Suberoylanilide hydroxamic acid sensitizes human oral cancer cells to TRAIL-induced apoptosis through increase DR5 expression

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Introduction

Squamous cell carcinoma of the oral cavity is the leading cause of cancer-related deaths in India and South Asian countries (1). Although treatment of early stage disease (stage I or II) is frequently successful, disease relapses still occur in about 20% to 30% patients, particularly local tumor or lymph node recurrence (2, 3). For patients with advanced disease (stage III or IV), standard therapy is far less successful. Fewer than 30% of them can be cured. Despite recent advances in surgery, radiotherapy, and chemotherapy, the overall 5-year survival rate for patients with late-stage oral squamous cell carcinoma is among the lowest of the major cancers and has not changed during the past two decades (4, 5). As standard treatments have not cured this disease, continued investigation of new chemotherapeutic agents is thus needed.

Most chemotherapeutic agents exert their cytotoxicity through the induction of an apoptotic response, and the efficiency of antitumor agents is related to the intrinsic property of the target tumor cells to respond to these agents by apoptosis (6). Initiation of apoptosis has been broadly separated into two major pathways, namely, the death receptor pathway and the mitochondria pathway (7). The death receptor pathway is mediated by interaction of death receptors (such as Fas, DR4, and DR5) with their cognate ligands [such as Fas ligand and tumor necrosis factor-related apoptosis–inducing ligand (TRAIL)], leading to recruitment of the Fas-associated death domain and caspase-8 activation. In type I cells, caspase-8 directly activates effector caspases (caspase-3, caspase-6, caspase-7), whereas in type II cells, caspase-8 cleaves Bid protein to t-Bid, which cause the release of cytochrome c in the mitochondria pathway. In the mitochondria pathway, diverse stimuli converge at mitochondria and cause cytochrome c release to cytoplasm, formation of a cytochrome c/Apaf-1/procaspase-9 complex, and activation of caspase-9, and the latter activates effector caspases, resulting in apoptosis.

Death ligands harbor potential as cancer-therapeutic agents because they can trigger apoptosis in many types of tumor cells (7, 8). However, some death ligands (e.g., Fas ligand, tumor necrosis factor-α) are of limited therapeutic use because of their severe toxicity toward normal tissues (7). Unlike Fas ligand and tumor necrosis factor-α, i.v. administration of nontagged TRAIL was well tolerated in two relevant nonhuman primate models (cynomolgus monkeys and chimpanzees) with no harmful symptoms even when doses up to 10 mg/kg/d were applied (8). Injection of TRAIL into athymic severe combined immunodeficient mice challenged with human mammary adenocarcinoma–, colon carcinoma–, or cholangiocarcinoma–induced tumor cell
apoptosis decreased the size of established tumors and improved survival substantially (7, 9). Such properties make TRAIL a promising candidate for cancer therapy. However, response to TRAIL is highly variable with resistance seen in many cancer types, and most of oral cancer cell lines are resistant to the TRAIL-induced cytotoxicity (10), suggesting that TRAIL alone may be ineffective for oral cancer therapy. Therefore, new strategies are necessary to overcome TRAIL resistance in oral cancer cells.

Recently, histone deacetylase inhibitors (HDACi) have been reported to selectively induce tumor apoptosis and enhance the sensitivity to other treatment regimens, such as radiation, etoposide, or TRAIL, in many cancer types (11). They are about as a promising new class of chemotherapeutic agents. We have recently found HDAC2 overexpression in 86% of oral squamous cell carcinoma cases (12), providing a good reason for treating oral squamous cell carcinoma with HDACis. One of the HDACis, suberoylanilide hydroxamic acid, is an orally administered HDACi that has been shown to induce apoptosis in a variety of tumor cells, but the signaling pathways involved seems to be dependent on cell type and context (13–19). It is now in phase I and II clinical trials for the treatment of various malignancies and has shown anticancer effects at doses that are tolerated by the patients (11). With respect to oral cancer, suberoylanilide hydroxamic acid has been reported to enhance the cisplatin induced apoptosis (20); however, the molecular mechanisms by which suberoylanilide hydroxamic acid induce apoptosis have not been fully investigated in oral cancer cells. A full understanding of the mechanism underlying suberoylanilide hydroxamic acid–induced apoptosis may help to design a more effective strategy to treat oral cancer and prolong life. In this report, we pursued a more detailed investigation of the mechanism of suberoylanilide hydroxamic acid–induced apoptosis and evaluated the ability of suberoylanilide hydroxamic acid to sensitize TRAIL-induced apoptosis in TRAIL-resistant oral cancer cells.

Figure 1. Effects of suberoylanilide hydroxamic acid treatment on human oral cancer cells and normal human oral keratinocytes. A, cells (1 × 10⁴) were treated with increasing concentrations of suberoylanilide hydroxamic acid (0–5 μmol/L) for 24 and 48 h. Viable cells were measured by MTT assay and expressed as a percentage of control (n = 9). All values are means of three independent experiments ± SE (bars). B, cell cycle analysis of SAS and Ca9-22 cells treated with suberoylanilide hydroxamic acid. Representative histograms of the DNA content in untreated cells and cells treated with 3 μmol/L suberoylanilide hydroxamic acid for 24 h. The percentages of sub-G1 populations increased in cells treated with 3 μmol/L suberoylanilide hydroxamic acid after 24 h of incubation. All values are mean ± SE (bars) of three independent experiments carried out in duplicate. C, TUNEL staining analysis of SAS cells treated with 3 μmol/L suberoylanilide hydroxamic acid for 24 h and visualized by fluorescence microscopy after staining with propidium iodide or TUNEL. Note the apoptotic cells with nuclear condensation and positive TUNEL signal compared with control cells. Right, percentages of cells undergoing apoptosis observed by TUNEL staining. Results are the means of three independent experiments. Values are mean ± SE. *, *P < 0.05 versus control.
Materials and Methods

Cell Culture and Materials

Human oral squamous cell carcinoma cell line SAS and Ca9-22 resistant to TRAIL-induced apoptosis were obtained from the Japanese Collection of Research Bioresources. Ca9-22 cells are known to have mutant \( p53 \) (21). SAS cells have a point mutation in one \( p53 \) allele, but it has been shown that they have an ability to induce \( p53 \)-dependent signal transduction (22). Normal human oral keratinocytes were gifts from Prof. Lin Shu-Chun at the National Yang-Ming University. Suberoylanilide hydroxamic acid (Alexis) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.01% in media without causing cytotoxicity. Human recombinant DR4/Fc, DR5/Fc, and Fas/Fc chimera protein were purchased from R&D Systems. Caspase inhibitors Z-IETD-FMK and Z-LEHD-FMK were from Calbiochem. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit was from Boehringer Mannheim. All tissue culture biologicals were obtained from Invitrogen.

Cell Viability (MTT) Assay

Cell viability was measured using the MTT-based cytotoxicity assay (Sigma), as previously described (23). Briefly, after seeding and overnight incubation, cells were treated with various concentrations of suberoylanilide hydroxamic acid for 24 or 48 h. On termination, the medium was replaced with fresh medium containing 0.5 mg/mL MTT. After incubation for 4 h at 37°C, medium and MTT were removed, and the MTT-formazan products were extracted with DMSO. The absorbance was read at 570 nm using an enzyme-linked immunosorbent array plate reader. Each data point is the average of results from nine wells.

Cell Cycle Analysis

Cell cycle analysis with flow cytometry was done as previously described (23). Briefly, after seeding and overnight incubation, SAS and Ca9-22 were treated with 3 \( \mu \)mol/L suberoylanilide hydroxamic acid. At the indicated time interval, cells were harvested by treatment with trypsin, washed in PBS, and fixed in ice-cold methanol/PBS (2:1 ratio) for storage at \(-80^\circ\)C until further analysis. Fixed cells were stained with propidium iodide (50 \( \mu \)g/mL), and their DNA content was analyzed with a Fluorescence Activated Cell Sorter (Becton Dickinson). The DNA histograms enable the calculation of percentages of cells in sub-G0, G0/G1, S, and G2/M phase following treatment.

TUNEL Assay

Suberoylanilide hydroxamic acid–treated and untreated cells were harvested, washed with PBS (pH 7.0), fixed with 2% paraformaldehyde, and subjected to TUNEL assay using the terminal incorporation of fluorescein-12-dUTP by terminal deoxynucleotidyl transferase into fragmented DNA in oral cancer cells, according to the manufacturer’s instructions as previously described (23, 24). The samples were washed and analyzed using fluorescence microscopy or Fluorescence Activated Cell Sorter. The cells that expressed FITC fluorescence were counted as apoptosis.

Immunoblotting

After treatment with suberoylanilide hydroxamic acid, the floating and adherent cells were harvested, sonicated, and lysed in lysate buffer, as previously described (23, 24). Equivalent amounts of protein (30 \( \mu \)g) were loaded onto a 10% SDS-PAGE, separated by electrophoresis, and electrotransferred onto a polyvinylidene difluoride membrane.
(Millipore). The membrane was then incubated with one of the following primary antibodies: Bcl-2 (1:1,000), Bcl-XL (1:1,000), Fas ligand (1:1,000), TRAIL (1:500), α-tubulin (1:10,000; Santa Cruz), Bax (1:250), caspase-8 (1:1,000), cytochrome c (1:1,000), Fas (1:250; BD Biosciences), t-Bid (1:1,000), caspase-9 (1:1,000), poly(ADP-ribose) polymerase (1:1,000; Cell Signaling Technology), β-actin (1:10,000), DR4 (1:500), and DR5 (1:500; Sigma). Primary antibodies were detected with goat anti-mouse or anti-rabbit horseradish peroxidase–linked secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories). After washing, the Western Lighting Chemiluminescence Reagent (Perkin Elmer) was added and then exposed with Kodak-X-Omat film.

**Statistical Analysis**

Group data are expressed as mean ± SEM. The unpaired Student’s *t* test (for control and study group comparisons) was applied to compare group differences. *P* < 0.05 was considered statistically significant.

**Results**

**Suberoylanilide Hydroxamic Acid Inhibits the Growth of Human Oral Squamous Cell Carcinoma Cells**

Effects of various concentrations of suberoylanilide hydroxamic acid (0.5–5 μmol/L) were examined by MTT assay in SAS, Ca9-22, and normal human oral keratinocytes. Figure 1A shows suberoylanilide hydroxamic acid strongly inhibited growth of SAS and Ca9-22 cells in a dose-dependent manner. The 50% inhibitory concentrations for SAS and Ca9-22 were 3 and 1.5 μmol/L, respectively, at 24 hours, and 1.5 and 0.75 μmol/L, respectively, at 48 hours. Conversely, no antiproliferative effect was observed on normal human oral keratinocyte after a 5 μmol/L suberoylanilide hydroxamic acid treatment for 48 hours. These results indicate that suberoylanilide hydroxamic acid potently and selectively inhibits the growth of oral squamous cell carcinoma cells.

**Suberoylanilide Hydroxamic Acid Induces Apoptosis in Oral Cancer Cells**

To examine the effect of suberoylanilide hydroxamic acid on cell cycle progression, SAS and Ca9-22 cells were treated with 3 μmol/L suberoylanilide hydroxamic acid for 0, 12, and 24 hours. The cell cycle phase distribution was then analyzed by flow cytometry (Fig. 1B). Treatment with 3 μmol/L suberoylanilide hydroxamic acid did not significantly change the pattern of cell cycle distribution. However, the number of cells having a hypodiploid DNA content (sub-G1 population) gradually increased. The percentage of accumulated sub-G1 apoptotic cells for SAS and Ca9-22 after 24 hours exposure to 3 μmol/L suberoylanilide hydroxamic acid was 25.8% ± 3.2% and 35.7 ± 2.5%, respectively. These results suggested that suberoylanilide hydroxamic acid–treated cells died by apoptosis rather than by necrosis.

To further investigate whether suberoylanilide hydroxamic acid–induced cell death in SAS cells was caused by apoptosis, TUNEL analysis of apoptotic DNA fragments was done. Cells positive for TUNEL staining were found to have condensed and/or fragmented nuclei (Fig. 1C). Approximately 28.8% of the cells were apoptotic by 24 hours after 3 μmol/L suberoylanilide hydroxamic acid treatment.

**Suberoylanilide Hydroxamic Acid Induces Apoptosis through Activation of DR5 Death Pathway**

To understand the mechanisms of suberoylanilide hydroxamic acid–induced apoptosis in oral cancer cells, we first investigated the levels of death receptor–related proteins in SAS cells after suberoylanilide hydroxamic acid treatment using Western blotting. As shown in Fig. 2A, treatment of SAS cells with 3 μmol/L suberoylanilide hydroxamic acid markedly increased Fas, Fas ligand, DR4, and DR5 protein expression. Furthermore, caspase-8 was found to cleave into the characteristic active fragments after suberoylanilide hydroxamic acid treatment.

We next investigated the role of mitochondria pathway in suberoylanilide hydroxamic acid–induced apoptosis. Figure 2B shows treatment of SAS cells with 3 μmol/L...
suberoylanilide hydroxamic acid caused cytochrome c to be released into the cytosol. Furthermore, caspase-9 was found to cleave into the characteristic active fragments after suberoylanilide hydroxamic acid treatment. The cytochrome c–initiated apoptotic pathway has been shown to regulate by the balance between proapoptotic and prosurvival Bcl-2 family proteins. Therefore, the influence of suberoylanilide hydroxamic acid on proteins of this family was examined (Fig. 2B). Suberoylanilide hydroxamic acid induced a down-regulation of Bcl-XL and an up-regulation of Bcl-2 in SAS cells after treatment. The expression of Bax was not changed. In addition, suberoylanilide hydroxamic acid treatment caused Bid cleavage into t-Bid in SAS cells after 6-hour treatment.

We further used caspase inhibitors, Fas/Fc, DR4/Fc, and DR5/Fc chimera proteins to study the mechanisms of suberoylanilide hydroxamic acid–induced apoptosis in more detail. Figure 3 shows suberoylanilide hydroxamic acid–induced apoptosis was almost completely inhibited by caspase-8–specific inhibitor Z-IETD-FMK and was slightly attenuated by caspase-9–specific inhibitor Z-LEHD-FMK. Blocking the interaction of Fas/Fas ligand or DR4/TRAIL by pretreatment with the Fas/Fc or DR4/Fc chimera protein failed to alter the suberoylanilide hydroxamic acid–induced apoptosis. However, blocking the interaction of DR5/TRAIL by DR5/Fc chimera protein significantly inhibited suberoylanilide hydroxamic acid–induced apoptosis. These results suggested caspase-8 is the initiator caspase in suberoylanilide hydroxamic acid–induced apoptosis and the DR5 pathway may be the major mechanism for the proapoptotic effects of suberoylanilide hydroxamic acid, at least in SAS cells.

Suberoylanilide Hydroxamic Acid Enhanced TRAIL-Induced Apoptosis

The ligand for DR5 is TRAIL. Chemotherapeutic agents that induce DR4 or DR5 expression usually enhance TRAIL-induced apoptosis. We hypothesized that suberoylanilide hydroxamic acid can enhance exogenous TRAIL-induced apoptosis through its ability to increase DR5 expression. Thus, we first tested the effect of suberoylanilide hydroxamic acid on DR5 expression in another oral squamous cell carcinoma cell line, Ca9-22. As shown in Fig. 4, suberoylanilide hydroxamic acid also induced DR5 protein expression in Ca9-22 cells. We then examined the combinatorial effect of suberoylanilide hydroxamic acid on DR5 expression in another oral squamous cell carcinoma cell line, Ca9-22. As shown in Fig. 4, suberoylanilide hydroxamic acid alone did not induce apoptosis at these concentrations. However, concurrent treatment with 1 μmol/L suberoylanilide hydroxamic acid and 20 ng/mL TRAIL dramatically induced apoptosis in both cell lines. These results indicated that suberoylanilide hydroxamic acid strongly sensitized exogenous recombinant TRAIL-induced apoptosis in a synergistic manner.

The Apoptosis Induced by Cotreatment with Suberoylanilide Hydroxamic Acid and TRAIL Is Blocked by DR5/Fc Chimeric Protein

To confirm that the induction of apoptosis by the combination of suberoylanilide hydroxamic acid and TRAIL is mediated through DR5, we used DR4/Fc and DR5/Fc chimeras to block the interaction of DR4/TRAIL or DR5/TRAIL. As shown in Fig. 5, DR5/Fc efficiently blocked apoptosis induced by cotreatment with suberoylanilide hydroxamic acid and TRAIL in SAS and Ca922 cells,
but not DR4/Fc. These results indicate that the synergistic enhancement of apoptosis caused by the combined treatment of suberoylanilide hydroxamic acid and TRAIL was mediated not by nonspecific toxicity but through specific interactions between TRAIL and DR5.

Discussion
Suberoylanilide hydroxamic acid has been shown to have antitumor activity in a variety of tumor cell line. In the present study, we showed that suberoylanilide hydroxamic acid inhibited oral cancer cell growth in a concentration-dependent manner. The 50% inhibitory concentration of suberoylanilide hydroxamic acid on SAS cells were comparable with those determined for other tumor cell lines. Normal human oral keratinocyte is resistant to suberoylanilide hydroxamic acid treatment. These results are comparable with those obtained by Gillenwater et al. (17) that suberoylanilide hydroxamic acid induced growth inhibition and apoptosis in head and neck squamous carcinoma cell lines but had limited effects on leukoplasia and normal oral cells. The differential toxicity of suberoylanilide hydroxamic acid on oral cancer cells and normal human oral keratinocyte is of value to its clinical application.

Apoptosis is an important mechanism by which most current anticancer agents induce cancer cell death (6). The definition of the apoptotic pathway activated by an anticancer drug not only is important to predict the efficacy but also to gain insights for improved design of therapeutic trials for that agent. It is known that suberoylanilide hydroxamic acid induces apoptosis in many human tumor cells, such as leukemia (13, 15, 19), multiple myeloma (18), breast cancer (14), and head and neck squamous carcinoma (17), but the mechanisms involved are different. In particular, the role of caspases in suberoylanilide hydroxamic acid–induced apoptosis is controversial. Suberoylanilide hydroxamic acid–induced apoptosis has been shown to be caspase-dependent in acute promyelocytic leukemia (13, 15), head and neck squamous carcinoma (17), breast cancer (14), lung, and prostate carcinomas (16). In contrast, Ruefli et al. (19) have shown that suberoylanilide hydroxamic acid induces caspase-independent apoptosis in acute T-cell leukemia cells. Mitsiades et al. also found suberoylanilide hydroxamic acid induced apoptosis independent of the activation of caspase-8, caspase-9, or caspase-3 in multiple myeloma cells (18). In this study, suberoylanilide hydroxamic acid–induced apoptosis was accompanied by the release of cytochrome c and activation of caspase-8 and caspase-9. Furthermore, suberoylanilide hydroxamic acid–induced apoptosis was almost completely inhibited by Z-IETD-FMK and was slightly attenuated by Z-LEHD-FMK. Therefore, in this model, the apoptosis seems to be caspase-mediated and caspase-8 is the initiator caspase in suberoylanilide hydroxamic acid–induced apoptosis, at least in SAS cells.

Gillenwater et al. (17) reported that suberoylanilide hydroxamic acid induced apoptosis in head and neck squamous carcinoma cells through activation of the Fas/Fas ligand death pathway in addition to the mitochondrial pathway. In those cells, suberoylanilide hydroxamic acid induced the expression of Fas and Fas ligand, but not DR4 or DR5. Interfering with Fas signaling blocked apoptosis induction and blunted growth inhibition by suberoylanilide hydroxamic acid. In the present study, we showed that suberoylanilide hydroxamic acid causes a significant increase in the levels of Fas, Fas ligand, DR4, and DR5 protein in SAS cells. Human recombinant DR5/Fc chimera protein but not DR4/Fc or Fas/Fc chimera protein significantly inhibited apoptosis induced by suberoylanilide hydroxamic acid, indicating that DR5 induction contributes to suberoylanilide hydroxamic acid–induced apoptosis. Thus, the death receptor apoptotic pathway, particularly that involving DR5, plays a critical role in mediating suberoylanilide hydroxamic acid–induced apoptosis in human oral squamous cell carcinoma SAS cells. The increased level of DR5 expression, leading to stimulation of the death receptor pathway, seems to be the cause of the activation of caspase-8.

It has been reported that overexpression of DR5 in TRAIL-resistant cancer cells restored TRAIL sensitivity (25). Increased DR5 expression has been highly correlated with sensitivity to TRAIL in some cell lines (26). On the other hand, some investigators reported that there is no correlation between TRAIL receptor expression and susceptibility to TRAIL-induced apoptosis in various tumor types (27, 28). Recent studies have shown that suberoylanilide hydroxamic acid can augment TRAIL-induced apoptosis through up-regulating the expressions of DR4 and DR5 in several types of tumor cells (29, 30). Therefore, we have determined whether a similar phenomenon can occur in oral squamous cell carcinoma cells. We observed TRAIL-resistant human oral squamous cell carcinoma cells exhibiting only close-to-baseline apoptotic levels after single TRAIL incubation, but we achieved pronounced apoptosis induction following cotreatment with subtoxic doses of suberoylanilide hydroxamic acid and TRAIL. A DR5-specific blocking protein but not DR4/Fc or Fas/Fc-competing protein efficiently blocked apoptosis induced by combined treatment with suberoylanilide hydroxamic acid and TRAIL in SAS and Ca9-22 cells, confirming that up-regulation of DR5 is functionally significant. Our present study shows for the first time that subtoxic doses of suberoylanilide hydroxamic acid are able to mediate a specific priming of TRAIL-resistant human oral squamous cell carcinoma cells to TRAIL-induced apoptosis by up-regulating DR5. This could provide an approach to treat oral squamous cell carcinomas that are resistant to TRAIL-induced apoptosis with TRAIL in combination with suberoylanilide hydroxamic acid.

Many cancer cells with p53 gene alterations resist chemotherapy or ionizing radiation-induced apoptosis (31). Introduction of wild-type p53 into different cell systems has been shown to enhance the efficacy of chemotherapeutic agents on cancer cells. In oral cancer cells, p53 is frequently inactivated. In some cell types, DR5 is a downstream target of p53 (32, 33). Some conventional
chemotherapeutic drugs, including etoposide or doxorubicin, can induce DR5 expression and enhance TRAIL-induced apoptosis in a p53-dependent manner in certain cancer cell types such as lung cancer and lymphocytic leukemia cells (32, 33). However, DR5 can be also regulated in a cell type–specific, trigger-dependent, and p53-independent manner (34), but the underlying mechanisms remain largely unclear. We found suberoylanilide hydroxamic acid increased DR5 expression and sensitized TRAIL-induced apoptosis not only in human oral cancer cells with wild-type p53 phenotype (SAS) but also in cells bearing a mutant p53 gene (Ca9-22). These results indicate that wild-type p53 is not required in suberoylanilide hydroxamic acid–induced DR5 up-regulation in oral cancer cells. The combined treatment with suberoylanilide hydroxamic acid and TRAIL maybe effective for oral cancer with resistance to cancer therapy caused by inactivated p53.

In conclusion, our results showed that suberoylanilide hydroxamic acid is more effective in inducing cytotoxicity in oral cancer cells compared with normal human oral keratinocyte as shown by cell growth inhibition assays after drug treatment. The differential toxicity of suberoylanilide hydroxamic acid on oral cancer cells and normal human oral keratinocyte is of value to its clinical application. Suberoylanilide hydroxamic acid induces apoptosis mainly through activation of DR5 pathway. Furthermore, we show for the first time that subtoxic dose of suberoylanilide hydroxamic acid markedly induces the expression of DR5 and synergistically acts with exogenous soluble recombinant human TRAIL to induce apoptosis in a p53-independent manner. Because oral cancer cells with mutant p53 showed resistance to multiple chemotherapeutic agents, our finding that suberoylanilide hydroxamic acid could up-regulate DR5 and sensitize TRAIL-resistant oral squamous cell carcinoma cells through a p53-independent mechanism would seem to be of potential importance in clinical use. The combination of suberoylanilide hydroxamic acid and TRAIL can be used as a new therapeutic approach for the treatment of oral cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Molecular Cancer Therapeutics

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