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Anti-proliferative and pro-apoptotic effects of *Uncaria tomentosa* aqueous extract in squamous carcinoma cells

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ABSTRACT

Uncaria tomentosa (Willd.) DC. (Rubiacee), also known as *uña de gato*, is a plant that grows wild in the upper Amazon region of Peru and has been widely used in folk medicine to treat several health conditions including cancer. We have produced an aqueous extract from *Uncaria tomentosa* (UT-ex) and analyzed its effects on squamous carcinoma cells and immortalized HaCaT keratinocytes. Squamous cell carcinoma (SCC) is an uncontrolled growth of abnormal cells arising in the skin's squamous layer of epidermis. When detected at an early stage, SCCs are almost curable, however, if left untreated, they can penetrate the underlying tissue and become disfiguring. We have evaluated cell proliferation, apoptosis and the level of reactive oxygen species following UT-ex treatment. UT-ex affected cell cycle progression and reduced cell viability in a dose and time-dependent manner. From a mechanistic point of view, this delay in cell growth coincided with the increase of reactive oxygen species (ROS). Furthermore, PARP1 cleavage was associated to the reduction of Y-box binding protein 1 (YB-1) 36 kDa, a nuclear prosurvival factor involved in DNA damage repair. These data indicate that UT-ex-induced cell death can be ascribed, at least in part, to its ability both to induce oxidative DNA damage and antagonize the mechanism of DNA repair relying upon YB-1 activity. They also show that non metastatic SCCs are more susceptible to UT-ex treatment than untransformed keratinocytes supporting the use of UT-ex for the treatment of precancerous and early forms of squamous cell carcinomas. Preliminary chemical investigation of UT-ex revealed the presence of hydrophilic low-medium molecular weight metabolites with anticancer potential towards squamous carcinoma cells.

1. Introduction

Squamous cell carcinoma (SCC) is an uncontrolled growth of abnormal cells arising in the squamous cells, which compose most of the skin's upper layers (Sapijaszko et al., 2015). SCCs may occur on any part of the body, including the mucous membranes and genitals, but are most common in areas frequently exposed to the sun, such as the neck, face, balding scalp, arms and legs. Squamous cell carcinomas detected at an early stage are almost always curable. However, left untreated, they eventually penetrate the underlying tissues and can become disfiguring. A small percentage may even metastasize to local lymph nodes, distant tissues, and organs and can become fatal. Actinic keratoses (AKs) are considered the earliest form of SCC and may be the first sign of squamous cell carcinoma (Boukamp, 2005); indeed, 40–60% of squamous cell carcinomas begin as untreated AKs.

Though considerable progress has been made in developing effective skin cancer treatments, surgery and radiation are still predominant and it would be beneficial to find new topical treatments for precancerous skin lesions to prevent their progression.

Nowadays, there is an increased demand for alternative concepts or approaches to cancer treatment. Compelling evidence from epidemiological and experimental studies highlight the importance of compounds derived from plants, "phytochemicals", for inhibiting the development and spread of tumors in pre-clinical studies.

Uncaria tomentosa (Willd.) DC., from the Rubiaceae family, is a thorny liana that grows wild in the upper Amazon region of Peru and

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neighboring countries. In Peru, *Uncaria tomentosa*, also known as *uña de gato*, is believed to have anti-inflammatory and anti-tumoral properties (Heitzman et al., 2005; Garcia Prado et al., 2007). The boiled extract of *Uncaria tomentosa* root/bark has been used over the centuries by the indigenous civilization of the Amazonian rain forest for the treatment of fever, rheumatism, arthritis, gastrointestinal disorders, cirrhosis, neurodegenerative diseases, microbial infections, cancer, skin impurities and inflammation (Keplinger et al., 1998; Ccahuana-Vasquez et al., 2007).

Many active compounds have been isolated from *Uncaria tomentosa* including antioxidants such as tannin, catechins, and procyanidins, sterols, triterpenes, flavonoids, carboxyl alkyl esters, indol and oxindol alkaloids (Heitzman et al., 2005; Wagner et al., 1985a). In particular, alkaloids, the major active components of Uncaria species were extensively studied for their potential use as anticancer agents and found to be effective against the proliferation of breast and bladder tumor lineages (Riva et al., 2001; Kaiser et al., 2013). From a mechanistic point of view, *Uncaria tomentosa* was found to affect tumor growth by inhibiting the Wnt or NF-kB/TNFα signaling pathways (Akesson et al., 2003; Gurrola-Diaz et al., 2011). Moreover, *Uncaria tomentosa* aqueous extract was reported to enhance DNA repair in human skin organ cultures irradiated with UV-B (Mammone et al., 2006) and to abate actinic keratoses and early squamous cell cancer in hairless mice exposed to UV-A radiation thereby suggesting its possible application as a product for skin cancer protection (Mentor et al., 2015).

The aim of the present study was to analyze, the biological effects of *Uncaria tomentosa* extract on human immortalized keratinocytes and squamous carcinoma cells in order to provide molecular evidence supporting its therapeutic use against this type of cancer.

2. Materials and methods

2.1. Cell culture and reagents

HaCaT, spontaneously immortalized keratinocytes from adult skin, were purchased from Service Cell Line (CLS, Germany) and cultured as described (Amoresano et al., 2010); A431, human epidermoid carcinoma cells were from American Type Culture Collection (ATCC, Manassas, VA). Head and neck cancer cell lines SCC011, SCC013 and SCC022 were originally derived from tumors in patients affected by squamous cell carcinoma of the upper aerodigestive tract (Resto et al., 2008). HaCaT and A431 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co, St. Louis, MO, USA), while all SCC lines were cultured in Roswell Park Memorial Institute medium (RPMI 1640 Sigma Chemical Co, St. Louis, MO, USA). All cell line cultures were supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT, USA), 1% L-glutamine, and 1% penicillin-streptomycin (ICN Biomedicals, Inc., Aurora, OH, USA) at 37 °C in humidified atmosphere of 5% CO_2.

2.2. Bright-field microscopy

HaCaT, A431, SCC011, SCC013 and SCC022 cells were plated at a density of 2.5×10^5 per well and treated with the indicated doses of Uncaria Tomentosa. After 24 h of treatment, images were acquired in phase-contrast at Nikon Eclipse TE-2000U.

2.3. Antibodies

The primary antibodies GAPDH (6C5), actin (I-19) and actinin (H-2) were purchased from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Primary antibodies YB-1 (ab12148), hnRNPA1 (ab177152) were from Abcam (Cambridge, UK). PARP1, p21-WAF (12D1), Bax (D2E11) and H2AX antibodies were from Cell Signaling (Beverly, MA). Anti-Ubiquitin antibodies were purchased from DAKO (Glostrup, Denmark).

2.4. Extract preparation

The water extract of *Uncaria tomentosa* bark (UT-ex) used for the experiments was prepared as follows: 15 g of dried bark were shredded and boiled for 30 min in 500 ml of bi-distilled H_2O, then cooled at room temperature (RT) and filtered through sterile gauze. The concentration (w/v) was estimated by the ratio between the weight of the bark, expressed in grams, and the final volume obtained at the end of the preparation:

$$15\ g/400\text{ml} = [00.0375\ g/\text{ml}]$$

UT-ex contains 1.28 mg/ml of proteins as determined by Bradford assay, 3.08 mg/ml phenols and 4.05 mg/ml carboidrates (2.08 pentoses and 1.97 hexoses).

The water extract was filtered through a 0.20 μm filter before being applied to the cells. An aliquot was lyophilized (SAVANT, SC110 Speed Vac Plus A) and resuspended in the initial volume of sterile H_2O in order to check for loss of properties during the sublimation process (ReUT-ex).

2.5. Cell viability assay

The effect of *Uncaria tomentosa* on cell viability was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2) 2,5-diphenyltetrazolium bromide (MTT) to formazan by mitochondrial dehydrogenase. Briefly, 9×10^3 cells were seeded on 96-well plates and exposed to increasing concentrations of *UT-ex* (from 0.3 to 10 mg/ml) for 24, 48 and 72 h. MTT/PBS solution (0.5 mg/ml) was then added to the wells and incubated for 3 h at 37 °C in a humidified atmosphere. The reaction was stopped by removal of the supernatant followed by dissolving the formazan product in acidic isopropanol. Optical density was measured with an ELISA reader (Bio-Rad) using a 570 nm filter. Under these conditions, undissolved formazan crystals were not observed. Cell viability was assessed comparing optical density of the treated samples compared to the control group.

2.6. Cell growth profile

HaCaT, A431 and SCC022 cells were plated at a density of 2.5×10^5 per well in a Corning 6 well plate and the counts were performed in a Neubauer chamber by Trypan Blue dye exclusion. Briefly, 20 μL cell suspension was diluted 1:1 with 0.4% solution of trypan blue in phosphate saline buffer pH 7.2. Cell counting was carried out at 24-hr intervals for 3 days.

2.7. Cell cycle analysis

Cells were seeded at 3×10^5 in 35 mm dishes and treated with UT-ex at 0.3 and 1.5 mg/ml for 24 h. Cells were then trypsinized and washed by centrifugation (1200 rpm, 4 min, 4 °C) in Phosphate Buffered Saline (PBS 1x). The cell pellet was resuspended in methanol, incubated in ice for 20 min and centrifuged at 1200 rpm, 5 min, 4 °C. After a wash in PBS, the cell pellet was incubated in PBS 1x, containing RNase 100 μg/ml for 20 min at RT. Propidium iodide was then added at a concentration of 50 μg/ml for 30 min at 4 °C. Cell cycle analysis was performed on the BD Accury™ C6 flow cytometer (BD Biosciences). Cell debris and aggregates were excluded from the analysis.

2.8. Western blot and immunoprecipitation analyses

Immunoblots (IB) and immunoprecipitations (Co-IP) were performed as previously described (Calabrò et al., 2004; Di Costanzo et al., 2011). *Uncaria tomentosa* treated and untreated cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, and protease inhibitors) as previously described (Lo Iacono