Synthetic Chenodeoxycholic Acid Derivative HS-1200-Induced Apoptosis of Human Oral Squamous Carcinoma Cells

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Bile acids and synthetic its derivatives induced apoptosis in various kinds of cancer cells and anticancer effects. Previous studies have been reported that the synthetic chenodeoxycholic acid (CDCA) derivatives showed apoptosis inducing activity on various cancer cells *in vitro*. It wasn't discovered those materials have apoptosis induced effects on YD9 human oral squamous carcinoma cells.

The present study was done to examine the synthetic bile acid derivatives(HS-1199, HS-1200) induced apoptosis on YD9 cells and such these apoptosis events. We administered them in culture to YD9 cells. Tested YD9 cells showed several lines of apoptotic manifestation such as activation of caspase-3, degradation of DFF, production of poly (ADP-ribose) polymerase(PARP) cleavage(HS-1200 only), DNA degradation(HS-1200 only), nuclear condensation, inhibition of proteasome activity, reduction of mitochondrial membrane potential(HS-1200 only) and the release of cytochrome c and AIF to cytosol. Between two synthetic CDCA derivatives, HS-1200 showed stronger apoptosis-inducing effect than HS-1199. Therefore HS-1200 was demonstrated to have the most efficient antitumor effect.

Taken collectively, we demonstrated that a synthetic CDCA derivative HS-1200 induced caspases-dependent apoptosis via mitochondrial pathway in human oral sqauamous carcinoma cells *in vitro*. Our data therefore provide the possibility that HS-1200 could be considered as a novel therapeutic strategy for human orall squamous carcinoma from its poweful apoptosis-inducing activity.

Key words : Apoptosis, Synthetic CDCA derivatives, HS-1200, Human oral squamous cell carcinoma

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Received: 2007-04-30
Accepted: 2007-06-23

*This work was supported by for two years Pusan National University research grant.

I. INTRODUCTION

Carcinoma of the oral cavity, especially oral squamous cell carcinoma(OSCC), are one of the most leading causes of cancer related death and affect nearly 500,000 patients annually world-wide. And OSCC is one of the most common malignancies that remain incurable with current therapies.[1)]

Apoptosis, or programmed cell death, is an essential physiological process that is required for the normal development and maintenance of tissue homeostasis. However, apoptosis also is implicated in a wide range of pathological conditions, including immunological diseases, allergy and cancer.[2,3)] During apoptosis, cells undergo specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA.[4,5)]
Bile acids are polar derivatives of cholesterol essential for the absorption of dietary lipids and regulate the transcription of genes that control cholesterol homeostasis. Depending on the nature of chemical structures, each kind of bile acid exhibits distinct biological effects.[6)] The natural bile salts were reported to inhibit cell proliferation and induce apoptosis in various cancer cells.[7,8)] After synthesis by the liver and excretion into the bile canaliculus and the digestive tract, the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are metabolized by enteric bacteria to produce secondary bile acids; primarily deoxycholic acid, ursodeoxycholic acid (UDCA), and lithocholic acid (LCA). Bile acids are conjugated to glycine, or taurine when the glycine conjugates predominate.[6)] Conjugation of bile acids to glycine and taurine is one mechanism by which an organism can decrease the hydrophobicity of a bile acid.[9,10)] The conjugation renders the molecules less cytotoxic at physiological concentrations.[11)] Numerous studies have shown that elevated concentrations of bile acid within the liver induce hepatocyte apoptosis. This provides a cellular mechanism for bile acid mediated liver injury.[12)] Bile acid hydrophobicity is correlated with induction of apoptosis and/or growth arrest.[13)]

To date, there is no report about the apoptotic effect of CDCA derivatives on YD 9 human oral sqauamous carcinoma cell line. Therefore this study was undertaken to examine the molecular mechanism underlying CDCA derivatives-induced apoptosis in human oral sqauamous carcinoma cells

II. MATERIALS AND METHODS

1. Reagents

CDCA was obtained from Dae-Woong Pharmaceutical Co.(Seoul, Korea) and Aldrich (Milwaukee, WI, USA). The synthetic bile acid derivatives, HS-1199 and HS-1200 were kindly provided by Professor Young-Hyun Yoo(Department of Anatomy, Dong-A University College of Medicine, Busan, Korea). The structure and methods of the synthesis of the synthetic bile acid derivatives were described by Im EO et al.[14)] HS-1199 is a conjugate form of CDCA with L-phenyl alanine benzyl ester(*N*-[(3α, 5β, 7α)-3,7-dihydroxyl-24-oxocholan-yl] L-phenyl alanine benzyl ester). HS-1200 is a conjugate form of CDCA with β-alanine benzyl ester(*N*-[(3α, 5β, 7α)-3,7-dihydroxyl-24-oxocholan-yl] β-alanine benzyl ester). These bile acids and their derivatives were dissolved in absolute ethanol, and dilutions were made in culture medium. The final concentration of ethanol in the medium was less than 0.1%(vol/vol) in the treatment range(10-100 μM) and showed no influence on cell growth(data not shown). The structures of CDCA and its conjugate forms(HS-1199 and HS-1200) are shown in Fig. 1.

The following reagents were obtained commercially: Rabbit polyclonal anti-human caspase-3 and anti-horse cytochrome c, and anti-human DNA fragmentation factor(DFF), and goat polyclonal anti-mouse AIF antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse polyclonal anti-human poly(ADP-ribose) polymerase (PARP) antibody was from

Oncogene (Cambridge, MA); FITC-conjugated goat anti-rabbit and horse anti-mouse IgGs were from Vector (Burlingame, CA); HRP-conjugated donkey anti-rabbit and sheep anti-mouse IgGs were from Amersham Pharmacia Biotech (Piscataway, NJ). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM) and FBS were from Gibco (Gaithersburg, MD). Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF, thiazolyl blue tetrazolium bromide and propidium iodide were from Sigma (St. Louis, MO); SuperSignal West Pico enhanced chemilumin, Gescence Western blotting detection reagent was from Pierce (Rockford, IL).

2. Cell culture

Oral squamous carcinoma cells(YD9 cells) (kindly provided by Professor Jin Kim, Department of Oral Pathology, Yonsei University College of Dentistry, Seoul, Korea) were maintained at 37℃ with 5% CO_2 in air atmosphere in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 ㎍/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 10% FBS. cells were matained in Dulbecco's modified Eagle's medium with 10% FBS.

3. MTT assay

Cells were placed in a 96-well plate and incubated 24 h. Then cells treated with 10, 25, 50, 100 μM of CDCA, HS-1199, and HS-1200 for 5 h. And then cells were treated with 500 μg/ml of thiazolyl blue tetrazolium bromide. Cells were incubated at 37℃ with 5% CO_2 for 4h. And then the medium was aspirated and formed formazan crystals were dissolved in the mixture solution of 75 μl DMSO and 75 μl absolute ethanol. Cell viability was measured by a ELISA reader(Sunrise Remote Control, Tecan, Austria) at 570 nm excitatory emission wavelength.

4. Hoechst staining

Cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 ㎍ /ml Hoechst 33342 for 30 min at 37℃ and fixed for 10 min in 4% paraformaldehyde.

5. DNA electrophoresis

2 x 10^6 cells were resuspended in 1.5 ml of lysis buffer(10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS) into which proteinase K(200 ㎍/ml) was added. After samples were incubated overnight at 48℃, 200 μl of ice cold 5 M NaCl was added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at -20℃ in 50% isopropanol and Rnase A-treated for 1 h at 37℃. The DNA from 10^6 cells(15 μl) was equally loaded on each lane of 2% agarose gels in Tris-acetic acid/EDTA buffer containing 0.5 ㎍/ml ethidium bromide at 50 mA for 1.5 h.

6. Proteasome activity

After treatment with 50μM of CDCA, HS-1199, and HS-1200 for 5h, cells were lysed in proteasome buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol, and 4 mM dithiothreitol (DTT)], sonicated, and then centrifuged at 13,000 g at 4℃ for 10 min. The supernatant (20 ㎍ of protein) were incubated with proteasome activity buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 50 μM Suc-LLVY-AMC] for 1 h 37℃. The intensity of fluorescence of each solution was measured by a modular fluorimetric system (Spex Edison, NJ, USA) at 380 nm excitatory and 460 nm emission wavelengths. All readings were standardized using the fluorescence intensity of an equal volume of free AMC solution (50 μM).

7. Western blot analysis

Cells (2 x 10^6) treated with CDCA, HS-1199 and HS-1200 were washed twice with ice-cold PBS, resuspended in 200 ㎕ ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 ㎕/ml aprotinin and 2 ㎕/ml leupeptin] and incubated at 4℃ for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4℃. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA) and 50 ㎍ of proteins were loaded onto 7.5-15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP(Alpha Innotech, San Leandro, USA).

8. Immunofluorescent staining

Cells were cytocentrifuged and fixed for 10 min in 4% paraformaldehyde, incubated with each primary antibody for 1 h, washed 3 each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 hr at room temperature. Cells were mounted with PBS. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany).

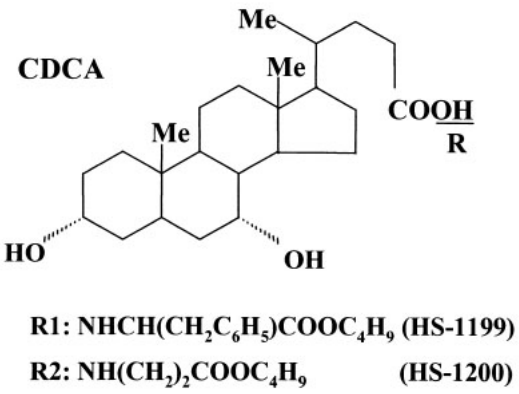

Fig. 1. Chemical structures of CDCA and its derivatives

9. Assay of mitochondrial membrane potential (MMP)

JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were resuspended in 10 ㎍/ml of methanol and incubated at 37℃ for 30 min. Flow cytometry to measure MMP was performed on a Epics XL (Beckman Coulter, FL, USA). Data were acquired and analyzed using EXPO32 ADC XL 4 color software. The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris.

Ⅲ. RESULTS

1. Synthetic CDCA derivatives reduced viability in YD9 cells.

As determined by MTT, HS-1200 at 50 μM for 5 h significantly reduced viability of YD9 cells compared to HS-1199. But CDCA at 50 μM did not

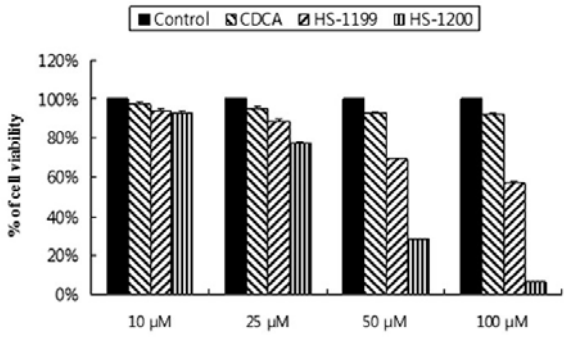

Fig. 2. YD9 cells treated with synthetic CDCA derivatives HS-1199 and HS-1200 at 10 μM, 25 μM, 50 μM and 100 μM for 5 h show the reduction of viability in a dose-dependent manner. Result is expressed as percentage of the vehicle-treated control ± SD of three separate experiments.

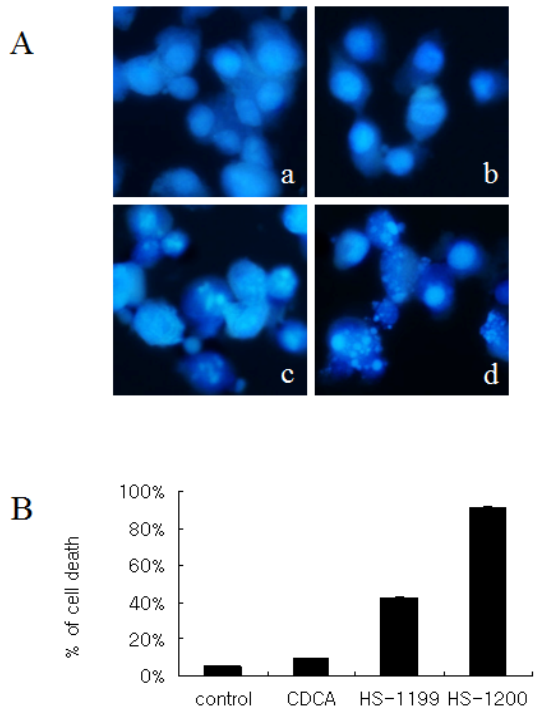

Fig. 3. YD9 cells treated with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM for 5 h show the nuclear condensation or fragmentation compared to the negative control or the CDCA-treated group. (A) Hoechst staining. (B) Quantification of the nuclear condensation determined by Hoechst staining. Result is expressed as percentage of the vehicle-treated control ± SD of three separate experiments.

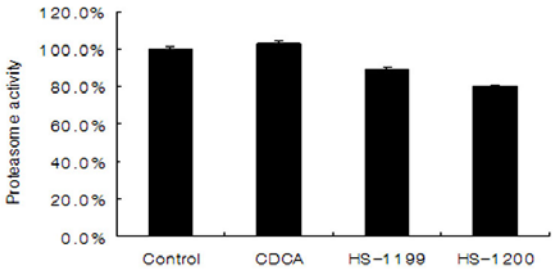

Fig. 4. OCS9 cells treated for 5 h with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM show the reduction of proteasome activity compared to the negative control or the CDCA treated group. Data are presented as the percent of the control.

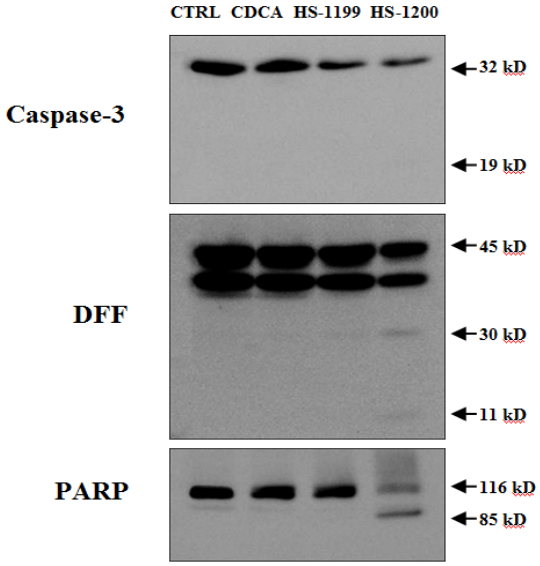

Fig. 5. YD9 cells treated for 5 h with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM produce nuclear events. Western blot analyses showing degradation of caspase-3, DFF and PARP. HS-1119 induced caspase-3 and DFF degradation. HS-1200 induced caspase-3, DFF and PARP degradtion, and produced 30 kD and 11 kd DFF cleavages and 85 kd PARP cleavage.

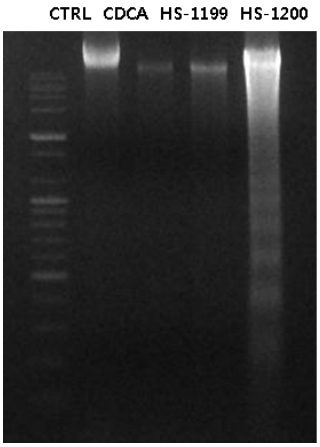

Fig. 6. DNA electrophoresis demonstration of YD9 cells treated for 5 h with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM. DNA electrophoresis evidently showed DNA ladder in YD9 cells treated with HS-1200.

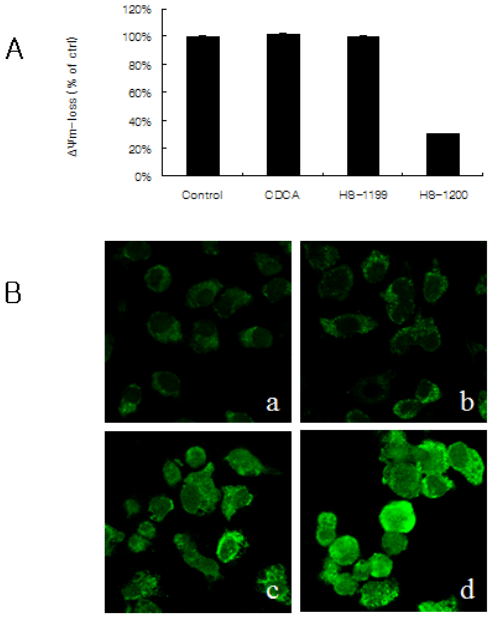

Fig. 7. YD9 cells treated for 5 h with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM induce mitochondrial events. (A) Loss of mitochondrial membrane potential (MMP) was significantly showed after treatment with HS-1200 but was not showed after treatment with CDCA and HS- 1119. (B) Confocal microscopy showing the release of cytochrome c from mitochondria into the cytosol after treatment with HS-1119 and HS-1200. a, control cells ; b, CDCA treated cells; c, HS-1119 treated cells; d, HS-1200 treated cells

Both synthetic CDCA derivatives decreased the viability of YD9 cells in a dose-dependent manner(Fig. 2).

2. Synthetic CDCA derivatives induced nuclear condensation and fragmentation in YD9 cells.

Hoechst staining proved that the reduction in viability resulted from apoptosis (Fig. 3A and 3B). HS-1200 at 50 μM showed stronger cytotoxic effect than HS-1199 at the same dose.

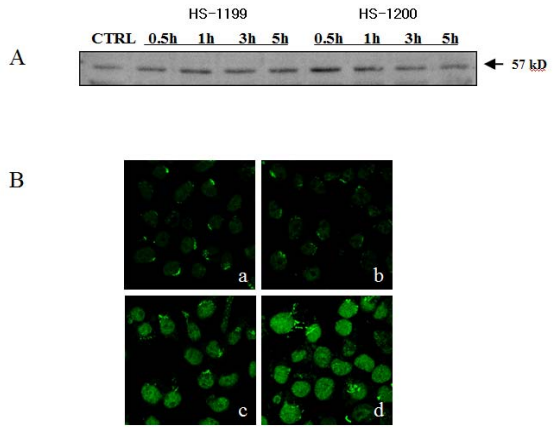

Fig. 8. AIF involves apoptosis in YD9 cells treated with synthetic CDCA derivatives HS-1199 and HS-1200 at 50 μM for 5 h. (A) Expression level of this protein slightly increased compared to the control. (B) Confocal microscopy showing that AIF was released from mitochondria, and that translocation onto nuclei was evident in HS-1119 and HS-1200 treated cells a, control cells ; b, CDCA treated cells; c, HS-1119 treated cells; d, HS-1200 trated cells

3. Synthetic CDCA derivatives inhibited proteasome activity in YD9 cells

Both synthetic CDCA derivatives caused decreases in proteasome activity of YD9 cells. HS-1200 showed stronger effect than HS-1199 at the same dose(Fig. 4).

4. Nuclear events were demonstrated in YD9 cells after synthetic CDCA derivatives treatment.

Western blot assay showed the activation of caspases-3, and degradation and cleavages of DFF and PARP in the treatment of HS-1200. In the treatment of HS-1199 at same dose, the degradation of caspase-3 and DFF showed whereas the PARP degadation and DFF cleavage production did not (Fig. 5). DNA electrophoresis showed a ladder

pattern of DNA fragments in the treatment of HS-1200 whereas did not show a ladder pattern of DNA fragments in the treatment of CDCA and HS-1199(Fig 6).

5. Synthetic CDCA derivatives induce apoptosis in YD9 cells via mitochondrial pathway.

Mitochondrial membrane potential(MMP) was remarkably reduced after treatment with HS-1200 but was not reduced after treatment with CDCA and HS-1119(Fig. 7*A*). Immunofluorescent study showed that synthetic CDCA derivatives led to the release of cytochrome c from mitochondria into the cytosol(Fig. 7B). Western blot assay and confocal microscopy were conducted to examine whether another mitochondrial apoptogenic factor AIF is involved or not. Expression level of this protein slightly increased after treatment of HS-1199 and -1200 (Fig. 8*A*). AIF was shown to release from mitochondria, and tranlocation onto nuclei was evident after treatment of HS-1199 and -1200 (Fig. 8B). These data support that HS-1199 and HS-1200 induce apoptosis via mitochondrial pathway in YD9 cells.

Ⅳ. DISCUSSION

Apoptosis is an evolutionarily conserved, innate process by which cells systemically inactivate, diassemble, 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.[15,16] 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.[15,16]

To date several genes involved in regulating apoptotic cell death have been identified. Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspase.[17] Among them caspase-3 has been studied the most intensively, which is activated proteolytically when cells are signalled to undergo apoptosis.[18] Several substrates of executive caspases have been demonstrated, including PARP. It is not known yet whether the cleavage of the substrates play a causal role in apoptosis.

A number of studies have been reported the antiproliferative efficacy of synthetic CDCA derivatives in various cancer cells by inducing apoptosis. Those studies demonstrated the decrease of proteasome activity, mitochondrial events, and nuclear condensation in synthetic CDCA derivatives induced apoptosis.[14,19-23]

Proteasome is a fundamental non-lysosomal tool that cells use to process or degrade a variety of short-lived proteins. Proteolysis mediated by the ubiquitin-proteasome system has been reported to be implicated in the regulation of apoptosis.[24] The proteasome pathway is mostly known to work upstream of the mitochondrial alterations and caspase activation[25] and can involve in different systems including NF-B, Bax and Bcl-2.[25-28] Proteasome inhibitors, as single or combined with other anticancer agents, are suggested as a new class of potential anticancer agents.[28-34] Also in previous studies a proteasome inhibitor, lactacystin augmented genistein-induced apoptosis of p815 mastocytoma cells.[35] HS-1200 not only produced decrease of proteasome activity, but also induced augmented apoptotic effect in the combination therapy of HS-1200 and lactacystin at low concentration.[20] In this study, synthetic CDCA derivative HS-1200 also produced the reduction of proteasome acitivity.

Mitochondria plays an important role in apoptosis, induction of the mitochondrial permeability transition play a key part in the regulation of apoptosis.[36-38] Permeabilization of the outer mitochondrial membrane (OMM) is modulated by members of the Bcl-2 family of proteins. Anti-apoptotic members, such as Bcl-2 and Bcl-

XL, inhibit protein release, whereas pro-apoptotic members, such as Bax and Bak, stimulate this release.[39] OMM becomes permeable to intermembrane space proteins such as cytochrome *c*[34] and AIF (apoptosis inducing factor) during apoptosis. Once released, cytochrome *c* promotes the activation of pro-caspase-9 directly within the apoptosome complex.[40] Cytochrome c release and disruption of MMP are in fact known features in apoptosis triggered by proteasome inhibition.[41,42] On induction of apoptosis, AIF translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation.[43] This study also showed that these representative mitochondrial events are involved in synthetic CDCA derivatives induced apoptosis of YD9 cells.

Common final event of apoptosis is nuclear condensation, and this event is controled by caspase, DFF, and PARP. DFF triggers both DNA fragmentation and chromatin condensation during apoptosis.[44] In our study cleavages or degradations of caspase-3, DFF, and PARP were shown in CDCA derivatives-treated YD9 cells.

In this study we analyzed whether a synthetic CDCA derivatives HS-1199 and HS-1200 have apoptotic effects on human oral sqauamous carcinoma cells. Conclusively, we demonstrated a synthetic CDCA derivative HS-1200 induced caspases-dependent apoptosis via mitochondrial pathway in human oral sqauamous carcinoma cells *in vitro*. But It remains an open question through which exact molecular mechanism synthetic CDCA derivatives exert anticancer activity. Furthermore, identification of the targets of synthetic CDCA derivatives in cancer cell apoptosis is needed. Future studies may provide important information for understanding the mechanism underlying synthetic CDCA derivatives induced apoptosis and their clinical application.

V. CONCLUSION

Bile acids and synthetic its derivatives induced apoptosis in various kinds of cancer cells and anticancer effects. Previous studies have been reported that the synthetic chenodeoxycholic acid (CDCA) derivatives showed apoptosis inducing activity on various cancer cells *in vitro*. It wasn't discovered those materials have apoptosis induced effects on YD9 human oral squamous carcinoma cells.

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국문요약

합성 Chenodeoxycholic Acid 유도체 HS-1200이 유도한 사람구강편평상피암종세포 세포자멸사 연구

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담즙산과 합성담즙산유도체가 여러 종류의 암세포에 세포자멸사(apoptosis)를 유도하고 항암효과가 있다고 알려져 있다. 합성 chenodeoxycholic acid (CDCA) 유도체가 여러 가지 암세포에 유도한 세포자멸사 *in vitro*

연구들이 보고되어져 왔다. 하지만 아직 까지 구강편평상피암종세포에 합성 CDCA 유도체가 유도한 세포자멸사 연구는 없었다. 그래서 본 연구는 합성 CDCA 유도체인 HS-1199와 HS-1200이 사람구강편평상피암종세포에 세포자멸사 효과와 세포자멸사 기작을 알기 위해서 수행되었다. 합성 CDCA 유도체로 처리된 사람구강편평상피암종세포(YD9 세포)에서 caspase-3의 활성화, DFF의 degradation, poly (ADP-ribose) polymerase(PARP)의 분절화(HS-1200 only), DNA 분절화(HS-1200 only), 핵 응축, proteosome 활성화의 저해, 사립체막전위(MMP)의 감소(HS-1200 only) 그리고 cytochrome c와 AIF의 사립체에서 세포질로의 유리와 같은 세포자멸사의 증거를 보였다. 그리고 두 개의 합성 CDCA 유도체 중에서 HS-1200이 HS-1199보다 더욱 더 강한 세포자멸사 효과를 보였다. 이 결과는 HS-1200이 YD9 세포에 항암효과를 가진다는 것을 증명한 것이다.

본 연구는 CDCA 유도체인 HS-1200이 사람구강편평상피암종세포에서 사립체 경로를 통한 caspase 의존적 세포자멸사를 강력하게 유도한다는 것을 증명했으며, 이러한 결과는 HS-1200이 사람구강편평상피암종의 치료적 전략으로서의 가능성이 높다고 생각한다.

주제어 : 세포자멸사, 합성 CDCA 유도체, HS-1200, 사람구강편평상피암종