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Combined cytotoxic action of paclitaxel and ceramide against the human Tu138 head and neck squamous carcinoma cell line

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Abstract  Purpose: Paclitaxel, a chemotherapeutic agent used in the treatment of recalcitrant ovarian and breast as well as other neoplasms, is being investigated for the treatment of squamous cell carcinoma of the head and neck. Our previous studies have demonstrated that exogenous addition of ceramide enhances apoptosis in paclitaxel-exposed human leukemic cells. In this study, we showed that exogenous ceramide augmented paclitaxel-induced apoptosis in Tu138 cells in vitro when added simultaneously in combination with the paclitaxel. Methods: The combined cytotoxic effects of paclitaxel and ceramide exposure against Tu138 cells were assessed by an MTT dye assay, cell cycle analysis, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, and isobologram analysis for synergistic activity. Results: The MTT dye assay results indicated augmentation of time- and concentration-dependent paclitaxel-mediated cell cytotoxicity by simultaneous ceramide treatment. Paclitaxel treatment of Tu138 cells also resulted in an accumulation of cells in the G2-M phase of the cell cycle. This paclitaxel-mediated G2-M phase accumulation decreased significantly with the addition of ceramide, indicating that combined paclitaxel/ceramide treatment resulted in the elimination of Tu138 cells from the S and/or G2-M phases of the cell cycle. Furthermore, ceramide enhancement of paclitaxel-mediated apoptosis was also detected by the TUNEL assay. Conclusion: Our results suggest that paclitaxel/ceramide combination therapy may be an attractive alternative to conventional methods of chemotherapy for head and neck cancer, and should be further explored.

Key words  Paclitaxel · Ceramide · Squamous cell carcinoma

Introduction

Squamous cell carcinoma of the head and neck is a particularly aggressive type of cancer, refractory to many current treatment regimens. Although chemotherapeutic agents have been routinely utilized in combination to arrest tumor cell growth, many have proven unsuccessful. Recent studies have demonstrated the promising efficacy of combination treatment with paclitaxel [17]. Paclitaxel is a chemotherapeutic agent that prevents microtubule depolymerization resulting in the arrest of proliferating cells in the G2-M phase of the cell cycle and leading to cell death [39]. Additionally, paclitaxel modulates a number of intracellular events which result in cellular apoptosis and ensuing nuclear degradation, including phosphorylation of Bcl-2 [21], or transient stimulation of c-Jun kinase (JNK) [2], activation of caspases, and cleavage of downstream caspase substrates such as poly-ADP ribose polymerase (PARP) [43].

Paclitaxel has been used alone and in conjunction with radiation and/or chemotherapeutic agents to treat a number of different cancers in clinical trials [18, 24]. Combination therapy with paclitaxel and radiation, cisplatin, or doxorubicin has been shown to induce high clinical response rates in patients with squamous cell cancer of the head and neck [12]. Despite the fact that
paclitaxel has been proven to be effective in the treatment of a number of aggressive types of neoplasms, including head and neck cancer, a major disadvantage of paclitaxel therapy is the broad range of severe hypersensitivity reactions associated with its administration, as well as neurotoxicity and myelosuppression [37]. As a result, numerous recent studies have focused on paclitaxel combination therapy with the ultimate goal of increasing therapeutic efficacy at a lower concentration of paclitaxel thus decreasing its toxicity.

Previous in vitro studies have indicated that ceramide is capable of enhancing paclitaxel-mediated growth inhibition and apoptosis, thus lowering the ED50 of paclitaxel and suggesting that its toxic effects could be reduced via dose reduction in vivo [33]. Ceramide, a compound primarily derived endogenously from sphingomyelin, a sphingolipid precursor which comprises part of the cell membrane, has been shown to have numerous regulatory effects on cell function, including cell growth and differentiation, cell cycle arrest, inflammation, and apoptosis [4, 25, 26, 34]. Sphingomyelin hydrolysis occurs after the subsequent activation of acid or neutral sphingomyelinase by any one of several exogenous mediators, including TNFα, endotoxins, interferon-γ, IL-1, Fas ligand, CD28, chemotherapeutic agents, heat and ionizing radiation, resulting in elevation of intracellular ceramide levels [22, 28]. Ceramide synthesis de novo has been implicated in lethal responses to several chemotherapeutic agents such as anthracyclines and ara-C [5, 45]. An in vitro model of paclitaxel/ceramide combination therapy is presented here. We demonstrated that apoptosis induced by clinically relevant concentrations of paclitaxel in the human Tu138 head and neck squamous cell carcinoma cell line can be enhanced by ceramide.

### Materials and methods

**Tumor cell line**

Tu138, an adherent head and neck squamous carcinoma cell line, was generously donated by Dr. Gary Clayman’s laboratory, University of Texas M D Anderson Cancer Center, Houston, TX [10, 31]. Tu138 cells were routinely maintained in T-75 culture flasks (Falcon, N.J.) at a plating cell density of 0.1 × 10^5/75 cm² surface area in complete DMEM/F-12 culture medium (10 ml) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Ga.), 2 mM glutamine (Gibco, N.Y.), 50 U/ml penicillin, 50 mg streptomycin (Gibco, N.Y.) and 20 mM HEPEs (Sigma, Mo.) at 37°C in an atmosphere containing 5% CO₂. Tu138 cell cultures were replenished with fresh complete culture medium and seeded twice weekly.

**Treatment of Tu138 tumor cells with paclitaxel and/or ceramide**

Prior to paclitaxel and/or ceramide exposure, Tu138 cells were trypsinized in 0.25% trypsin-EDTA, washed twice in complete DMEM/F-12, and plated in 96-well culture plates at 50 × 10^3 cells/ml in a final volume of 0.2 ml in complete DMEM/F-12. Cells were incubated in the absence or presence of different concentrations of paclitaxel (0–6000 ng/ml; Bristol Myers Squibb, N.J.) and/or C6 ceramide (N-hexanoyl-N-sphingosine, 0–25 µg/ml; Sigma Chemicals, Mo.). The highest paclitaxel concentration utilized was selected to be within the achievable range for clinical treatment. Cells were subjected to (a) tetrazolium-based dye assay of cell survival, (b) flow cytometry analysis of cell cycle progression, and (c) TUNEL assay for the measurement of apoptosis.

**MTT assay**

Cellular cytotoxicity was measured by the addition of 50 µl of a 0.2% solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] dye (Sigma Chemicals, Mo.) to Tu138 cells [7] after treatment with paclitaxel and/or ceramide. MTT-treated cultures were then incubated for 4 h at 37°C. Culture plates were then centrifuged at 300 g for 2 min and the culture supernatants removed. MTT formazan crystals formed by cells undergoing coupled respiration were solubilized by the addition of 150 µl DMSO after removal of the culture medium. Optical density was determined spectrophotometrically (Model EL 311, Biotek) at 540 nm.

**Cell cycle measurements and TUNEL assay**

Tu138 cells were exposed to paclitaxel (600 ng/ml) and/or ceramide (25 µg/ml), plated in six-well culture dishes at 0.5 × 10⁷ cells/ml in complete DMEM/F-12, and incubated at 37°C for either 24 or 48 h. Cells were then trypsinized and washed in complete DMEM/F-12.

Cell cycle measurements were made following the addition of 0.5 ml 0.1% propidium iodide solution (New Concepts Scientific, Niagara Falls, N.Y., USA) containing 0.1% sodium citrate and 0.1% NP-40. RNase (1 µg) was added to each sample and cells were incubated at 4°C for 30 min. Acquisition of at least 1 × 10⁴ cells was recorded using a FACScan (Becton Dickinson, Calif.) flow cytometer. A precalibration DNA QC kit (Becton Dickinson) was utilized. The acquired cells were then subjected to cell cycle analysis using ModFit LT software (Becton Dickinson).

Apoptosis was measured by flow cytometry using an in situ cell death detection kit (Boehringer Mannheim, Ind.).

**Results**

Paclitaxel- and ceramide-induced cytotoxicity as a function of time of exposure

The aim of the first set of experiments was to observe the cytotoxic effects of paclitaxel and ceramide at different concentrations separately on Tu138 cells as measured by the MTT dye assay. As shown in Fig. 1A, paclitaxel-induced cytotoxicity was 37% and 64% at 600 and 6000 ng/ml, respectively, whereas a 72-h exposure to ceramide at 12.5 and 25 µg/ml resulted in 19% and 46% cytotoxicity, respectively (Fig. 1B). These experiments (n = 6) indicated that the ED50 values for paclitaxel and ceramide were 1920 ± 1200 ng/ml and 22 ± 5 µg/ml, respectively.

The enhancement of cellular cytotoxicity by paclitaxel and ceramide in combination was evaluated by incubating Tu138 cells with paclitaxel (600 ng/ml) and/or ceramide (25 µg/ml). This paclitaxel concentration is within the clinically achievable range using a 24-h infusion in patients. Cell viability was estimated using the MTT dye assay at 24-h time intervals, concluding at 72 h. As shown in Fig. 2, paclitaxel alone at 600 ng/ml reduced cell viability by from 0.9% at 24 h to 21.8% at 72 h. Treatment with ceramide also reduced cell viability...