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Oxidative stress, polarization of macrophages and tumour angiogenesis: Efficacy of caffeic acid

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ABSTRACT

Macrophage polarization is a process when macrophage expresses different functional programs in response to microenvironmental signals and two extreme forms exist; M1 and M2 macrophages. M1 macrophages are highly microbicidal and anticancer with enhanced ability to kill and phagocytose pathogens, upregulate pro-inflammatory cytokines and reactive molecular species, and present antigens; M2 macrophages and the related tumour associated macrophages (TAMs) regulate tissue remodelling and promote tissue repair and angiogenesis and can amplification of metabolic pathways that can suppress adaptive immune responses. It is demonstrated that ROS production, critical for the activation and functions of M1 macrophages, is necessary for the differentiation of M2 macrophages and TAMs, and that antioxidant therapy blocks TAMs differentiation and tumorigenesis in mouse models of cancer. In order to study how caffeic acid (CA), a natural antioxidant, affects macrophage function, polarization, angiogenesis and tumour growth we injected mice with Ehrlich ascites tumour (EAT) cells and treated them for 10 days with CA in a dose of 40 and/or 80 mg kg^{-1}. Macrophage polarization was further characterized by quantifying secreted pro- and anti-inflammatory cytokines, nitric oxide and arginase 1 activity. CA may increase the cytotoxic actions of M1 macrophages and inhibit tumour growth; inhibitory activity on TAMs may be mediated through its antioxidative activity. Taken together, we conclude that the antitumour activity of CA was the result of the synergistic activities of different mechanisms by which CA acts on proliferation, angiogenesis, immunomodulation and survival. The continuous administration of CA efficiently blocked the occurrence of TAMs and markedly suppressed tumorigenesis in mouse cancer models. Targeting TAMs by antioxidants can be a potentially effective method for cancer treatment.

1. Introduction

Reactive oxygen species (ROS) are associated with multiple cellular functions such as cell proliferation, differentiation, apoptosis and angiogenesis. ROS are key signalling molecules that play an important role in the progression of inflammatory disorders, including cancer. Oxidative stress and chronic inflammation are the most critical factors involved in the development of approximately 15–20% of malignancies Worldwide [1]. It is believed that persistent inflammatory cell recruitment, repeated generation of ROS and pro-inflammatory mediators, and continued proliferation of genomically unstable cells contribute to neoplastic transformation and ultimately result in tumour invasion and metastasis [2].

Macrophages are ubiquitous cells that secrete a number of potent bioactive inflammatory mediators such as growth factors, cytokines, proteolytic enzymes, proteoglycan, lipid mediators and prostaglandins [3,4]. They play a critical role in the initiation, maintenance, and resolution of inflammation. Furthermore, macrophages are important cells in the primary response to pathogens, maintenance of tissue homeostasis, inflammation, and immunity [3]. Macrophages, also with other inflammatory cell types, provide

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a wide range of bioactive molecules that cause marked changes in the inflammatory loci by interacting with epithelial, mesenchymal and vascular endothelial cells. They are dynamic cells that might modify their functional profiles in response to a variety of stimuli polarizing to functionally different phenotypes, including classically activated (M1) and alternatively activated (M2) macrophages. While M1 macrophages are highly pro-inflammatory, microbicidal and anticancer; M2 macrophages and the related tumour associated macrophages (TAMs) regulate tissue remodelling and angiogenesis and can display immunomodulatory activity [5].

It is demonstrated that ROS production, critical for the activation and functions of M1 macrophages, is necessary for the differentiation of M2 macrophages and TAMs, and that antioxidant therapy blocks TAMs differentiation and tumorigenesis in mouse models of cancer [6]. The role of macrophages in tumours is still controversial but many data indicate that macrophages are key players in the development of tumours and that increased numbers of TAM are significantly associated with poor prognosis. This population of TAM promote production of growth factors, proteases, angiogenic factors and reactive oxygen species, which support the progression of cancer. In particular, TAMs that are recruited to the tumour have a key role in the angiogenic switch and malignant transition of cancer. Consequently, tumour progression is correlated with tumour angiogenesis [5].

Therefore, drugs that can specifically target tumour-supporting macrophages and tumour-specific inflammatory responses could be useful in preventing tumour angiogenesis, growth and metastasis. Therapeutic agents that target inflammatory signals, such as NF-κB and COX-2, may also be useful in suppressing tumour angiogenesis [1,7–9]. Thus, natural products-based anticancer drugs including green tea polyphenols, resveratrol, limonene, epigallocatechin gallate, capsaicin, taxol, berberine, curcumin and organosulfur compounds have been shown to cause strong inhibition of COX-2 and NF-κB [8,9].

Caffeic acid (CA), the major hydroxycinnamic acid, is a widespread phenolic acid that occurs naturally in many agricultural products such as fruits, vegetables, wine, olive oil, coffee and honeybee propolis [8–10]. Recent experimental evidence suggests that caffeic acid is a potent antioxidant [10–12] and might have beneficial health impact *in vivo* [11,12]. Caffeic acid has several biological and pharmacological properties, such as antiviral [13], antioxidant [10–12], anti-inflammatory [8,9], anticarcinogenic [8,9,14–16], and immunomodulatory activity [14]. It has been shown that caffeic acid inhibits both lipoxygenase activity and suppresses lipid peroxidation [17]. Caffeic acid completely blocks the production of reactive oxygen species (ROS) and the xanthine/xanthine oxidase system [17]. In addition, CA efficiently inhibits ceramide-induced NF-κB binding activity [18] and UVB-induced COX-2 expression [9,19]. Further, several studies have identified caffeic acid to be an inducer of apoptosis in cancer cell lines and capable of tumour growth inhibition and regression in animals [15,20,21].

Although caffeic acid has antioxidant effects, its effect on tumour angiogenesis, macrophage polarization and its effect on nitric oxide synthase and arginase, and proinflammatory cytokines has not yet been investigated.

The imbalance between the generation and clearance of reactive oxygen/nitrogen species (ROS/RNS) aids the development of the tumour mainly by inducing genomic instability. Furthermore, ROS could trigger the secretion of the most potent angiogenic factor VEGF, in many cell types and induce proliferation, fluid accumulation, migration, cytoskeletal reorganization and new blood vessel formation, as well as triggering the differentiation of M2 macrophages and TAMs which also contribute to tumour angiogenesis. We believe that the suppression of ROS by a strong antioxidant such as caffeic acid, can block the macrophage polarization and reduce the incidence of tumour associated macrophages, suppress tumour growth and angiogenesis.

2. Materials and methods

2.1. Animals

Male Swiss albino inbred mice, weighing 20–25 g, approximately 2 months old, obtained from the Department of Animal Physiology, Faculty of Science, University of Zagreb, were used in this study. In all experiments, mice were of the same sex. The animals were kept not more than 5 per cage and were maintained on a pellet diet (Standard Diet 4RF 21 GLP certificate, Mucedola, Italy) and water *ad libitum*. Experimental groups comprised 15 mice each. Animal studies were performed in compliance with the guidelines in force in the Republic of Croatia (the Croatian Animal Protection Law ("Narodne Novine", 135/2006 and 37/2013) and the Directive of The European Parliament and of the Council (2010/63/EU)) and according to the Guide for the Care and Use of Laboratory Animals, DHHS Publ. (NIH) 86–123, 1985.

2.2. Tumour cells

Ehrlich ascites tumour (EAT) is transplantable, poorly differentiated and fast growing malignant tumour which appeared originally as a spontaneous breast carcinoma in a mouse. EAT cells were maintained in male Swiss albino mice in ascitic form by serial intraperitoneal inoculation at 7 or 9 day intervals. After harvesting and preparation of cells, their total number and viability were determined by counting in a Bürker-Türk chamber using Trypan Blue dye. Viability was always found to be at least 95%. Mice were inoculated *ip* on day 0 with 2.5×10^6 viable tumour cells per mouse in a volume of 0.5 mL 0.9% sodium chloride solution.

2.3. Caffeic acid (CA)

Caffeic acid (CA)–3,4-di-hydroxycinnamic acid, (purity≥98%) was purchased from Aldrich-chemie, Milwaukee, WI, U.S.A. Before use, CA was dissolved in 0.9% saline. Test components were given to mice *ip* at dose of 40 and 80 mg kg^{-1} body weight.

2.4. Ehrlich ascites tumour (EAT) model and experimental design

Mice were injected intraperitoneally (*ip*) with 2.5×10^6 viable EAT cells. Day of tumour implantation was assigned as day '0'. On day 1, the animals were randomized and divided into three groups; one control and two experimental groups (n = 15 each). Animals were treated *ip* with 0.9% saline (control EAT) or CA at a dose of 40 and 80 mg/kg/d/bw for 10 consecutive days. The CA doses were selected based on the previous literature [14–17]. The animals with ascitic tumour were weighed every five days. Seven animals of each group were sacrificed on the 13th day, 24 h after the last dose of CA and ascitic fluid was collected for the evaluation of tumour growth, differential count of the cells present in the intraperitoneal fluid, macrophage spreading test and nitric oxide (NO) and arginase 1 activity assay from peritoneal and spleen macrophages. Ascites fluid was used for analysis VEGF secretion and Th1, Th2, Th17 cytokines (Multi-Analyte ELISArray kit, Qiagen). In addition to ascites fluid, VEGF was also measured in the tumour cells and macrophages isolated from the peritoneal cavity of total ascites. Percent tumour growth inhibition was calculated by comparing the total number of tumour cells present in the peritoneal cavity of treated groups and the control group. The liver, spleen, kidney and tumour cells were collected 24 h after the last dose of the CA and used to