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Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems

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ABSTRACT

Oxidative stress is involved in many of the stages of tumorigenesis, and the administration of exogenous antioxidants seems to modulate them. Thus, it has been described that oleuropein and hydroxytyrosol exert important anti-cancer activities. We analyse *in vivo* the anti-tumor properties of both phenolic compounds in an animal model of glioma, and their effects on oxidative stress, enzymatic and non-enzymatic antioxidant defence systems and on several biochemical biomarkers. Hydroxytyrosol, but not oleuropein nor the mixture of both compounds, inhibits tumor growth through mechanisms that involve enzymatic and non-enzymatic antioxidant defences, as demonstrated by a decrease in lipid peroxidation and protein oxidation levels. Furthermore, hydroxytyrosol maintains the non-enzymatic antioxidants as in healthy animals, and positively modifies the enzymatic antioxidants. However, hydroxytyrosol probably acts not as an antioxidant, but through other mechanisms that only indirectly modify the redox status. Finally, these compounds yield few adverse effects related to changes in hepatic enzymes.

1. Introduction

Tumorigenesis represents a multistage, multistep process involving a number of molecular and cellular events that lead to the transformation of a normal cell into a malignant cell. Free radicals seem to be involved in many steps of several of these stages, triggering lipid peroxidation of cellular membranes and oxidation of proteins and DNA, leading to changes in chromosome structure, genetic mutations and/or modulation of cell growth (Visioli, Bellomo, & Galli, 1998). In fact, tumor development has been associated with oxidative stress and reduced responses of antioxidant defense systems. However, reactive oxygen species (ROS) generation is a constant feature

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of oxygen metabolism in cells. An imbalance between ROS generation and the efficiency of antioxidant mechanisms leads to oxidative damage (Acharya, Das, Chandhok, & Saha, 2012; Herrera et al., 2012). In this manner, the central nervous system is especially vulnerable to free-radical damage (Liang & Patel, 2006). However, cancer cells may also be destroyed by ROS, which block key steps in the cell cycle and promote apoptosis through mechanisms that remain to be elucidated (Watson, 2013). Furthermore, several chemotherapy drugs that enhance apoptosis of cancer cells act by lowering antioxidant levels, and their actions cease if antioxidant compounds are administered concomitantly (Kirshner et al., 2008).

Therefore, although administration of exogenous antioxidants has received particular attention due to the potential of these agents to modulate oxidative stress and to act as putative antitumor compounds, an important controversy exists. A vast number of nutritional intervention trials have shown that antioxidants are not obviously effective in preventing cancer (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Watson, 2013), whereas other epidemiological evidence has indicated that increased consumption of fruits and vegetables containing antioxidants is associated with health improvements in terms of cancer risks (Charoenprasert & Mitchell, 2012).

Phenolic compounds derived from olives and virgin olive oils, especially oleuropein and its major metabolite hydroxytyrosol, exert important anti-inflammatory, cardioprotective and anticancer activities both *in vitro* and *in vivo* due to their antioxidant properties (Carluccio et al., 2007; Covas et al., 2006; Visioli et al., 1998; Visioli, Poli, & Gall, 2002), reducing the risk of mutagenesis and carcinogenesis (Waterman & Lockwood, 2007). Thus, in the present report, we analyzed the antitumor properties of the phenolic compounds oleuropein and hydroxytyrosol *in vivo* in an animal model of glioma. We also assessed their effects on oxidative stress biomarkers (lipid peroxidation and protein oxidation), on both non-enzymatic and enzymatic antioxidant defense systems and on several biochemical serum biomarkers to analyze their putative adverse effects on several physiological functions.

2. Materials and methods

2.1. Cell culture

C6 glioma cells were grown in 5% fetal bovine serum (FBS)-supplemented DMEM/HAM F-12 medium (Sigma-Aldrich, Madrid, Spain) without antibiotics. Cells were incubated at 37 °C in a modified atmosphere of 5% CO_2/95% air until confluence. Absence of mycoplasma contamination was assessed regularly using Hoechst 33258 (Invitrogen, Madrid, Spain).

2.2. Colorimetric cytotoxic assay

To set up a colorimetric cytotoxic assay (CCA), cells were trypsinized from a monolayer and diluted to 4×10^4 cells/mL. They were in the exponential phase of growth during the entire experiment. One-milliliter aliquots of cells were pipetted into wells of 24-well tissue culture plates and incubated for 24 h. Oleuropein and hydroxytyrosol were then added to the wells at a volume of 1 mL per well and a range of concentrations (25, 50, 100, 200, 400 and 500 mM), with each dose being used in at least four replicate wells. After 3 days of incubation, the medium was removed, and the cultures were washed with phosphate-buffered saline (PBS) prior to fixation with 10% trichloroacetic acid (TCA) for 30 min at 4 °C. Next, the cultures were washed with tap water to remove the TCA. The plates were air dried and then stored until use. TCA-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of staining period, the SRB was removed, and the cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried, and the bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density (OD) was measured with a Tecan Genios Plus plate reader at 492 nm. The photometer response was linear according to the dye concentration, and it was proportional to the cell numbers counted in parallel with an automated cell counter (TC10, BioRad). The fractions of cell growth affected (Fa) by the compound dosage were used to compute the doses required for the 50% inhibition of cell growth or IC_{50} (Dm) value, which is the coefficient of the sigmoidicity of the dose–effect curve (m) and the linear correlation coefficient of the median-effect plot (*r*).

2.3. Animals and treatments

Forty male adult Wistar rats (350 ± 3.24 g body weight) were used in this study. The animals were provided by Harlan Ibérica S.A. and maintained at the University of Jaen in an animal house in a controlled environment at a constant temperature (25 °C) with a 12 h-light/12 h-dark cycle. The rats were housed in cages and given free access to standard laboratory rat food and water. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). The protocols were approved by the Bioethical Committee of the University of Jaen (PEJA 4957M). The animals were randomly divided into five groups of eight rats each. Four groups were subjected to C6 glioma cell implantation (tumor groups), and one group remained as a non-tumor healthy control group. Ten days after C6 glioma cell implantation, animals in three of the tumor groups received daily subcutaneous injections of 100 μg oleuropein, 100 μg hydroxytyrosol or 100 μg oleuropein plus 100 μg hydroxytyrosol disolved in 500 μL saline solution for 5 days. The other tumor group and the non-tumor healthy control group received vehicle-only injections (saline solution) for the same time period.

2.4. Implantation of C6 glioma cells

Five million C6 glioma cells suspended in 25 μL of culture medium without FBS were injected subcutaneously in both dorsal flanks of the rats using a Hamilton syringe with a 26-gauge needle. The non-tumor group received the same procedure without cells. The characteristics of this glioma model have been previously described (Mayas, Ramirez-Exposito, Carrera, Cobo, & Martinez-Martos, 2012).

2.5. Measurement of tumor volume and sample collection

The size of the abdominal tumor was measured with slide calipers at 10 days after C6 glioma cell implantation, just before