studies depicted that the therapeutic effect of a variety of chemotherapeutic agents on osteosarcoma depends on the induction of apoptosis (Lu & Yagi 1999, Seki *et al.* 2000).

This study was undertaken to investigate the mitochondrial event in synthetic bile acid derivatives-induced apoptosis in osteosarcoma cells. This study shows that mitochondrial events implicate this type of apoptosis but alteration of the mitochondrial permeability transition was not shown.

MATERIALS AND METHODS

1. Materials

1) Antibodies

The following reagents were obtained commercially: Rabbit polyclonal anti-horse cytochrome c and Smac /DIABLO were from Santa Cruz Biotechnology (Santa Cruz, CA); FITC-conjugated goat anti-rabbit IgG antibody was from Vector (Burlingame, CA).

2) Fluorescence probes

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1), MitoTracker Red CMXRos, 3,3′-Dihexyloxacarbocyanine iodide (DiOC-6) and Rhodamine123 were obtained from Molecular Probes (Eugene, OR).

3) Other reagents

Permeability transition pore (PTP) blocker Bongkrekic acid (BKA) was obtained from Calbiochem (San Diego, CA). Caspase inhibitor, z-VAD-fmk, was obtained from Kamiya Biomedical Co. (Seattle, WA). Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF, eugenol and propidium iodide were obtained from Sigma (St. Louis, MO).

2. Cell culture

HOS-human osteosarcoma cell line was purchased from the ATCC (Rockville, Maryland). Cells were maintained at 37°C with 5% CO_2 in air atmosphere in DMEM with 4 mML-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

3. Preparation of synthetic bile acid derivatives

Conjugate from of CDCA with β-alaninbenzyl ester (*N*-[(3α,5β,7α)-3,7-dihydroxy-24-oxocholan24-yl]β-alanine benzyl ester: HS-1200) was synthesiz-

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ed as follows. To a solution of 1.0 g (2.76 mM) of CDCA in 20 mL of DMF, 631 mg (3.06 mM) of DCC and 400 mg (2.96 mM) of HOBT were added at 4°C. After stirring at 4°C for 40 min, 1.0 g (4.64 mM) of β-alanine benryl ester *p*-toluenesulfonate salt in 10 mL of DNF and 590 μL of TEA (4.20 mM) was added. After stirring overnight at room temperature, the reaction mixture was diluted with 200 mL of ethyl acetate, and washed with magnesium sulfate and concentrated under reduced pressure The residue was purified by flash chromatography to give 1.05 g (67%) of *N*-([3α,5β,7α]-3,7-dihydroxy-24-oxocholan24-yl) β-alanine benzyl ester (HS-1200) as a yellow foam: R_f 0.33 (SiO_2, 80% ethyl acetate-hexane); ^1H NMR (200MH_Z, $CDCL_3$) δ7.34 (br s, 5H), 6.00 (br s, 1H), 5.13 (s, 2H), 4.11 (q, 2H, *J* = 7.33), 3.55-3.47 (m, 2H), 2.57 (t, 2H, *J* = 6.59), 2.29-0.89 (m, 34H), 0.63 (s, 3H); ^{13}C NMR (50 MH_Z, $CDCL_3$) δ173.5, 172.3, 135.5, 128.4, 128.2, 128.0, 72.2, 71.7, 68.1, 66.3, 60.2, 55.7, 50.2, 42.5, 41.4, 39.6, 39.3, 35.3, 34.9, 34.7, 34.5, 34.0, 33.3, 32.6, 31.6, 30.5, 23.5, 22.6, 20.8, 20.4, 18.2, 14.0, 11.6.

The structures of CDCA and its novel synthetic derivatives are shown in Fig. 1.

4. Bile acid derivatives treatment

After cells were subcultured for twenty four hours, the original medium was removed. Cells were washed with PBS and then incubated in the same fresh medium. Each type of synthetic bile acid derivatives from a stock solution was added to the medium to obtain various dilutions of the drug. The concentration of ethanol, 0.1～0.5% (v/v), used in this study both as a vehicle for bile acids and as a control, had no effect on HOS cell viability in our preliminary studies.

5. Determination of viability by HS-1200 treatment

Cells treated with UDCA, CDCA and their synthetic derivatives were harvested after 24 h, stained with trypan blue and then counted using a hemocytometer. Since HS-1200 showed the most efficient antitumor effect and the dose required for half-maximal inhibition of viability 7 h after treatment was about 35 μM (as will be presented in results section), this single concentration was utilized for further *in vitro* assessment of apoptosis.

6. Morphological assessment of apoptosis

Cells were observed and photographed under DIC optics. Then cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/mL Hoechst 33342 for 30 min at 37°C, fixed for 10 minutes in 4% paraformaldehyde, and observed and photographed under an epifluorescence microscope. Samples were observed under an epifluorescence microscope. For each time point, the number of cells, which showed condensed or fragmented nuclei with Hoechst staining, was determined by a blinded observer from a random sampling of 250～300 cells per experiment. Four independent experiments were conducted.

7. Measurement of mitochondrial membrane potential (MMP)

Changes in MMP 0, 1, 2, 3, 5 and 7 h after treatment with HS-1200 were measured by flow cytometry.

JC-1: JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated at 37°C for 15 min.

MitoTracker: MitoTracker Red CMXRos was directely to the cell culture medium (450 nM final concentration) and incubated at 37°C for 30 min.

DiOC-6: DiOC-6 was directely to the cell culture medium (40 nM, final concentration) and incubated at 37°C for 30 min.

Rhodamine123: Rhodamine123 was directely to the

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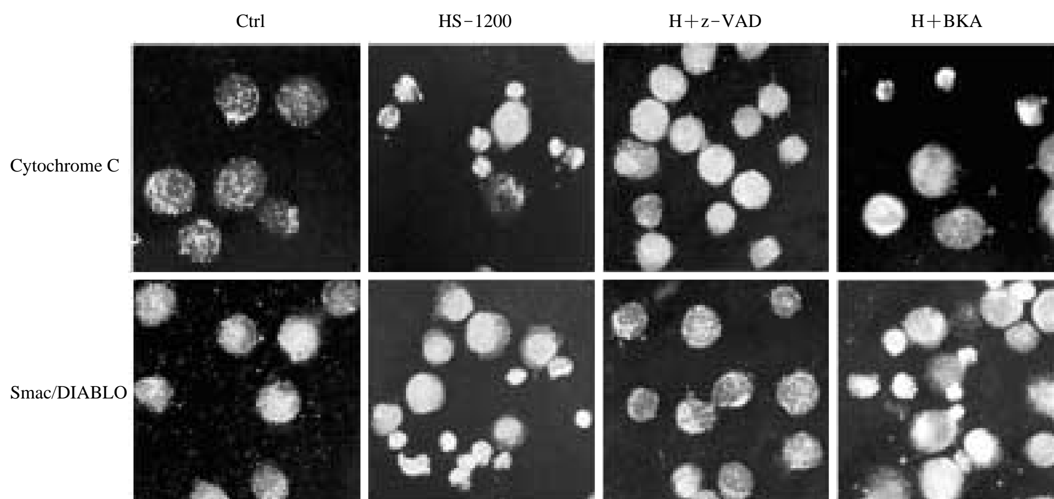

Fig. 1. Immunofluorescent micrographs showing the release of cytochrome c in upper panel and Smac/DIABLO in lower panel from mitochondria in 35 μM HS-1200 treated HOS cells. In contrast to the control, both cytochrome c and Smac/DIABLO are released from mitochonria after HS-1200 treatment. zVAD-fmk prevented the release of Smac/DIABLO but not cytochrome c. BKA prevented neither cytochrome c nor Smac/DIABLO. A representative data from four independent experiments were presented.

cell culture medium (10 μg final concentration) and incubated at 37°C for 60 min.

Flow cytometry was performed on a Epics XL (Beckman Coulter, FL, USA). Data were acquired and analyzed using EXPO32 ADC XL 4 color software.

8. Immunofluorescent staining

Cells were harvested and cytocentrifuged. Samples were incubated with anti-cytochrome C or Smac/DIABLO antibody for 1 h, washed 3X each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS, and observed and photographed under an epifluorescence microscope.

9. Statistical analysis

Four independent experiments were carried out. The results of the experiment and control groups were tested for statistical significance by a one-way ANOVA.

RESULTS

To demonstrate the involvement of mitochondrial events, release of two well known mitochondrial apoptogenic factors, cytochrome c and Smac/DIABLO, was observed by immunofluorescent microscopy. Whereas control cells had a bright, punctuate cytoplasmic cytochrome c or Smac/DIABLO distribution, in cells treated with HS-1200, both stainings was diffuse and uniform throughout the cytoplasm, indicating that both had been released from the mitochondria (Fig. 1). Next, it was studied whether the release of the factors are caspase-dependent. A pancaspase inhibitor zVAD-fmk blocked the release of Smac/DIABLO but not cytochrome c. In the next step, a PTP blocker BKA was examined whether inhibited the release of both factors from mitochondria. BKA did not prevent release of both factors. These data show that Smac/DIABLO but not cytochrome c is

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released from mitochondria dependently of caspase. However, both factors are released independently of PTP. Blocking cytochrome c release by zVAD-fmk finally prevented the nuclear condensation (Fig. 2).

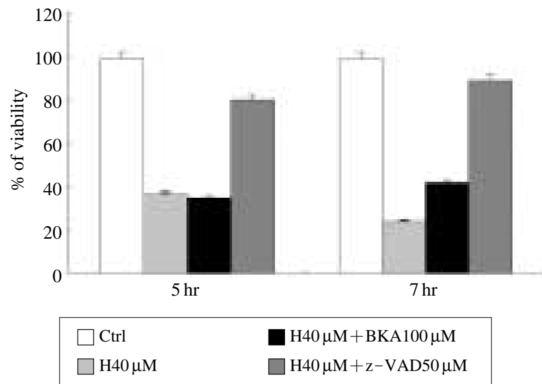

Fig. 2. The viability as determined by Hoechst staining in 35 μM HS-1200 treated HOS cells. zVAD prevented nuclear condensation. A representative data from four independent experiments was presented.

Next, changes of MMP was studied. Interestingly, flow cytometry employing JC-1, which is the most commonly used dye for measurement of MMP, did not show the reduction of MMP (Fig. 3). Next, three additional dyes were utilized to measure MMP. The dyes, *MitoTracker, DiOC-6, Rhodamine*123, also did not demonstrate the reduction of MMP (Fig. 4). Although the mean ± the standard deviation of the means obtained from each independent experiment were tested by a one-way ANOVA, no statistical significance was observed in MMP.

To explore whether no reduction of MMP is apoptotic stimulus specific or cell specific, HOS cells were treated with 50 μM genistein which was another apoptotic stimulus for HOS cells. Importantly, no reduction of MMP was observed in HS-1200 treated HOS cells (Fig. 5).

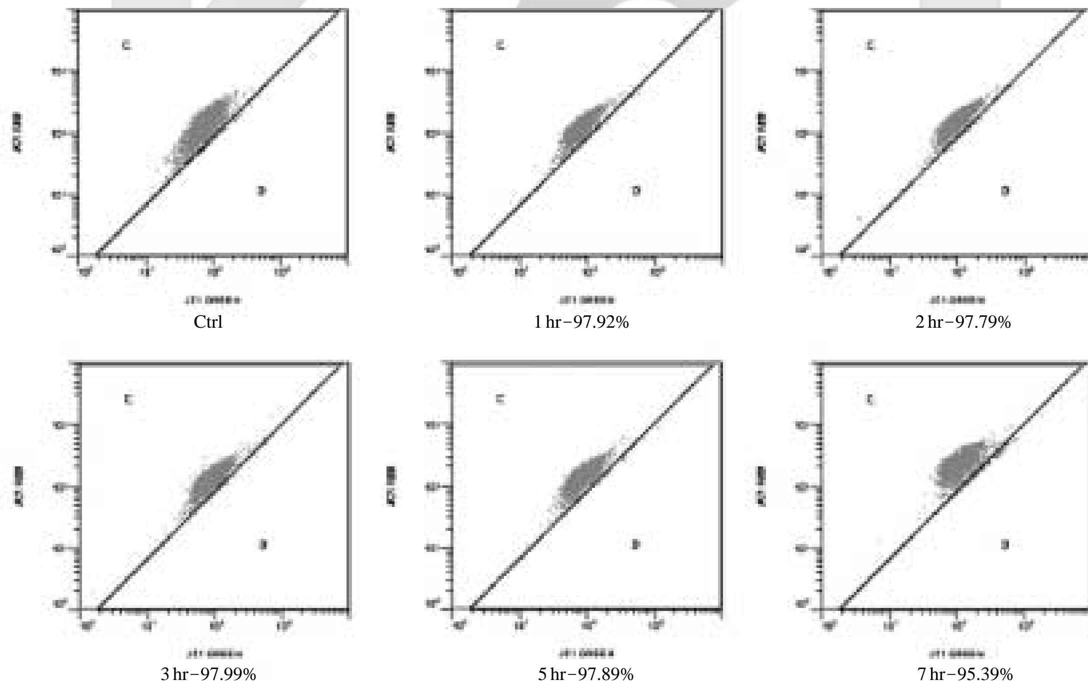

Fig. 3. Flow cytometry data showing no reduction of MMP in 35 μM HS-1200 treated HOS cells. JC-1 dye was employed. A representative data from four independent experiments was presented.

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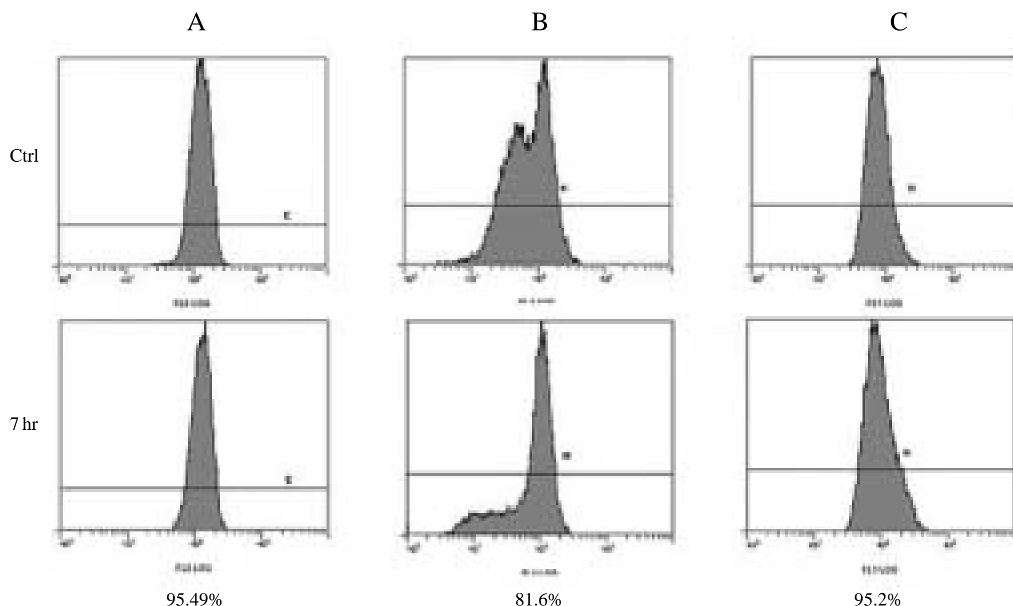

Fig. 4. Flow cytometry data showing no reduction of MMP in 35 μM HS-1200 treated cells. MitoTracker (A), DiOC-6 (B) and Rhodamine123 (C) dye were employed. A representative data from four independent experiments was presented.

Taken together, apoptotic stimuli to HOS cells induced the release of apoptogenic factors without reduction of MMP.

DISCUSSION

The division of apoptosis into three functionally distinct phases (Kroemer *et al.* 1995a, Kroemer 1997, Kroemer *et al.* 1997, Kroemer *et al.* 1998, Susin *et al.* 1998), though artificial, provides an attractive theoretical framework for the molecular dissection of apoptotic pathways. The pre-mitochondrial initiation phase would include diverse signal transduction and damage pathways which are private in the sense that they are not activated in universal fashion and rather depend on the death-inducing primary stimulus and/or cell type. These pathways would converge on the mitochondrion, which integrates them into a common pathway. The common mitochondrial phase is characterized by an irreversible loss of mitochondrial membrane barrier function (Kroemer *et al.* 1995, Kroemer 1997, Kroemer *et al.* 1998, Susin *et al.* 1998). Thus, the inner mitochondrial transmembrane potential is frequently lost during the early phase of apoptosis (Zamzami *et al.* 1995a, Zamzami *et al.* 1995b, Castedo *et al.* 1996, Marzo *et al.* 1998), and the outer mitochondrial membrane is permeabilized, leading to the release of soluble intermembrane proteins into the cytosol (Liu *et al.* 1996, Kluck *et al.* 1997, Yang *et al.* 1997, Susin *et al.* 1999a). It is only after this process, beyond the point-of-no-return, that downstream caspases (e.g. caspase-3 and -6) are activated and endonucleases come into action, leading to the acquisition of the biochemical and morphological hallmarks of apoptosis (Liu *et al.* 1996, Susin *et al.* 1996, Zamzami *et al.* 1996, Liu *et al.* 1997, Susin *et al.* 1997, Zhou *et al.* 1997, Susin *et al.* 1999b).

According to this model, the mitochondrion would function as a sort of Pandoras Box, in which the release of proteins that are usually well secluded in the mito-

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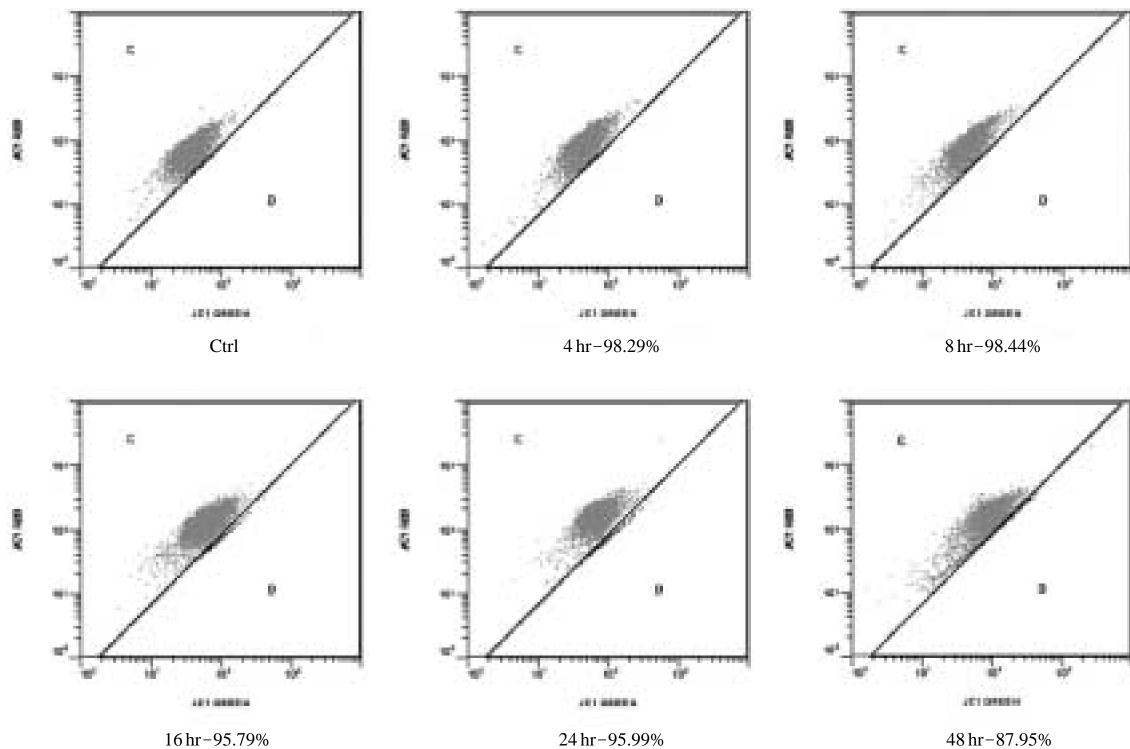

Fig. 5. Flow cytometry data showing no reduction of MMP in 50 μM genistein treated cells. JC-1 dye was employed. A representative data from four independent experiments was presented.

chondrial intermembrane compartment determines the cells fate. Most authors agree upon the fact that mitochondrial intermembrane proteins such as cytochrome c are generally released during apoptosis, at least in mammalian cells, although some rare exceptions have been reported (Chauhan *et al*. 1997, Adachi *et al*. 1998, Tang *et al*. 1998). However, the importance of this release is subject to rather divergent interpretations. Some data may be interpreted to mean that the release of mitochondrial intermembrane proteins would be a decisive, perhaps obligatory step of the apoptotic cascade (Kroemer *et al*. 1995, Kroemer 1997, Kroemer *et al*. 1997, Green & Kroemer 1998, Green & Reed 1998, Kroemer *et al*. 1998, Penninger & Kroemer 1998, Susin *et al*. 1998, Susin *et al*. 1999a), or at least an amplifying mechanism accelerating cell death (Kuwana *et al*. 1998). Much evidence has been accumulated to suggest that release of cytochrome c from mitochondria is an important step in apoptosis (Reed 1997). In response to a variety of death-promoting stimuli, cytochrome c is released from its normal position within the intermembrane space of mitochondria, in association with changes in mitochondrial permeability, membrane potential, and ultrastructure (Heiskanen *et al*. 1999). Once in the cytosol, cytochrome c binds to Apaf-1/cytochrome c in complexes that activate procaspase-9 (Zhou *et al*. 1997). Finally, active caspase-9 is released from the Apaf-1/cytochrome c complex and activates down-stream caspases such as caspase-3, -6 and -7 (Li *et al*. 1997, Zhou *et al*. 1997).

Although much emphasis has been laid on the role

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of cytochrome c as a molecule linking mitochondrial membrane permeabilization to the activation of cytosolic caspases (Liu *et al*. 1996, Kluck *et al*. 1997, Liu *et al*. 1997, Yang *et al*. 1997, Zhou *et al*. 1997), it is clear that additional mitochondrial intermembrane proteins including Smac/Diablo (Du *et al*. 2000) and mitochondrial caspases (Mancini *et al*. 1998, Susin *et al*. 1999b) can participate in the apoptotic process. Moreover, the metabolic consequences of mitochondrial membrane permeabilization are likely to have a major impact on the death process. Recent studies have shown that cytochrome c is not the only mitochondrial protein involved in the activation of caspases. Inhibitors of apoptosis (IAPs) suppress cell death by directly binding to and inhibiting caspaes (Deveraux & Reed 1999). Therefore, during apoptosis the inhibitory effects of IAPs must be suppressed, a process that is mediated by another mitochondrial intermembrane protein called Smac/Diablo (Du *et al*. 2000). Upon induction of apoptosis Smac/Diablo is release from the mitochondria into the cytosol. Whereas cytochrome c activates caspase-9 through Apaf-1, Smac/Diablo interacts with a variety of IAPs thus relieving their inhibitory effect on initiator caspases (such as caspase-9) as well as effector caspases (such as caspase-3) (Wu *et al*. 2000). This study showed that HS1200 treatment elicited the release of cytochrome c and Smac/DIABLO from mitochondria. The release of Smac/DIABLO was prevented by zVAD-fmk, suggesting that its release depends on caspase.

Cytochrome c release preceded the loss of $\Delta\Psi m$ (Rodriguez & Lazebnik 1999), whereas other studies reported the reverse, cytochrome c release following mitochondrial swelling, outer membrane rupture, and loss of $\Delta\Psi m$ (Zamzami *et al*. 1995b). Mitochondrial swelling is related to formation of the permeability transition (PT) pore, a complex that contains the voltage-dependent anion channel, and the adenin nucleotide translocation (Marchetti *et al*. 1996, Zamzami *et al*. 1996) in addition to other cytosolic and mitochondrial proteins.

This study showed that alteration in mitochondrial membrane transition was not involved in apoptosis of HOS cells induced by synthetic bile acids. Neither alteration of mitochondrial membrane transition was not observed in genistein-induced apoptosis of HOS cells. Although several methods measuring mitochondrial membrane potential were employed to exclude the technical errors, any alteration of mitochondrial membrane transition was not shown.

Although currently favored hypothesis for the induction of apoptosis is that perturbations of mitochondria allow the release of cytochrome c and much evidence has accumulated to suggest that release of cytochrome c from mitochondria is an important step in apoptosis (Reed 1997), it is controversial still that the release of cytochrome c is essential for the induction of all type of apoptosis. It is not still unclear exactly how cytochrome c release is achieved. Although opening of a permeability transition pore was proposed previously as one possible means of enabling cytochrome c release to the cytosol (Kroemer *et al*. 1997) cytochrome c release has been observed in situations where no loss in mitochondrial transmembrane potential was observed (Bossy-Wetzel *et al*. 1998). Several studies rather reported the mitochondrial hyperpolarization in apoptosis (Khaled *et al*. 1999, Kahled *et al*. 2001a, b, Kim *et al*. 2003). Thus, many important issues of this type of osteosarcoma cell death remain to be elucidated.

In conclusion, mitochondiral events involves in synthetic CDCA derivatives-induced apoptosis of osteosarcoma cells.

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합성담즙산유도체에 의한 뼈육종세포의 세포자멸사에서 사립체 관련 현상의 특징

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간추림 : 담즙산 유도체 HS-1200에 의한 골육종세포의 세포자멸사에서 사립체 관련 현상을 연구하였다. HS-1200은 사립체의 주요 세포자멸사 관련 인자인 cytochrome c와 Smac/DIABLO의 방출을 유도하였다. 이런 형태의 세포자멸사에서 cytochrome c 방출은 caspase 억제제로 막아지지 않았으나 Smac/DIABLO의 방출은 막아졌다. PTP 억제제인 BKA는 cytochrome c와 Smac/DIABLO 모두의 방출을 막지 못하였다.

흥미롭게도 JC-1 dye를 사용하여 측정한 결과 MMP의 변화가 관찰되지 않았다. 이를 확인하기 위하여 MitoTracker, DiOC-6 및 Rhodamine123 등을 사용하여 MMP를 측정하여도 감소는 관찰되지 않았다. 이런 현상이 세포자멸사 신호에 특이한지 혹은 세포에 특이한지를 조사한 결과 genistein 투여 후에도 MMP 감소가 관찰되지 않아 세포 특이한 반응으로 생각한다.

결론적으로 HS-1200는 뼈육종세포에서 사립체 경로를 통하여 세포자멸사를 유도하지만 MMP의 감소는 없이 cytochrome c와 Smac/DIABLO의 방출이 유도되며 이중 Smac/DIABLO의 방출은 caspase 의존적이다.

찾아보기 낱말 : 담즙산유도체, 세포자멸사, 사립체막전위, 뼈육종세포

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