Project PN-II-ID-PCE-2011-3-0800, no. 342/2011

Study on the molecular and cellular mechanisms of action of EGCG and quercetin as potential chemotherapeutic agents in human leukemia Jurkat T-cells

Scientific context and motivation.

During the past decades there has been a steadily growing interest in the benefits of natural flavonoids. These compounds which are ubiquitously occurring in fruits, vegetables and tea, possess chemo-preventive, cardioprotective, anti-cancer, anti-inflammatory, anti-allergenic and anti-microbial properties. Epigallocatechine-3-gallate (EGCG) and quercetin (QC; 3,5,7,3',4'-pentahydroxyflavone) are two well-investigated flavonoids which inhibit cell proliferation and induce apoptosis in various cancer cell types [1-9]. Both EGCG and QC can exert a dual, pro- and anti-oxidant effect, depending on dosage and time of treatment and numerous studies have indicated that malignant cells are more susceptible than normal cells to the cytotoxicity of these two flavonoids [2,7-9]. At present, only a few agents are known to possess such potential for selective/preferential elimination of cancer cells while exerting cytoprotective effects on normal cells [2]. Therefore, this property could be exploited to prevent leukemia or to increase the efficiency of leukemia chemotherapies. However, in its current stage, this area of investigation has been limited to pre-clinical studies in cellular and animal models, inasmuch as there is great concern regarding the toxic effects on normal cells at high doses of flavonoids. Establishment of the safe therapeutic range for EGCG or QC supplementation remains still a critical task to be achieved. Another serious concern is that failure to eliminate cells that have been exposed to genotoxic agents by apoptosis has been associated with the development of secondary cancer and resistance to anticancer therapy. These issues have prompted for searches of new pharmacological approaches targeted at sensitizing cancerous cells to apoptosis, hence reducing the risk of inflammation and potential complications of both chemo- and radio-therapy. Additional important benefits may derive from developing therapies which use compounds with greater specificity for cancerous cells while protecting healthy tissues. In this project we will employ the Jurkat cell system as a cellular model for human acute lymphoblastoid leukemia and will investigate the effects of EGCG and QC as potential enhancers of MD-induced apoptosis. At the moment, the antiproliferative effects of EGCG and QC and their dose-dependence in Jurkat T-lymphoblasts are largely unknown. It has been shown that QC can accumulate in large quantities inside the mitochondria [10] and inhibit Complexes I and III of the mitochondrial respiratory chain (MRC) [11]. High doses of QC enhance the cellular production of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) [9,12-13], whereas at low doses (~10 μM) QC exercises protective effects against H_2O_2 but not MD in Jurkat cells [14]. In Jurkat cells low EGCG concentrations (~10 μM) exhibit a protective, antioxidant effect, whereas high concentrations (~100 μM) have a prooxidant, cyto- and genotoxic effect, inducing DNA lesions even in the absence of exogenous oxidant agents [1]. In Jurkat cells, EGCG (50 μM) produces intracellular H_2O_2 which can in turn induce apoptosis [5]. In a different cell type, EGCG has been shown to associate with mitochondria and other yet unidentified cytoplasmic organelles [15]. A clinically important chemotherapeutic agent used in the treatment of leukemia is menadione (vitamin K_3) [16, 17]. Menadione (MD) reduction at Complex I of the mitochondrial respiratory chain (MRC) [18, 19], which accounts for ~50% of MD metabolism [19], can readily divert the electron flow from Complex I [19] thus producing superoxide. In MRC Complex I, the two electrons delivered by reduced nicotinamide adenine dinucleotide (NADH) to flavine mononucleotide (FMN) are individually transferred between eight

consecutive iron-sulfur clusters and eventually reach the ubiquinone. Rotenone (ROT) is largely used in pre-clinical studies as an effective molecular tool in delineating the effects of various compounds at the level of the respiratory Complex I [20,21]. Rotenone binds to a specific site on MRC Complex I and inhibits the electron transfer from iron-sulfur centers to ubiquinone. As a direct consequence, the electrons are deviated from Complex I to the surrounding molecular oxygen, thereby producing superoxide. EGCG, QC, MD and ROT can activate the apoptotic program in various cell types via a Ca^{2+}-dependent mitochondrial pathway, by promoting elevation of cytosolic Ca^{2+} levels and collapse of the mitochondrial membrane potential [7,11,13,16,20-25]. However, the current available data on the effects of these compounds on the cell cycle or apoptosis/necrosis in Jurkat cells are extremely limited. We have shown that QC is a potent inducer of apoptosis and is also able to enhance MD-induced apoptosis in Jurkat cells [14]; however, these effects were only investigated for a limited set of doses. In addition, we have not found any reports regarding their effects on clonogenic survival, which is a critical indicator for the antiproliferative efficiency of anti-cancer drugs as well as for the likelihood of relapse after chemotherapy. We intend to undertake a systematic study of these effects under treatments of different time and dosage of the compounds. We also intend to investigate the relationship between calcium and apoptosis induced by flavonoids and/or menadione in Jurkat cells. To the best of our knowledge, there are no previous reports on the effects of EGCG or QC on calcium signaling in Jurkat cells. Calcium ions play a central role in multiple signal transduction pathways to accomplish a variety of biological functions involved in fertilization, cell proliferation, apoptosis, muscle contraction, secretion or memory [25, 26]. Ca^{2+} is released from the endoplasmic reticulum upon activation of inositol 1,4,5-trisphosphate (IP_3) or ryanodine (Ry) receptors. It has been found that the solely ryanodine receptor expressed in Jurkat T-cells is the type 3 RyR (RyR3), which is responsible for sustaining the Ca^{2+} signal required for clonal expansion [27]. We recently found that quercetin at 50 μM evokes a strong, sustained Ca^{2+} signal in Jurkat cells [28]. In further experiments in which we used different antagonists of this receptor, we have obtained preliminary results indicating that RyR3 is responsible for the quercetin-induced Ca^{2+} release signal in Jurkat cells and that binding of quercetin to the receptor can be assayed by spectrofluorimetric means. So far, there are only two electrophysiological studies showing that EGCG and QC can act as direct modulators of the activity of the RyR/Ca^{2+} release channel [29,30]. Our group has experience in the field of IP_3R and RyR regulation. We have developed previously a quantitative model which describes the allosteric regulation of the type-2 RyR by caffeine and quercetin [31,32]. Having in view that the current interest in promoting delayed luminescence (DL) spectroscopy as a valuable tool in the diagnosis of mitochondrial disorders or cancer [33-37], a third goal of our project is to investigate for the first time the correlation between delayed luminescence and the mitochondrial metabolism. DL represents a very weak, long-time scale light emission following exposure to pulsed light or UV radiation. We have a close collaboration [14,38,39] with the Italian group at LNS-INFN Catania which has developed a highly sensitive instrumentation to study the DL properties. Recently [14] we evaluated the effects on DL of MD, H_2O_2 and QC at relevant doses in Jurkat cells and found a strong anti-correlation between apoptosis and DL on a specific DL time-scale (100 μs - 1 ms). Moreover, our data suggested that electron transfer at the level of MRC Complex I is an important source for DL.

Objectives.

(1) We intend ***to quantify the dose-differential effects of EGCG and quercetin in human leukemia Jurkat cells*** with particular stress on apoptosis, clonogenic survival, cell cycle and mitochondrial metabolism. We want to define the optimal parameters for the treatment time and dosage in order to produce significant enhancement by the two flavonoids of menadione-

induced apoptosis in cultured cells while minimizing the applied dose of menadione and flavonoids. We will assess clonogenic survival, cell cycle progression and apoptosis/necrosis, mitochondrial membrane potential, and the mitochondrial levels of superoxide, NADH and FMN in Jurkat cells treated with EGCG, QC, MD or ROT. (2) We want ***to characterize the capacity of EGCG and QC, alone or in combination with MD, to promote apoptosis via*** Ca^{2+} ***release*** from intracellular stores and to gain new insights into the regulation of the ryanodine receptor by these flavonoids. We will assess the kinetic variations in the intracellular concentration of Ca^{2+} induced by EGCG, QC and/or MD, in the absence or the presence of ruthenium-red or dantrolene, two inhibitors of the RyR receptor. We will investigate to what extent the calcium signal is affected by the reduction in calcium concentration of the extracellular medium or the intracellular medium (by clamping extracellular Ca^{2+} and incubation with the calcium ionophore ionomycin or with the calcium chelator BAPTA/AM). In QC treatments, the relative level of internalized quercetin associated with mitochondria or other cytoplasmic organelles will be determined by kinetic recordings of quercetin fluorescence at specific excitation/emission wavelengths [28]. (3) We will ***investigate the correlation between delayed luminescence and the mitochondrial metabolism described by relevant parameters mentioned above***. DL of Jurkat cells will be measured after treatments with EGCG, QC, MD and ROT. The DL quantum yield will be calculated on three time scales (11 - 100 μs, 100 μs - 1 ms and 1 - 10 ms) which are characteristic to different electron transfer steps. The correlation between the quantum yield and the mitochondrial parameters will be established for each DL component.

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