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Norcantharidin-induced apoptosis in oral cancer cells is associated with an increase of proapoptotic to antiapoptotic protein ratio

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Abstract

Norcantharidin (NCTD), the demethylated analogue of cantharidin, has been used to treat human cancers in China since 1984. It was recently found to be capable of inducing apoptosis in human colon carcinoma, hepatoma and glioblastoma cells by way of an elusive mechanism. In this study, we demonstrated that NCTD also induces apoptosis in human oral cancer cell lines SAS (p53 wild-type phenotype) and Ca9-22 (p53 mutant) as evidenced by nuclear condensation, TUNEL labeling, DNA fragmentation and cleavage of PARP. Apoptosis induced by NCTD was both dose- and time-dependent. We found NCTD did not induce Fas and FasL, implying that it activated other apoptosis pathways. Our data showed that NCTD caused accumulation of cytosolic cytochrome *c* and activation of caspase-9, suggesting that apoptosis occurred via the mitochondria mediated pathway. NCTD enhanced the expression of Bax in SAS cells consistent with their p53 status. Moreover, we showed that NCTD downregulated the expression of Bcl-2 in Ca9-22 and Bcl-X_L in SAS. Our results suggest that NCTD-induced apoptosis in oral cancer cells may be mediated by an increase in the ratios of proapoptotic to antiapoptotic proteins. Since oral cancer cells with mutant p53 or elevated Bcl-X_L levels showed resistance to multiple chemotherapeutic agents, NCTD may overcome the chemoresistance of these cells and provide potential new avenues for treatment.

Keywords: Norcantharidin; Oral cancer; Apoptosis; Bcl-2

1. Introduction

Oral cancer, most commonly squamous cell carcinoma, is the leading cause of cancer-related deaths in India and South Asian countries [1]. Although early stage disease is highly curable, more than 50% of oral cancer patients present with

Abbreviation: NCTD, norcantharidin.

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advanced disease and fewer than 30% of them can be cured [2]. Studies in the past decade showed that concurrent chemoradiotherapy programs have the potential to improve the overall survival of patients with squamous cell carcinoma of the head and neck [3]. Continued investigation of new chemotherapeutic agents is thus needed.

Cantharidin, a 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid derivative, is a natural toxin extracted from blister beetles and the active ingredient of the purported aphrodisiac Spanish fly [4]. It has been demonstrated to have strong inhibitory effect on protein phosphatases type 2A (PP2A) [5,6]. Clinical trials indicated that cantharidin had effects on patients with primary hepatoma, but the application was limited by its severe toxicity for mucous membranes, mainly in the gastrointestinal tract, ureter and kidney [7]. A series of bioactive analogues have been synthesized in an attempt to increase the utility and to reduce the toxicity of cantharidin. Norcantharidin (NCTD), the demethylated analogue of cantharidin, appeared to cause the least nephrotoxic and inflammatory side effects. Although NCTD was shown effective in inhibiting tumor cell proliferation and has been used to treat human cancers in China since 1984 [7], there were, however, very few reports describing the cytotoxic effects of NCTD on tumor cells. In our earlier study [8], NCTD showed differential cytotoxicity to KB and normal buccal keratinocytes. The IC_{50} of 24 h NCTD treatment for KB and keratinocytes were 15.06 and 216.29 μg/ml, respectively. Therefore, NCTD may be of value in treating oral cancers.

Apoptosis, a mode of cell death, is a physiologic event that regulates cell number and eliminates damaged cells. Recent studies have implicated that apoptosis is a common mechanism through which chemotherapeutic agents exert their cytotoxicity and that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [9]. Furthermore, apoptosis genes can improve tumor chemosensitivity and treatment outcome [10–12]. In vitro studies have shown that NCTD treatment can induce apoptosis in human colon carcinoma [13], hepatoma [14,15] and glioblastoma cells [16]. However, the mechanisms by which NCTD exert its cytotoxic effect on oral cancer cells are not well understood. Therefore, we undertook this study to investigate the apoptotic pathways associated with NCTD treatment in two oral cancer cell lines SAS and Ca9-22.

2. Materials and methods

2.1. Cell lines and cell cultures

Two oral cancer cell lines SAS and Ca9-22 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). Ca9-22 cells are known to have mutant *p53* [17]. SAS cells also have point mutation in one *p53* allele, but it has been shown that they have an ability to induce p53-dependent signal transduction [18,19]. The cancer cells were cultivated in 5% CO_2 at 37 °C in Dulbecco modified Eagles' medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). For all experiments, cells were seeded in 35 mm Petri dishes at an initial density of 1×10^4 and allowed to attach for 24 h before NCTD treatments. The culture medium was replaced with fresh medium containing various concentrations of NCTD and incubated for indicated periods of time. All tissue culture biologicals were obtained from Gibco Laboratories (Life technologies, Grand Island, NY, USA).

2.2. Hoechst staining and TUNEL assay

Apoptotic cells were quantified using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as previously described [16]. Briefly, cells were spun onto slides coated with adhesive agent (VectaBond, Vector Laboratories, Burlingame, CA, USA), fixed with 4% paraformaldehyde in PBS (pH 7.4), permeabilized and then incubated in TUNEL reaction mixture (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were then washed, counterstained with Hoechst 33342 (Sigma, St. Louis, MO, USA), mounted with mounting medium (Vectashield, Vecta) and visualized under a fluorescence microscope. At least 500 cells/sample in different fields were evaluated. Apoptosis was assessed as the fraction of cells displaying positive TUNEL staining with nuclear condensation and fragmentation. Each experiment was repeated three times.