Apoptotic Response of Human Oral Squamous Carcinoma Cells to Etoposide

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Anti-cancer drugs have been shown to target diverse cellular functions in mediation cell death in chemosensitive tumors. Most antineoplastic drugs used in chemotherapy of leukemias and solid tumors induce apoptosis in drug-sensitive target cells. However, the precise molecular requirements that are central for drug-induced cell death are largely unknown. Etoposide is used for the treatment of lung and testicular cancer. This study was performed to examine whether etoposide promote apoptosis in human oral squamous carcinoma cells (OSC9) as well as in lung and testicular cancer.

Etoposide had a significant dose- and time-dependent inhibitory effect on the viability of OSC9 cells. TUNEL assay showed the positive reaction on condensed nuclei. Hoechst stain demonstrated that etoposide induced a change in nuclear morphology. The expression of p53 was increased at 48 hour, suggesting that the nuclear of OSC9 cell was damaged, thereby inducing apoptosis. Etoposide treatment induced caspase-3 cleavage and activation. Intact PARP protein 116-kDa and 85-kDa cleaved product were observed. The activated caspase-3 led cleavage of the PARP. These results demonstrate that etoposide-induced apoptosis in OSC9 cells is associated with caspase-3 activation.

Key words : Apoptosis, etoposide, human oral squamous carcinoma cells

I. INTRODUCTION

Apoptosis or programmed cell death is an active process in cell auto-destruction which is essential for tissue homeostasis[1]. Diverse death signals activate different pathways that converge toward a conserved execution machinery composed of specific apoptotic proteases[2]. The hierarchical activation of these proteases provokes the cleavage of key nuclear and cytoplamic proteins as well as the activation of DNases, leading the nuclear and cytoplasmic lesions characteristic of apoptotic cell death[3].

Apoptotic nuclear modifications are characterized by a progressive condensation of chromatin at the periphery of the nucleus[1]. Condensed chromatin masses further bleb off the nuclear surface and ultimately disperse into the cytoplasm. In parallel with these morphological changes, chromatin is cleaved into nucleosomal and polynucleosomal fragments due to the activation of a specific nuclease[4,5].

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Multiple lines of evidence indicate that apoptosis can be triggered by the activation of caspases[6]. Among them caspase-3 has been studied the most intensively, which is activated proteolytically when cells are signalled to undergo apoptosis[7]. Several substrates of caspase-3 have been demonstrated, including poly (adenosine diphosphate ribose) polymerase (PARP) and DNA fragmentation factor (DFF). It is not known yet whether the cleavage of the substrates plays a causal role in apoptosis.

Cellular stresses that can induce p53-mediated apoptosis include DNA damage[8], deregulated oncogene expression[9], hypoxia[10], ribonucleotide depletion[11], and oxidative stress[12]. The most well-studied gene in terms of its impact on DNA damage-induced cell death is p53[13]. The p53 gene is one of the most frequently altered genes in a wide variety of tumor types, indicating its importance in cell growth control and tumorigenesis[14]. The loss of wild type p53 activity removes important controls on cell cycle regulation, apoptosis, and maintenance of genomic integrity in cultured cells[15], and contributes to tumor development[16]. Some targets of p53 appear to negatively regulate its function through regulation of the half-life of p53.

Chemotherapy and radiotherapy are ideal for killing malignant cells in cancer patients, because such therapies induce apoptosis of these cells[17], and because the therapy-induced apoptotic cells are believed to be safely scavenged by phagocytoic cells such as marcophages[18]. Apoptosis is regulated by a set of proteins including p53, whose mutation appears to explain much of the failure of such therapies[19].

Etoposide is one of the chemotherapeutic agents which are currently used in clinics, and is well known to inhibit topoisomerase II, thereby inducing apoptosis of a variety of cells[20, 21]. The drug is used for the treatment of lung and testicular cancer. This study was performed to examine whether etoposide has cytotoxic effects and promote apoptosis in human oral squamous carcinoma cells (OSC9) as well as in lung and testicular cancer.

II. MATERIALS AND METHODS

1. Cell culture

OSC9 cell line that established from oral squamous carcinoma patient was used in this experiment. The cells were cultured in Dulbecco's modified Eagles medium (DMEM/F12, 3:1) supplement with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 100 $\mu g/m\ell$ penicillin/streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2.

2. Antibodies

The following reagents were obtained commercialy: anti-human p53, anti-human caspase-3 and PARP antibodies were from Santa Crux Biotechnology (Santa Cruz, CA, USA) ; Peroxidase labelled anti-mouse antibody was from Amersham Biosciences (Buckinghamshire, UK)

3. Agents

Etoposide, dimethyl sulfoxide (DMSO), leupeptin, aprotinin, propidium iodide, phenyl-methylsulfonyl fluoride (PMSF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), hoechst 33342, Ponceau S were purchased from Sigma (St Louis, MO). ECL western blotting detection reagents and Hybond nitrocellulose membrane from Amersham Biosciences (Buckinghamshire, UK). TUNEL reaction mixture kit was purchased from Boehringer Mannheim (Mannheim, Germany).

4. Cell viability assay

The viability of cultured cells was estimated by MTT assay. In the MTT assay, cells were placed in a 96-well plate and incubated for 24 h. Cells were treated with different concentrations of etoposide for proper times. And then, the media was aspirated and replaced with 0.2 ml of MTT (1 mg/ml in PBS), followed by incubation at 37℃ for

4 h. MTT was then aspirated and the wells were air dried for 5 min. The crystal were dissolved with 200 $\mu\ell$ DMSO, until the solution turned purple and absorbance analyzed in an enzyme-linked immunosorbent assay (ELISA) plate reader (Quant, Bio-Tek, Highland Park, USA) at 540 nm. The assay was performed in triplicate.

5. Hoechst stain

Cells were washed with cold PBS and incubated with 4 μg/ml of hoechst 33258, a DNA-binding fluorescent dye, for 30 min at 37℃. And then fixed in 4% paraformaldehyde for 10 min. The morphological characteristics of apoptotic cells were identified with the aid of a fluorescent microscope (ECLIPSE E800, Nikon, Tokyo, Japan) with excitation at 540 nm. The cells with fragmented and/or condensed nuclei were classified as apoptotic cells.

6. TUNEL assay

To detect DNA breaks *in situ*, the TUNEL assay was employed with an TUNEL reaction mixture kit. After etoposide treatment, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, and applied permeabilisation solution for 2 min at 4℃, and washed again with PBS. This was followed by *in situ* end labeling according to the manufacturer′s instructions. Apoptotic cells were detected under a fluorescent microscope with excitation at 450 nm.

7. Western blot analysis

For protein analysis, cells were lysed with RIPA buffer (10 mM Tris/HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 1 h. Lysate were clarified by centrifugation at 12,000 revolution for 20 min at 4℃, and then the supernatant was obtained. The protein contents of the lysate were determined using the

Bio-Rad Protein Assay (Bio-Rad laboratoris Hercules, CA). The 30 μg protein was mixed with equal volume of electrophoresis buffer (10 nM Tris/HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating, the protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane. After transfer, the membranes were blocked with blocking reagent (5% non-fat milk in distilled water) for 1 h and then the membranes were incubated with primary antibody. The membranes were incubated for 1 h with the corresponding secondary antibody, diluted in the above blocking reagent. After three final washes, the membranes were treated with chemiluminescence reagent. All the procedures were done at room temperature.

III. RESULTS

1. The cytotoxic effect of etoposide on cell viability

The effect of the exposure to etoposide on the cell viability of OSC9 cells, as assessed by the MTT test, is shown in Fig. 1. After cells were incubated for 24 h in medium (control) with various concentrations of etoposide (0~100 μg/mℓ), the
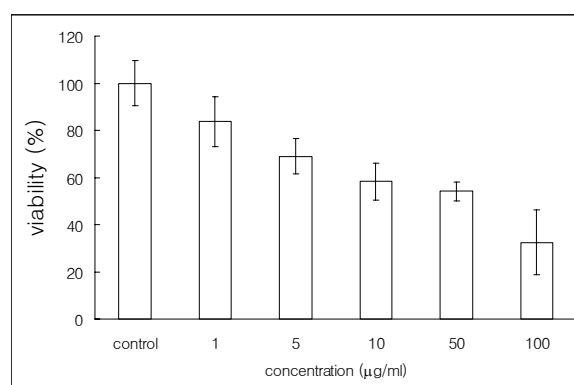

Fig. 1. Effect of etoposide on cytotoxicity in OSC9 cells. Cells were treated with various concentration of etoposide (0~100 μg/mℓ) for 24 h and the cells were tested for viability by MTT assay.
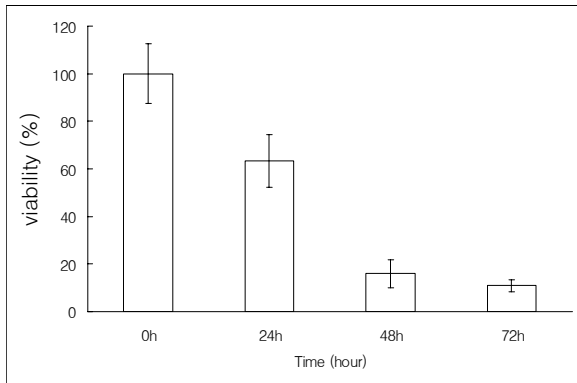
Fig. 2. Time course of 10 μg/mℓ etoposide-induced apoptosis of OSC9 cells. Cells were treated with 10 μg/mℓ etoposide for indicated time periods and then, cell numbers were measured by MTT assay.

Fig. 3. Immunofluorescent micrographs after Hoechst staining. Control cells showing typical round nuclei (a). Cells treated with 10 μg/mℓ etoposide show atypical fragmented nuclei (b).

number of cells was decreased according to increasing concentrations. The growth of OSC9 cells was inhibited in time-dependent manner by same concentration of etoposide (Fig. 2). At 10 μg/mℓ of etoposide, the viability dropped to 63.3%, 16% and 10.9% of the control on treatment times 24 h, 48 h and 72 h, respectively.

2. Morphological changes of cells treated with etoposide

The incubation of OSC9 cells with 10 μg/mℓ etoposide resulted in several morphological associated with apoptosis. Hoechst stain demonstrated that etoposide induced a change in nuclear morphology (Fig. 3). Compared to the typical round nuclei of the control cells, apoptotic OSC9 cells displayed condensed and fragmented nuclei. In other to confirm nuclear event on etoposide-induced apoptotic cells, TUNEL stain was conducted (Fig. 4). TUNEL stain showed that the percentage of cell death was increased compared to control cells and the positive reaction on condensed nuclei in the cells treated with etoposide.
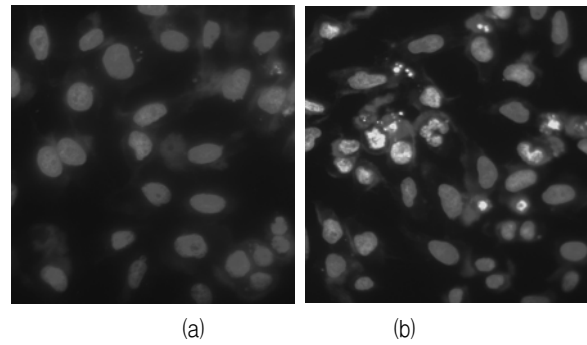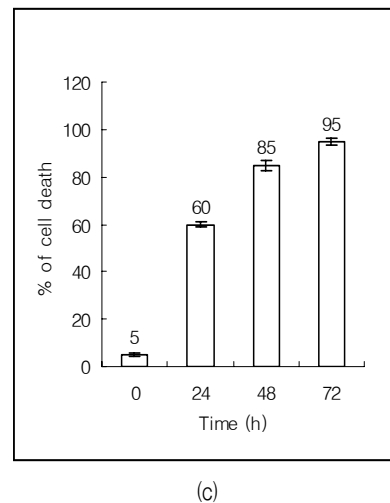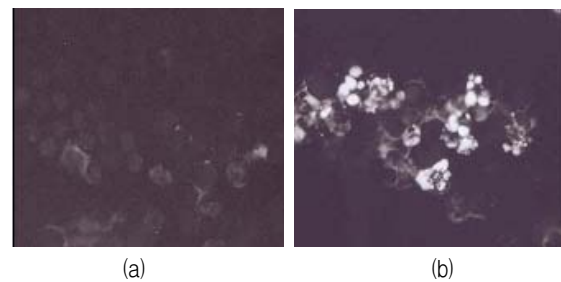
Fig. 4. TUNEL staining. OSC9 cells were stimulated with etoposide (10 μg/mℓ) for 24 h. Cells were fixed and analyzed by TUNEL staining. While control cells showing negative reaction (a), cells treated with etoposide show positive reaction (b). The time course quantification of etoposide-induced cell death of OSC9 cells is depicted (c).
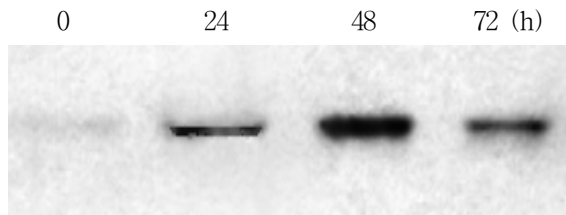
234

Fig. 5. The expression of p53 protein levels. Cells were treated with 10 $\mu g/m\ell$ etoposide for 24, 48 and 72 h.

3. Western blot analysis

To establish a relationship between the change in the various molecules and apoptotic pathways, we examined tumor suppressor gene p53 and other proteins such as caspase-3 and PARP by western blot analysis. In order to investigate the change in molecules expression according to time, OSC9 cell was treated with 10 $\mu g/m\ell$ etoposide and then incubated for 24 h, 48 h and 72 h. The expression of p53 protein was increased at 48 h in OSC9 cells (Fig. 5). We observed the proteolytic activation of procaspase-3 induced by etopside by Western blot (Fig. 6). 32 kDa precursor of caspase-3 was degraded, and 17 kDa cleavage products were produced. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Treatment of OSC9 cells with 10 $\mu g/m\ell$ etoposide induced proteolytic cleavage of 116 kDa PARP with accumulation of the 89 kDa cleaved products.

IV. DISCUSSION

Anti-cancer drugs have been shown to target diverse cellular functions in mediation cell death in chemosensitive tumors. Cytotoxic drugs which are currently used for the treatment of malignant diseases such as cytarabine, doxorubicin, methotrexate, etoposide and cisplatin are thought to exert their effects through inhibition of DNA-polymerase (cytarabine), DNA-intercalation (doxorubicin), antagonization folic acid (methotrexate), inhibition of topoisomerase II (etoposide) and DNA-crosslinking
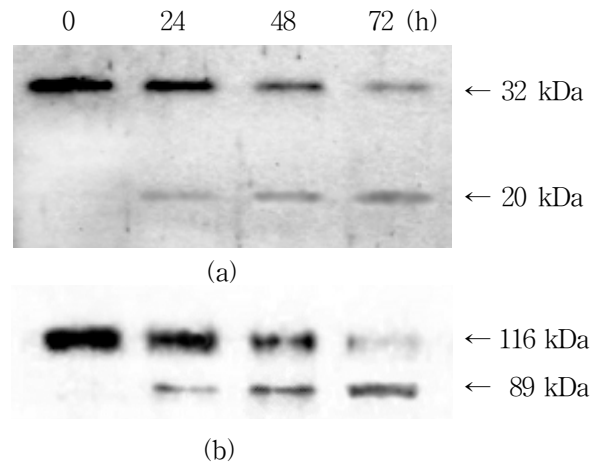

(a)

(b)

Fig. 6. Effect of etoposide on processing of procaspase-3 (a) and proteolytic cleavge of PARP (b) in OSC9 cells. Cells treated with 10 $\mu g/m\ell$ etoposide for 24, 48 and 72 h showed caspase-3 and PARP degradation.

(cisplatin)[22]. In addition, it has been recently shown that most antineoplastic drugs used in chemotherapy of leukemias and solid tumors induce apoptosis in drug-sensitive target cells.

Apoptosis is typical accompanied by the activation of a class of death proteases (caspase) and widespread biochemical and morphological changes to the cell[23]. These changes almost invariably involve chromatin condensation and its margination at the nuclear periphery, extensive double-stranded DNA fragmentation, and cellular shrinkage and blebbing[23]. However, apoptosis can also occur in the absence of DNA fragmentation[24]. It can be activated intracellulary through a genetically defined developmental program or extracellulary by endogenous proteins, cytokines and hormones, as well as drugs, xenobiotic compounds, radiation, oxidative stress, and hypoxia[25, 26].

This study focused on the etoposide-induced apoptosis in oral carcinoma cells as well as lung and testicular cancer. Etoposide-induced apoptosis variously confirmed by MTT assay, Hoechst stain, TUNEL stain and western blot analysis. Etoposide had a significant dose- and time-dependent inhibitory effect on the viability of OSC9 cells.

235

Hoechst stain demonstrated that etoposide induced a change in nuclear morphology and TUNEL assay showed the positive reaction on condensed nuclei in cells treated with etoposide.

Apoptosis has been shown to be a significant mode of cell death following cytotoxic drug treatment in a variety of tumour types. In this process, p53 plays a key role[26]. p53 is a regulatory protein that determines the cell regulation in response to DNA damage and other cellular stresses[27]. Under DNA damage and other stimulation the wild type p53 level rapidly increases in the cell and this increased level is required for the functioning of p53 as a guardian of the genome. In the present study, the expression of p53 was increased at 48 hour, suggesting that the nuclear of OSC9 cell was damaged, thereby inducing apoptosis p53-dependently.

Caspases are synthesized as inactive proenzymes that are processed in cells undergoing apoptosis of self-proteolytic cascade, capable of cleaving and activating specific substrates, including an enzyme involved in DNA repair and genomic maintenance, PARP and DFF, etc. PARP plays the active role of "nick senor" during DNA repair and apoptosis, when it synthesizes ADP-ribose from NAD+ in the presence of DNA strand breaks. Moreover, PARP becomes a target of apoptotic caspases, which originate two proteolytic fragments of 89 and 24 kDa[28]. Since the specific proteolytic cleavage of PARP is considered to be a biochemical characteristic of apoptosis. Figure 4B shows that PARP is cleaved into 89 kDa fragment suggesting that capase-3 is activated. In this study, etoposide treatment induced caspase-3 cleavage and activation. Intact 116-kDa and 89-kDa cleaved product were indicated. The data of Figure 4 give evidences that etoposide-induced cell death is apoptosis.

In summary, we present evidence that incubation of OSC9 cells with etoposide leads to an activation of the caspase-3 and indcution of apoptosis. However, based on presented evidence, it is impossible to differentiate direct epoposide action from indirection as a stage in caspase's cascade. Although more investigation is needed, etoposide can be considered as a good candidate with specific activity on oral squamous carcinom cells.

V. CONCLUSIONS

Etoposide showed apoptotic effect on OSC9 cells. The death of cells was further demonstrated to be due to apoptosis characterized by chromatin condensation and nuclear fragment by Hoechst staining and TUNEL method. Apoptosis-related factors were analyzed by Western blot. Etoposide-induced apoptosis in OSC9 cells is associated with caspase-3 activation.

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국문요약

Etoposide에 대한 사람구강편평상피암종세포의 세포자멸사 반응

부산대학교 치과대학 및 구강생물공학연구소 구강해부학교실[1], 구강보존과학교실[2], 구강내과학교실[3]

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항암제의 연구는 화학물질에 민감한 암세포를 죽음에 이르게 하는 세포자멸사와 같은 다양한 세포기능에 초점을 맞추어 왔다. 그러나 약물이 유도한 세포의 죽음에 있어서 핵심적인 분자적 기작은 아직 잘 이해되지 않고

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있다. Etoposide는 폐암과 고환암에 사용되는 항암제로서, 본 연구는 etoposide가 사람구강편평상피암종세포 (OSC9)에도 세포독성효과와 세포자멸사를 일으키는지를 알아보기 위해 실행하였다.

이 실험에서 etoposide는 농도와 시간 의존적으로 OSC9 세포의 생존율를 현저하게 저해시켰다. TUNEL 염색과 Hoechst 염색을 이용한 핵의 형태학적 관찰에서는 etoposide에 의해 핵이 응축되고 분절되었다. p53의 발현은 48 시간에 증가했으며, etoposide 처리로 인해 caspase-3의 활성을 관찰할 수 있었으며, 그 기질에 해당되는 PARP 단백질은 116-kDa과 89-kDa으로 분절되었다. 위의 결과들은 OSC9 세포에서 etoposide가 유도한 세포자멸사는 caspase-3의 활성과 관련됨을 설명하고 있다.

주제어 : Apoptosis, etoposide, human oral squamous carcinoma cells