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Carboplatin resistant human laryngeal carcinoma cells are cross resistant to curcumin due to reduced curcumin accumulation

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ABSTRACT

Curcumin is a natural compound that exhibits a wide range of beneficial effects, among them the antitumor activity. Recently it was shown that curcumin may be efficient against drug resistant tumor cells. The goal of our investigation was to examine if human laryngeal carcinoma cells resistant to carboplatin display sensitivity to curcumin, as compared to parental cells, and if this sensitivity is altered, to determine the molecular mechanisms that are responsible for it. We found that carboplatin resistant 7T cells were also cross resistant to curcumin. After the treatment with equimolar concentration of curcumin, 7T cells exhibited lower intracellular accumulation of curcumin which coincided with reduced formation of reactive oxygen species (ROS), diminished lipid and DNA damage followed by reduced induction of apoptosis and expression of heat shock protein 70 (Hsp70), as compared to parental HEp-2 cells. However, after the treatment with equitoxic concentration of curcumin, intracellular accumulation and all the explored downstream effects were similar in both cell lines suggesting that resistance of 7T cells to curcumin was based on its reduced intracellular accumulation. Since curcumin accumulates mostly in the membranes, we explored the fatty acid composition of both cell lines, but we did not find any difference between them.

1. Introduction

Curcumin (diferuloylmethane) is a yellow pigment derived from the rhizome of the plant *Curcuma longa*. The powdered rhizome of this plant, called turmeric, has been used in traditional Chinese and Hindu medicine for centuries in the treatment of different diseases (Chattopadhyay et al., 2004). Although curcumin has shown a wide range of beneficial pharmacological effects, its anticancer properties have attracted a great interest. It has been shown that curcumin can induce the death of cancer cells and inhibit the formation of tumors in animal models of carcinogenesis (Kunnumakkara et al., 2008; Lopez-Lazaro, 2008; Teiten et al., 2010). Since curcumin influences multiple components of intracellular signaling pathways and molecular targets involved in the development and progression of cancer (Lopez-Lazaro, 2008; Teiten et al., 2010), it is a promising drug for the treatment of cancer as a multifactorial disease. The ongoing clinical trials should determine the possible beneficial effect of curcumin in cancer treatment, when given alone or in combination with conventional anticancer drugs (Teiten et al., 2010).

There are numerous reports about the production of reactive oxygen species (ROS) during curcumin-induced apoptosis in different tumor cell lines (Atsumi et al., 2005; Chen et al., 2010; Fujisawa et al., 2004; Hail, 2008; Kang et al., 2005). These free radicals can cause serious damage to lipids, proteins, and DNA (Halliwell and Gutteridge, 1999) and they have been suggested as some of the initiators in apoptotic signaling (Carmody and Cotter, 2001; Circu and Aw, 2010). Unfortunately, tumor cells can often evade apoptosis through many different mechanisms. Overexpression of some antiapoptotic proteins like Bcl-2, Bcl-XL and Smac protected cancer

Abbreviations: AO, acridine orange; BHT, butylated hydroxytoluene; BSO, L-buthionine-sulfoximine; DCF, 2′,7′-dichlorofluorescein; EtBr, ethidium bromide; GSH, glutathione; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PI, propidium iodide; PARP, poly (ADP-ribose)polymerase; PS, phosphatidylserine; ROS, reactive oxygen species.

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cells from curcumin treatment (Karunagaran et al., 2005), while some curcumin resistant cell lines showed higher production of Hsp70 (Khar et al., 2001; Rashmi et al., 2003, 2004).

Resistance of tumor cells to chemotherapy is the major obstacle that limits the effectiveness of cancer treatment. Therefore, novel strategies based on new compounds that could be efficient against drug resistant tumor cells would be a promising way to increase the effectiveness of cancer treatment. Literature data suggest that curcumin exerts the same anticancer effects in several drug resistant cell lines as in their sensitive parental counterparts. This was shown for multidrug resistant myeloid leukemia cells (Lu et al., 2012), cisplatin resistant ovarian cancer cells (Weir et al., 2007), multiple myeloma cells resistant to dexamethasone, doxorubicin and melphalan (Sung et al., 2009), as well as hormone independent, multidrug resistant breast cancer cells (Labbozzetta et al., 2009).

Drug resistance is a general problem in cancer treatment. Since curcumin has been shown to be very efficient as anticancer agent in some chemoresistant cell lines, the knowledge about the mechanisms involved in modified sensitivity of drug resistant cells to this natural compound could be important in future attempts of using curcumin as antitumor agent in clinical applications, especially in treatment of drug resistant cancers. The goal of our investigation was to examine if human laryngeal carcinoma cells resistant to carboplatin display sensitivity to curcumin, as compared to parental cells, and if this sensitivity is altered, to determine the molecular mechanisms that are responsible for it.

2. Materials and methods

2.1. Cell culture and treatment with curcumin

Human laryngeal carcinoma HEp-2 cells were obtained from cell culture bank (Gibco, Grand Island, NY). Development of their subline resistant to carboplatin has been published previously (Osmak et al., 1995). These cells were propagated for 26 passages without reversion in sensitivity to carboplatin and are now called 7T. HEp-2 and 7T cells were grown at 37 °C with 5% CO_2 as a monolayer culture in Dulbecco's modified Eagle's medium, DMEM (Gibco), supplemented with 10% newborn calf serum (Gibco). The doubling times for HEp-2 and 7T cells were 22 h and 26 h, respectively. Curcumin (Sigma, St. Louis, MO) was prepared in dimethylsulfoxide (DMSO) as 50 mM stock solution and stored at −20 °C for no longer than two months. After overnight incubation, cells were treated with curcumin in the dark (due to its photosensitivity) and diluted in culture medium (final concentration of DMSO being lower than 0.08%). After the treatment both adherent and floating cells were collected (adherent by tripsinization) centrifuged, counted and used in most of experiments, except for those in which the ROS induction was measured. In these experiments the cells were treated with curcumin in suspension.

2.2. Cytotoxicity assay

The sensitivity of cells to curcumin was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay (Mickisch et al., 1990). Briefly, 2.500 HEp-2 cells and 3.500 7T cells were seeded in 180 μl medium in quadruplicate in 96-well tissue culture plates and following overnight incubation treated with curcumin. After 72 h MTT was added and the absorbance of the formazan product was measured on a microplate reader (Awareness Technology Inc, Palm City, USA) at 600 nm. To examine the influence of ROS induction on survival of curcumin treated cells, they were pretreated for 1 h with 10 mM *N*-acetyl-L-cysteine (NAC; Sigma) and than co-treated with curcumin for additional 72 h. Survival of cells treated with both NAC and curcumin was calculated according to survival of cells treated only with NAC (with approximately 100% survival for both HEp-2 and 7T cells). In addition, cells were pretreated with 22.2 μg/ml L-buthionine-sulfoximine (BSO; Sigma) for 24 h and co-treated with curcumin for the next 48 h. Survival of cells treated with both BSO and curcumin was calculated according to survival of cells treated with BSO only (with the survival of more than 92% for both, HEp-2 and 7T cells).

Because the cytotoxic potency (as well as the intracellular accumulation) of a highly lipophilic curcumin *in vitro* depends on the composition of the *in vitro* system, i.e. the relation between cell number (volume) and medium volume and the binding protein and lipid content of the medium, we have done all our experiments in the medium supplemented with 10% of newborn calf serum. In addition, we have examined the influence of cell number and the medium volume on the cytotoxicity of curcumin. As shown in Fig. S1, the alteration in cell number and in the medium volume that we used in our experiments, did not influence the cytotoxicity of curcumin. In addition, in order to have the same seeding density at the time of the treatment, we always kept the same ratio between the cell number and surface of the dish that we used.

2.3. Accumulation of curcumin

HEp-2 (4×10^5/10 ml medium) and 7T cells (6×10^5/10 ml medium) were seeded in 10 cm Petri dishes and after overnight incubation treated with curcumin for different time periods, collected, washed twice with cold PBS and held on ice until analysis. Due to the fact that curcumin exhibits a green fluorescent signal (Khopde et al., 2000; Wang et al., 2006), cells were analyzed using the FL1 channel of a FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) equipped with laser emitting at 488 nm wavelength. Fluorescence emission (above 530 nm) from 10,000 cells was collected and analyzed with BD CellQuest Pro software (BD Biosciences, San Jose, CA).

2.4. ROS measurement

The intracellular accumulation of ROS was determined by flow cytometry using the 2′,7′-dichlorofluorescein diacetate probe (H_2DCFDA; Molecular Probes Invitrogen, Eugene, OR). H_2DCFDA behaves as a nonfluorescent molecule until acetate groups are removed by intracellular esterases. Following oxidation, it becomes a highly fluorescent dye 2′,7′-dichlorofluorescein (DCF). HEp-2 (1.2×10^6/12 ml medium) and 7T (1.7×10^6/12 ml medium) cells were seeded in 10 cm Petri dishes and following overnight incubation, they were collected, resuspended (2×10^5 cells/ml) in warm PBS without Ca^{+2} and Mg^{+2} ions and loaded with 20 μM H_2DCFDA for 30 min at 37 °C. After centrifugation, cells were suspended in 1 ml DMEM (supplemented with 10% newborn calf serum (Gibco) without phenol red (Gibco) and treated with curcumin for 15 min at 37 °C. Thereafter they were washed with PBS without Ca^{+2} and Mg^{+2} ions and the intracellular florescence of DCF was monitored by flow cytometry. Fluorescence emission above 530 nm was analyzed from 10,000 cells. In each experiment three negative controls were used: (a) untreated cells, in order to determine the contribution from autofluorescence, (b) curcumin treated cells, in order to determine the contribution from curcumin fluorescence (similarly to DCF curcumin emits fluorescence above 530 nm) and (c) cells treated with H_2DCFDA, in order to determine the level of intracellular ROS present before curcumin treatment.