APOPTOSIS AND DELAYED LUMINESCENCE OF HUMAN LEUKEMIA JURKAT T-CELLS AFTER PROTON-IRRADIATION AND TREATMENTS WITH OXIDANT AGENTS AND FLAVONOIDS

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Abstract

We investigated apoptosis and delayed luminescence (DL) of human leukemia Jurkat cells under oxidative stress and irradiation conditions. Irradiation with 2 Gy but not 10 Gy of protons produced a significant increase in the apoptotic rate at 48 h after irradiation. Both doses consistently blocked the cell cycle at the G_2/M phase within 24 h after irradiation, and there was a consistent decrease in the G_2/M cell fraction 48 h after irradiation with 2 Gy but not 10 Gy. DL spectroscopy indicated that irradiation with 10 Gy of protons had a rather modest effect on mitochondrial respiration and production of reactive oxygen species in Jurkat cells. The oxidant agents menadione (MD), hydrogen peroxide and the flavonoid quercetin (QC) potently induced apoptosis and G_2/M arrest in Jurkat cells and decreased DL considerably. A significant enhancement of apoptosis induced by MD was obtained by pre-incubating Jurkat cells with 5 μM QC or 0.5 μM epigallocatechin gallate (EGCG) for 24 h, as well as with 10 μM QC for 1 h, but not with 0.5 μM QC for 24 h. Moreover, in the EGCG-MD combination the G_2/M blockage persisted for at least 48 h. A significant enhancement of apoptosis induced by H_2O_2 was obtained by pre-incubation with 0.5 μM EGCG for 24 h, whereas a short incubation with 10 μM QC for 1 h exerted protective effects against H_2O_2. QC and MD at high doses exhibited virtually identical effects on DL over a wide time-interval (100 μs - 10 ms), whereas EGCG exhibited a fairly uniform reduction of DL on the entire DL time-scale (11 μs - 10 ms).

INTRODUCTION

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 and anti-allergenic properties. Epigallocatechine-3-gallate (EGCG) and quercetin (QC; 3,5,7,3',4'-pentahydroxyflavone) are two well-investigated flavonoids which can inhibit cell proliferation and induce apoptosis in various cancer cell types [1-7]. 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 [2,5-7]. Therefore, this property could be exploited to prevent leukemia or to increase the efficiency of leukemia chemotherapies. A clinically important chemotherapeutic agent used in the treatment of leukemia is menadione (vitamin K_3) [8], which can produce large amounts of superoxide at the level of Complex I of the mitochondrial respiratory chain (MRC) [9]. MD, H_2O_2, QC and EGCG can activate the apoptotic program via a Ca^{2+}-dependent mitochondrial pathway [1-11]. However, the current available data on the effects of these compounds on the cell cycle or apoptosis/necrosis in Jurkat T-cells are extremely limited. We also investigated the effects of proton irradiation on the cell cycle and apoptosis in this cell line. It is generally known that the sensitivity to radiation of Jurkat cells is relatively high and high doses (≥10 Gy) of X or γ radiation can induce significant apoptosis in a time- and dose-dependent manner [12-14].

Having in view the growing interest of using delayed luminescence (DL) spectroscopy in clinical applications [15-17], a second goal of our studies was to provide new insights into the biochemical mechanisms responsible for DL, as well as to provide new data regarding the relation between DL and the cell status. Delayed luminescence represents a very weak, long-time scale light emission following exposure to pulsed light or UV radiation.

Here we report some preliminary results regarding apoptosis and DL in human leukemia Jurkat cells under

oxidative stress and irradiation conditions. A more comprehensive study on these issues is presented in [4].

MATERIALS AND METHODS

Cell cultures

Human leukemia Jurkat T cell lymphoblasts were cultured in MegaCell RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Hydrogen peroxide 30% solution and stock solutions of menadione sodium bisulphite dissolved in phosphate buffer saline (PBS), or dihydrated quercetin and epigallocatehin gallate dissolved in dimethyl sulfoxide (DMSO) were used. DMSO was 0.1% (v/v) in all cultures. After the treatment, cells were washed twice with PBS and resuspended in PBS (for DL samples, ~40 × 10^6 cells/ml) or in complete medium for apoptosis assessment (~0.2 × 10^6 cells/ml). DL samples were analyzed immediately by DL spectroscopy. Cell density, viability and morphology were examined with a CCD camera Logitech QuickCam Pro 4000 connected to an Olympus CK30 phase contrast microscope.

Irradiation conditions

Cell suspensions (7 ml) were irradiated in 50 ml-centrifuge tubes in vertical position. Clinical 62 MeV proton beams accelerated by the superconducting cyclotron at LNS-INFN, Catania (Italy) were used in wide-spread Bragg peak configuration at a dose rate of 11.76 Gy/min. A plane-parallel advanced PTW 34045 Markus ionization chamber was adopted as a reference dosimeter. The dose measurements were performed in a water phantom, according to International Atomic Energy Agency (IAEA) TRS 398. The absorbed dose to water per monitor unit (cGy/M.U.) was measured at isocenter, at the depth corresponding to the middle of the modulated beam, with the reference circular collimator (diameter = 25 mm).

Flow cytometry

24 and 48 h after the treatment, samples containing 10^6 cells were fixed in 70% ethanol and frozen at -20°C. For flow-cytometer determinations, the ethanol-fixed samples were washed with PBS, incubated with a propidium iodide PI/RNAse staining buffer (PHARMINGEN 550825) for 30 min. at 37°C in the dark and analyzed with a Becton Dickinson FACS Calibur flow-cytometer. Apoptosis was evaluated as the fraction of hypodiploid cell fragments (the sub-G_0/G_1 cell fraction). The G_0/G_1, S and G_2/M cell fractions were calculated for the non-apoptotic cell population, by excluding the hypodiploid events from cell cycle analysis. All the data are presented as mean ± standard error of the mean.

Delayed Luminescence Spectroscopy

We used an improved version of the ARETUSA set-up [18], a highly sensitive equipment able to detect single photons. The cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100 ± 5 µJ/pulse). A multi-alkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector for photoemission signals in the wavelength range 400-800 nm, in single photon counting mode. The detected signals were acquired by a Multi-channel Scaler (Ortec MCS PCI) with a minimum dwell-time of 200 ns. DL measurements were done on at least 3 different drops from each cell sample (drop volume 15-25 µl) at room temperature (20 ± 1°C). PBS luminescence was subtracted from all recordings. Photoemission was recorded between 11 µs and 10 ms after laser-excitation. DL intensity (I) was obtained as the number of photons recorded within a certain time interval divided to that time interval and to the number of living cells in the drop. The quantum yield was calculated in three time-domains of the DL emission: 11-100 µs (DL-I), 100 µs - 1 ms (DL-II) and 1-10 ms (DL-III), as the ratio between the integral of I and the energy of the laser.

RESULTS AND DISCUSSION

Irradiation with 2 Gy but not 10 Gy of protons produced a significant increase in the apoptotic rate at 48 h after irradiation (Fig. 1). However, both doses consistently blocked the cell cycle at the G_2/M phase within 24 h after irradiation, suggesting the presence of severe DNA damage (Fig. 1). There was a consistent decrease in the G_2/M cell fraction at 48 h after irradiation with 2 Gy but not 10 Gy, suggesting that a part of the cells receiving 2 Gy of protons were able to initiate apoptosis from the G_2/M phase arrest whereas those receiving 10 Gy were too damaged to trigger apoptosis. Indeed, trypan blue exclusion tests confirmed high necrotic rates of 18.4 ± 3.2% and 46.6 ± 6.8% at 24 h and 48 h after irradiation with 10 Gy of protons, respectively, indicating that this dose induces massive necrosis in this cell system, similar to other reports for X- or γ- irradiation [12-14]. An increase of ~12% in the apoptotic rate at 24 h after irradiation with 10 Gy of gamma rays has been reported [14].

Quercetin induced apoptosis in Jurkat cells in a dose- and time-dependent manner (Fig. 1). 50 µM QC delivered for 1 h or 24 h produced at 48 h after the treatment an apoptotic rate of 14.1 ± 2.1% or 81.3 ± 2.0%, respectively, as compared with the corresponding rate of 7.4 ± 2.2% in control cells. QC arrested Jurkat cells in the G_2/M phase and this blockage is reversible (Fig. 2). In addition, the data suggest that after the most severe treatment (50 µM QC for 24 h) a part of the G_2/M-arrested cells most likely initiated apoptosis after 1 day.

250 µM menadione delivered for 20 min. produced consistent apoptosis and arrested the cell cycle in G_2/M in the first 24 h after the treatment (Fig. 1). A significant enhancement of apoptosis induced by MD was obtained by pre-incubating Jurkat cells with 5 µM QC or 0.5 µM EGCG for 24 h, as well as with 10 µM QC for 1 h, but not with 0.5 µM QC for 24 h. Moreover, in the EGCG-

MD combination the G_2/M blockage persisted even after 48 h following the treatment.

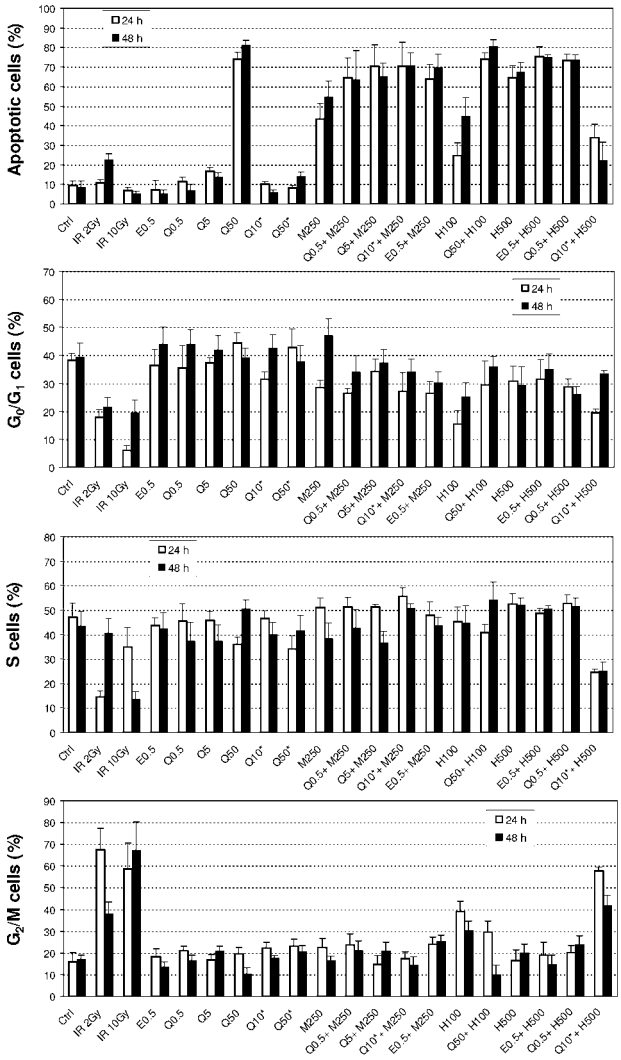

Figure 1: Apoptosis and cell-cycle distributions 24 and 48 h after treatment of Jurkat cells with 0.5, 5 or 50 µM QC for 24 h (Q0.5, Q5, Q50), 10 or 50 µM QC for 1 h (Q10*, Q50*), 0.5 µM EGCG for 24 h (E0.5), 250 µM MD for 20 min. (M250), 100 or 500 µM H_2O_2 for 20 min. (H100 or H500), or after combined treatments (QC or EGCG pre-incubation followed by addition of 250 µM MD or 100/500 µM H_2O_2 for 20 min.), and after irradiation with 2 Gy or 10 Gy of protons (IR 2Gy, IR 10Gy).

Hydrogen peroxide also induced apoptosis in Jurkat cells in a dose- and time-dependent manner (Figs. 1, 2). Interestingly, 100 µM H_2O_2 but not 500 µM H_2O_2 could significantly increase the G_2/M cell fraction, suggesting that the highest dose produced sufficient cell damage to inhibit the activation and/or the maintenance of the G_2/M checkpoint. Application of 100 µM H_2O_2 after pre-treatment with 50 µM QC for 24 h produced an apoptotic cell fraction closely similar to the quercetin treatment alone, but had different effects on the cell cycle in the first 24 h after the treatment, with a significant increase in the G_2/M cell fraction. However, after 48 h the cell cycle distribution became similar to that produced by quercetin alone. A significant enhancement of apoptosis induced by H_2O_2 was obtained by pre-incubation with 0.5 µM EGCG for 24 h, whereas a short incubation with 10 µM QC for 1 h exerted protective effects against H_2O_2. Thus, the apoptotic cell fraction produced by 500 µM H_2O_2 decreased 2 times, and this process was correlated with a consistent G_2/M block (Fig. 1), indicating that the short pre-incubation with quercetin can protect the cells against the deleterious effects of H_2O_2 and improve the repair capacity of the cells.

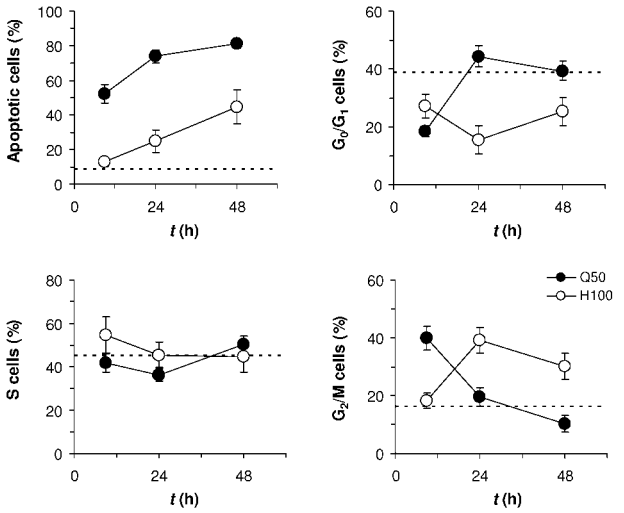

Figure 2: Time course of the apoptotic rate and cell-cycle distributions after treatment of Jurkat cells with 50 µM QC for 24 h (Q50) or 100 µM H_2O_2 for 20 min. (H100). The dashed line represents the average obtained from control cell samples.

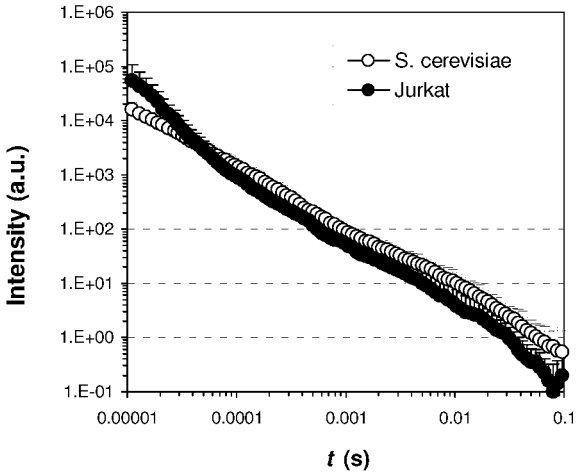

Figure 3: Time course of the DL intensity of untreated Jurkat and *Saccharomyces cerevisiae* cells.

DL of Jurkat cells irradiated with 10 Gy of protons exhibited different characteristics when probed at 1 h or 24 h after irradiation. Hence, a reduction of 34.1 ± 9.6% in the DL-III quantum yield was observed after 1 h from irradiation, and the DL-II quantum yield exhibited an increase of 27.3 ± 8.5% in cell samples probed 24 h after irradiation, while all the other components of the DL emission were not significantly different from the control.

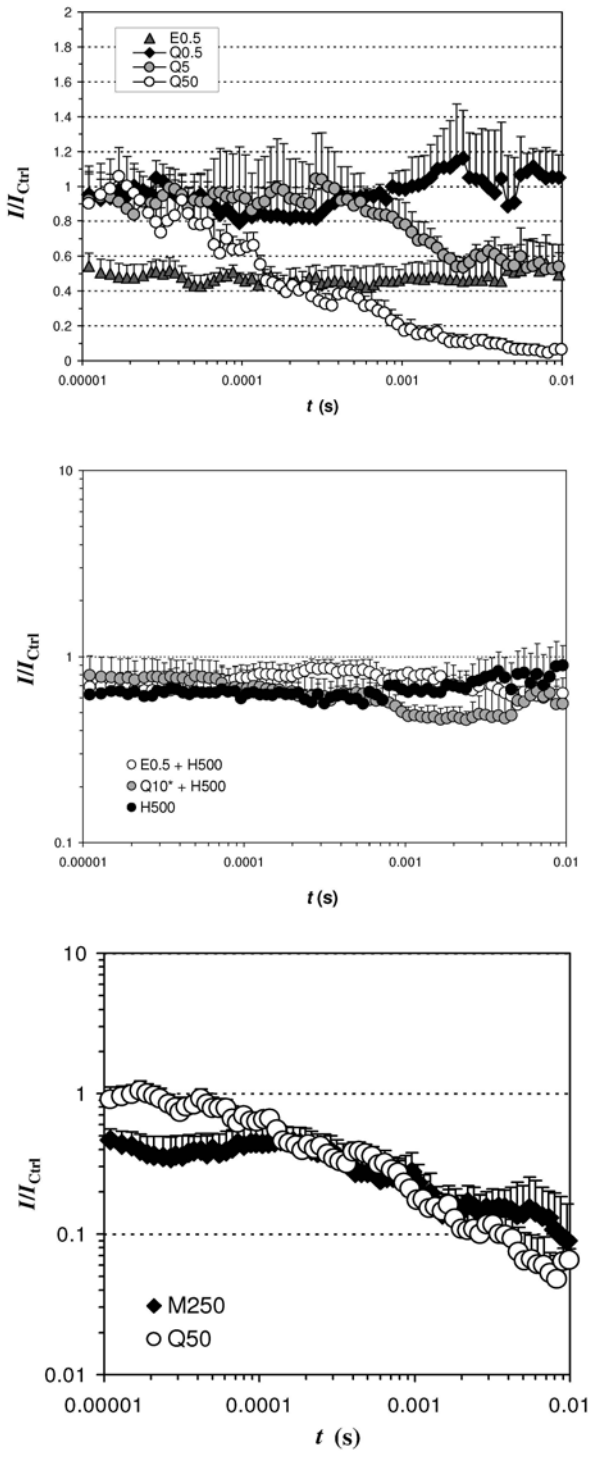

Figure 4: Kinetics of DL emission of Jurkat cells after various treatments labeled as in Fig. 1. The intensity of light emission (I) is normalized to the DL intensity of untreated cultures (I_{Ctrl}).

Similar results were obtained previously with a different cell system [19], the budding yeast *Saccharomyces cerevisiae*, which presented closely similar kinetics of the DL photoemission in untreated Jurkat cells (Fig. 3). Our previous studies have indicated that DL is correlated with the activity of the Complex I of the mitochondrial respiratory chain (MRC) but not with the existence of DNA strand breaks [3, 4, 19]. Hence, our data strongly suggest that irradiation with high doses (10 Gy) of protons had a rather modest effect on mitochondrial respiration and production of reactive oxygen species.

At increasing doses, quercetin inhibited DL progressively (Fig. 4). The most sensitive DL region was DL-III, which decreased by one order of magnitude after the treatment with 50 µM QC for 24 h, whereas DL-I was only slightly affected by QC. QC and MD at high doses exhibited virtually identical effects on DL over a wide time-interval, from 100 µs to 10 ms after laser-excitation (Fig. 4), consistent with the idea that their similar effects on DL were caused by superoxide production and inhibition of Complex I of the mitochondrial respiratory chain [3]. EGCG exerted a qualitatively different effect on DL, with a fairly uniform reduction of the photoemission intensity along the entire time-scale. H_2O_2 reduced DL significantly over the regions DL-I and DL-II (Fig. 4). Pre-treatment with 10 µM EGCG for 24 h was able to induce a significant recovery of DL-II emission, whereas 10 µM QC for 1 h further reduced DL-III intensity of cells exposed to H_2O_2.

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