사이클로스포린 A 투여 시 불멸화구강각화세포와 구강 편평세포암종 세포주에서 docetaxel 유도 아팝토시스 항진효과

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〈ABSTRACT〉

Cyclosporin A Enhance Docetaxel Induced Apoptosis in Immortalized Human Oral Keratinocyte and Human Oral Squamous Cell Carcinoma Cell Line

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Oral squamous cell carcinoma (SCC) is one of the leading causes of cancer mortality worldwide. It is generally thought that adjuvant chemotherapy provides modest prolongation of survival in various carcinoma. Docetaxel (Taxotere, TXT) play a significant role in the treatment of various solid tumors of epithelial origin. CsA (immunosuppressive drug) was widely used as adjunct for the treatment of cancer. Thus, it is important to pursue the apoptosis of IHOK and oral SCC induced by TXT combined with CsA related to the pathogenesis of oral SCC. But TXT combined CsA effect on IHOK and oral SCC remains unclear. After cultured IHOK and HN 22 oral squamous cell carcinoma cell line treated by 10 nM TXT and 1 μM, and caspase inhobitor, respectively, apoptosis index, cytochrome c and caspase-3, -8, -9 mRNA expression by RT-PCR, and procaspase-3 protein amount by immunoslot blotting was prepared. The purpose of this study were to examine the TXT-induced apoptosis pathway via caspase activation by CsA enhancement, and to apply these results to an effective therapeutic treatment plan for oral SCC by TXT combined CsA . 10 nM TXT showed about 60%, 55% cellular apoptosis of IHOK and HN 22, cell line, respectively, while CsA alone did not induce apoptosis in IHOK and HN 22 cell line, respectively, while CsA combined with 10 nM TXT showed increased apoptosis in IHOK and HN 22 cell line through caspase-3 and cytochrome c expression, while could not effect on caspase-8 and -9. Caspase inhibitor suppressed apoptosis of IHOK and HN 22 cell line induced by a combination of 1 μM CsA and 10 nM TXT. Immunoslot blotting showed procaspase-3 activation by a combination 1 μM CsA and 10 nM TXT, while caspase inhibitor inhibited activation. It suggested that a combination of CsA and TXT might induce increased apoptosis of IHOK and HN 22 oral squamous cell carcinoma cell line through caspase-3 activation. This treatment with a combination of TXT and CsA may be an effective therapeutic strategy for oral squamous cell carcinoma.

Key words : Apoptosis, Cyclosporin A, Docetaxel, Human Oral SCC Cell Line, IHOK

I. INTRODUCTION

Oral squamous cell carcinoma is one of the leading causes of cancer mortality worldwide. It is generally thought that adjuvant chemotherapy provides modest
prolongation of survival in some cases. Docetaxel is of the chemotherapy drug class: taxane, and is a semi-synthetic analogue of paclitaxel (Taxol), an extract from the rare Pacific yew tree Taxus brevifolia. Due to scarcity of paclitaxel, extensive research was carried out leading to the formulation of docetaxel (an esterified product of 10-deacetyl baccatin III) which is extracted from the renewable and readily available European yew tree. Docetaxel (TXT) is a unique anticancer agent that poisons mitotic spindles by stabilizing microtubules, thus inhibiting their depolymerization to free tubulin. TXT, as well as other types of anticancer drugs, induces caspase activation in various carcinoma cells and oral squamous cell carcinoma (SCC).

Cyclosporine A (CsA) is a highly lipophilic cyclic undeca peptide, produced by the fungus Trichoderma polysporum (Link ex Pers.) Rifai, Cylindrocarpon lucidum Booth, and Tolypocladium inflatum Gams. CsA is a clinically important immunosuppressive drug that is widely used to prevent graft rejection after organ or bone marrow transplantation.

It is known to possess a variety of biologic and physiologic actions such as antiparasitic, fungicidal, anti-inflammatory effects, immuno-suppressive properties, and a cure for several cancer cells.

Since then it is interesting to focus on the combined drugs affecting oral squamous cell carcinoma. It has been widely accepted that IHOK could be an intermediate stage to squamous cell carcinoma in vitro. Thus it is important to examine the biological basis for the observed effects of TXT combined CsA on IHOK and oral SCC. But there was not published reports on the direct effects of TXT combined CsA on IHOK and oral SCC in culture.

The purpose of this study were to examine the TXT-induced apoptosis pathway via caspase activation by CsA enhancement, and to apply these results to an effective therapeutic treatment plan for oral SCC by TXT combined CsA.

II. MATERIALS and METHODS

1. Cell Culture Condition

IHOK and HN 22 cells were incubated at 37°C under 5% CO₂, trypsinized with trypsin-EDTA, and subcultured in KBM bullet kit (Clonitecs, USA). Then the cells were seeded onto a 24 well plate (6 × 10³ cell per well). The cultures were fed every 3 to 4 days with fresh culture medium until the cultures become almost confluent under 0.15 mM Ca++. Immortalized human oral keratinocyte (IHOK) and HN 22 cells (oral squamous cell carcinoma cell line) was cultured under the same condition. CsA (Sandimmun, Japan) was diluted in absolute ethanol (stock solution, 50 mg/ml), which was used as 1, 5, and 50 μM. TXT (Aventis Pharma Ltd., Japan) was used 5, 10, and 100 nM.

2. Apoptosis Index

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis–benzimide stain (Hoechst 33342; Molecular Probes, Inc. USA). Briefly, IHOK and HN 22 cells (1 × 10⁴ cells/well) were grown in flat–bottomed 96–well plates and cultured in the presence or absence of 10 nM TXT with 1 μM CsA for 3–24 hrs at 37°C and then stained with Hoechst 33342. Cells with apoptotic chromatin changes were counted with a fluorescence microscope. Cells with condensed or fragmented nuclei were considered apoptotic. Apoptosis index (%) was calculated as apoptotic cells/all cells under the mean number of apoptotic nuclei in three fields (× 200).
3. Caspase Inhibitor

Caspase inhibitor zVAD–FMK (Enzyme Systems Products, USA) dissolved in DMSO at a stock concentration of 10 mM, 1 × 10^4 HN 22 cells were grown in 96-well plates and treated at 37°C for 18 hrs with a mixture of CsA and TXT with or without zVAD–FMK (10 μM).

4. Total RNA Extraction and RT–PCR Analysis

To demonstrate whether mRNA expression is consistent with the protein level detected in IHOK and HN 22 cell grown in KBM bullet kit were harvested and washed in cold PBS.

The Guanidinium thiocyanate method was used to isolate total RNA. Precipitated specimen was dissolved in DEPC solution, spectrophotometer (Simatzu, Japan) was used at 260 nm to measure the amount of RNA. After dispensing, it was kept at -70°C Deep freezer (Mybio, Japan) to be used in the next experiment. To confirm total RNA, 1% agarose gel made with 10 × 3–(N–morpaholino) propane sulfonic acid (MOPS), 50 mM sodium acetate, 0,5 M EDTA (pH 8,0), 10 N NaOH (pH 7,0) solution were used, 3 μl RNA, 1,25 μl 10x MOPS, 2 μl formaldehyde and 6,25 μl formamide were mixed together, heated for 5 minutes, and cooled down in ice. It was mixed with 1,25 μl gel RNA loading solution, and 1xMOPS was used as the buffer solution. At 120 V/cm, electrophoresis was done until the blue color of bromophenol blue reached 2/3 of the total gel.

5. RT–PCR reaction for cDNA

The reverse transcription reaction of the RNA was performed 2 hrs at 42°C using AMV (avian myeloblastosis virus), and 1,0 μl of this was used for temp–plates of the all PCR, The PCR condition was 2 min at 95°C (one cycle for denaturation), each 30 second at 95°C, 55°C, and 72°C (25 cycles) and 10 min at 72°C (one cycle) with 32P–dCTP labeling using PCR machine. To detect Caspase–9 sense (GAGGGAGTCAGGCTCTTCC) and antisense (TCACCA AATCCCTCCAGAAC), Caspase–3 sense (TTTTTCAGAGGGGATCGTTG) and antisense (CGGCTCCACTGGGTATTT), Caspase–8 sense (TCGAGGGTCTCAGCTGTGTTG) and antisense (CA AAAATCAGCCCATGTGG), cytochrome c sense (TGTGCCAGCGACTAA AAAAGA and antisense (ATGCGCCGCTTGGGTAGAGG)), Human β–actin as positive control sense (ATCCTCCACAOAACCTGGTCGAGCTCCT), and antisense (CGCTCATTCTCCTGTGCTGTGGATACCATOGG) were used as positive control for RT–PCR reactions. Each mRNA level was divided and calculated by mRNA level of Human β–actin. Data was representative of three experiments. Caspase and cytochrome c gene expression levels were evaluated semi–quantitatively.

6. Immunoslot Blotting

The cultured cells under KBM bullet kit were harvested with 0,1 M Tris–acetate (pH 7,5), 1 mM EDTA, protease inhibitors. Protein amount of those extracts was quantified at 495 nm with spectrophotometer, serially diluted in the range of 10 μg to 1 μg, and applied into slot chambers containing nitrocellulose membrane, Slot chambers were twice washed with TBS. The membrane was processed as the following western blotting methods. The membrane was treated with blocking serum, incubated with primary antibodies and secondary antibodies, The antibodies for the antigens were purchased from the following sources: actin (clone AC–15, Sigma), and procaspase–3 (Santacruz, USA). This was developed by an enhanced chemino luscence (ECL) method, and examined by
densitometer in triplicate. The protein concentrations were determined by Bio–Rad Protein Assay.

III. RESULTS

Effect of TXT combined with CsA on apoptosis in oral squamous cell carcinoma cells was analyzed. TXT induced apoptosis in IHOK and HN 22 cells in a dose–dependent manner and CsA alone did not induce apoptosis in this study (Fig. 1, 2). When 1 μM CsA was combined with 5 or 10 nM TXT, apoptosis in HN 22 cells was induced to a greater extent than that seen with TXT only, and it was increased in a time–dependent manner (Fig. 3). And also a combination of 5 μM CsA plus 10 nM TXT induced higher detectable apoptosis in IHOK. Although HN 22 cells showed resistance to 1 μM TXT, the presence of 5 μM CsA significantly increased 10 nM TXT–induced apoptosis.

zVAD–FMK (a broad–range caspase inhibitor) was applied to determine whether the activation of caspase is essential for nuclear fragmentation of IHOK and HN 22 cells treated with a combination of TXT and CsA. Apoptosis induced by a combination of TXT and CsA was blocked completely by 10 μM zVAD–FMK in HN 22 cells (Fig. 4).

We then investigated whether caspase–3 is activated by exposure to TXT alone or only by a combination of TXT plus CsA in IHOK and HN 22 cells (Fig. 5a). Activation of caspase–3 by TXT and CsA in combination was seen. Activation of caspase–3 was determined by decreased expression of procaspase–3 mRNA expression in IHOK & HN 22.
based on Immunoblot blot analysis (Fig. 6a). The density of the procaspase-3 band was reduced more by exposure to CsA plus TXT by exposure to caspase inhibitor (Fig. 6a, b). Caspase-8 mRNA expression was not detected in IHOK and HN 22 cells treated with TXT + CsA or TXT + CsA or TXT + CsA + caspase inhibitor, while caspase-9 expression and cytochrome c was slightly increased (Fig. 5a).

IV. DISCUSSIONS

Docetaxel differs from paclitaxel at two positions in its chemical structure. It has a hydroxyl functional group on carbon 10, whereas paclitaxel has an acetate ester and a tert-butyl substitution exists on the phenylpropionate side chain. The carbon 10 functional group change causes docetaxel to be more water soluble than paclitaxel \((20)\). We sought to determine whether cyclosporin A (CsA) enhances docetaxel (Taxotere, TXT)–induced apoptosis in IHOK and human oral squamous cell carcinoma cell line, and, if so, to determine the relation between this apoptosis and caspase activation. Apoptotic cell death was verified morphologically by nuclear fragmentation assay with Hoechst staining. It was reported that a combination of CsA and TXT significantly enhanced apoptotic cell death in the carcinoma cells and cell lines through cytochrome c. It was thought that CsA could enhance TXT–induced apoptosis in cancer cells using several gastric carcinoma cell lines and freshly isolated gastric carcinoma cells \((20)\), and oral squamous cells \((9–11)\). It was demonstrated that CsA enhanced TXT–induced apoptosis in cancer cells but not in normal HUVECs cells due to the inhibition of NF-κB, an antiapoptotic transcription factor. In vivo model, it showed that a combination of CsA and TXT was more effective in the inhibition of tumor development than each agent alone in mouse peritoneal tumor models \((21)\). In this study a combination of CsA plus TXT induced marked apoptosis in HN 22 cells, even when a low concentration of TXT was used although in the HN 30 and HN 12 xenograft models, supradose levels of docetaxel produced distinct protein expression patterns for regulators of the cell cycle (cyclins A and B, p21, and p27), apoptosis (cleaved caspase-3 and cleaved PARP) \((9)\). It was suspected that CsA blocks the development of resistance to apoptosis induced by chemotherapeutic agents \((21)\). It has been shown that this enhance the anticancer effects of chemo-therapeutic agents \((21)\), and it has been reported that TXT induced caspase-3 activation in several types of malignant cells. When the human oral SCC cell line HSC–3 was exposed to docetaxel, activation of caspase cas-
caspase-3, -8, and -9). This data also showed that TXT induced only caspase-3 activation at mRNA level and procaspase-3 protein activation in IHOK and HN 22 cells. From these observations, it suggested that CsA enhances TXT-induced apoptosis by activating TXT-induced activation of caspase-3. Therefore, caspase-3 activation could be associated with CsA enhancement of TXT-induced apoptosis. In this study, however, TXT did not induce expression of caspase-8 in IHOK and HN 22 cells at the mRNA level. Collectively, these results suggested that CsA enhanced TXT-induced apoptosis mainly through caspase-3 dependent apoptosis pathway in IHOK and HN 22 cells.

Although enhanced apoptosis induced by a combination of CsA and TXT was blocked completely by caspase inhibitor (zVAD-FMK), apoptosis induced by TXT alone was not affected. These findings lead us to propose a mechanism for CsA/TXT-induced apoptosis. It was reported that the pan-caspase inhibitor z-VAD-fmk prevented apoptosis induced by docetaxel showing participation of caspases in this process8). When tumor cells are exposed to TXT alone, caspase-dependent and -independent apoptosis pathways and a CsA-sensitive antiapoptosis pathway are activated simultaneously22). Because the CsA-sensitive antiapoptosis pathway inhibits the caspase-dependent apoptosis pathway, apoptosis is induced through the caspase independent apoptosis pathway23). When CsA is combined with TXT, the CsA-sensitive antiapoptosis pathway is blocked. As a result, TXT combined CsA might induce a high level of apoptosis through both the caspase-independent and -dependent pathways. However, the identity of the antiapoptosis substances acting in the CsA sensitive antiapoptosis pathway is unknown. In vivo experiments, although it was reported that Docetaxel inhibited the expression of growth factors and receptors in tumor cells using the HN6, HN12, and HN30 HNSCC xenograft model10). Treatment with a combination of CsA and TXT caused a larger antitumor effect than treatment with CsA or TXT alone21). CsA enhanced TXT-induced apoptosis in tumor formation of nude mouse through tumor cell inhibition by TXT-induced caspase-3 activation22). Additional studies will be needed to clarify the in vivo effect of the combination. It suggested that treatment with a combination of TXT and CsA would be a useful therapeutic strategy for oral cancer patients, especially for oral squamous cell carcinoma.

V. REFERENCES
