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Green tea polyphenol "epigallocatechin-3-gallate", differentially induces apoptosis in CLL B-and T-Cells but not in healthy B-and T-Cells in a dose dependant manner

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ABSTRACT

B-cell chronic lymphocytic leukaemia (CLL) is characterized by an accumulation of CD5-positive monoclonal B-cells due in large part to a failure of apoptosis. The ability to study CLL B-cells in vitro has always been a challenge and hampered by the low viability of the CLL B-cells in cell culture systems. In this study, we present a multicellular cell culture system to maintain CLL B-cells viable in culture for 60 h in the presence of a stromal cell feeder layer in combination with a whole white blood cell preparation. Using this optimized system, we tested and showed that the addition of epigallocatechin-3-gallate (EGCG) at concentrations ranging from 25 to 100 μg/ml induced apoptosis in CLL B-cells whilst not affecting healthy control B-cells. Moreover, the results showed that in contrast to healthy controls, T-cells from CLL patients underwent apoptosis in the presence of EGCG. This study demonstrated that the combination of a cell feeder layer with a whole white blood cell preparation maintained B-cell viability *in vitro* over an extended period of time. In addition, the study showed that EGCG differentially induces apoptosis in CLL B-and T-Cells but not in healthy B-and T-Cells in a dose dependent manner.

1. Introduction

Chronic lymphocytic leukaemia is the most common type of leukaemia in Western countries with an annual incidence of approximately 4/100,000 population [1]. The median age at diagnosis is approximately 70 years and it is more common in men. The disease is characterized by a clonal proliferation of CD5-positive B-cells in blood, bone marrow and lymphatic organs. Typically, the clonal B-cells display features consistent with a failure of apoptosis and this is considered a hallmark of malignant CLL B-cells [2,3]. CLL follows a variable clinical course with some patients having indolent disease not requiring treatment for many years while others progress more rapidly. Treatment is reserved for symptomatic disease and there is no defined benefit for treating in the earlier stages of disease. However, an intervention which could delay disease progression for those patients with early stage disease would be valuable, particularly if the side-effect profile was favourable and, in this context, green tea compounds have been studied [4–7].

Epigallocatechin-3-gallate (EGCG), the most predominant and biologically active catechin found in green tea (*Camellia sinessis*) has been postulated as an anti-cancer therapy agent [8,9] with in-vitro studies showing its anti-proliferative and pro-apoptotic capabilities [10–12]. EGCG inhibits cell growth or induces cell death in various tumour cell lines by affecting many different biochemical pathways such as the ability to induce apoptosis in human fibrosarcoma HT-1080 cells [12], suppression of proliferation in human A431 epidermoid carcinoma cells [13] and down-regulation of vascular endothelial growth factor expression in human colon cancer cells [14]. In addition, EGCG can modulate epidermal growth factor receptor and platelet-derived growth factor-BB, which have been both implicated in tumourigenesis [13,15–18]. Alternatively, EGCG can induce apoptosis through various mechanisms, including modulation of the expression of BCL-2 proteins [19–26], the induction of caspase independent apoptosis [11] or by permeabilisation of the lysozomal membrane [27]. In addition, animal models have demonstrated a protective effect of EGCG with oral administration inhibiting prostate cancer cell growth [28].

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In the present study, we developed an *in vitro* model to assay the effect of compounds such as EGCG against CLL patient B-cells over a 60 h period. In doing so, we showed several factors from different cell compartments are required to maintain *in vitro* B-cell viability over an extended period of time. In addition, we demonstrated the apoptosis-inducing effect of EGCG is specific to CLL patient B-cells in the presence of these same cell compartments. However, our data further suggests that CLL impairments may not be limited to the B-cell compartment and that further studies are needed to determine to what degree other white blood cells are impaired.

2. Materials and methods

2.1. Patients

Approval to conduct the study was obtained from the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC# 2012-045). Peripheral blood samples from 4 patients with untreated CLL were obtained from the Charles Day Tissue Bank (HREC # 2008-108). Peripheral blood samples collected in EDTA vacutainer tubes were processed to remove red blood cells. Briefly, red blood cells were lysed for 10 min in BD Pharm Lyse (BD, USA) then washed twice in Hank's Balance Salt Solution (HBSS) (Thermo Fisher Scientific, USA). White blood cells were cryogenically stored in liquid nitrogen for later use. Control samples were obtained from healthy volunteers following informed consent and processed and stored using the same protocol.

2.2. Cell lines

The HS-5 human bone marrow stromal cell line was used in this study. The cell line was maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, USA) containing 1.5 g/L $NaHCO_3$, 10% Foetal calf serum and 1% Penicillin-Streptomycin in a 37 °C, 5% CO_2 incubator. Collection of HS-5 conditioned media (HS-5CM) was performed by replacing the media with fresh RPMI 1640 (Thermo Fisher Scientific, USA) media containing 1.5 g/L $HaHCO_3$, 10% Foetal calf serum and 1% Penicillin-Streptomycin when the cell line had reached 80% confluency and collecting the media after 24 (24hrHS-5CM), 48 (48hrHS-5CM) and 72 (72hrHS-5CM) hours.

2.3. B-cell isolation

Isolation of patient and healthy control B-cells was carried out using the B-CLL Cell Isolation Kit by Miltenyi Biotec (Germany). Briefly, cryogenically preserved CLL white blood cells (WBC) were thawed rapidly and counted. Up to 1×10^7 B-cells were negatively selected for from 5×10^7 white blood cells by magnetic separation using antibodies against non B-cells (CD2, CD4, CD11b, CD16, CD36, Anti IgE and CD235a). B-cell purity was determined by staining cells for CD3-FITC (clone UCHT1), CD5-PE (clone UCHT2), CD45-PerCP-Cy5.5 (clone HI30), CD19-PE-Cy7 (clone HIB19) and CD20-APC (clone 2H7). Cells were incubated for 30 min at room temperature in the dark, washed in HBSS and cell surface expression was measured using a BD FACSCanto II flow cytometer and DIVA software.

2.4. Apoptosis analysis

HS-5 cells were plated in RPMI 1640 media at a concentration of 1×10^6 cells/well in a 12-well plate or 5×10^5 cells/well in a 24-well plate and grown for 8 h. An equal number of wells were prepared with RPMI 1640 media alone. Following incubation either isolated B-cells or total WBCs were added to the wells at a final whole cell concentration of either 2×10^6 cells/well or 1×10^6 cells/well in a 12-well or 24-well plates respectively. EGCG was added to the wells to give a final concentration of 0, 25, 50, 75 or 100 μg/ml. At various time points, both adherent and non-adherent cells were collected and stained with CD3-APC (clone SK7), CD56-PE-Cy7 (clone B159), CD14-APC-H7 (clone MφP9), CD19-V450 (clone SJ25C1), FITC-Annexin V and Propidium Iodide (PI) for 15 min. Cells were analysed by using a BD FACSCanto II flow cytometer and DIVA software. Lymphocytes were first gated for by size (FSC) and granularity (SSC). Specific lymphocyte populations were identified and gated by expression of CD3 (T cells), CD56 (NK cells), and CD19 (B cells). AnnexinV/PI staining was used to gate the various lymphocyte subpopulations into viable, early apoptotic or late apoptotic cells.

2.5. Statistics

Data was presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used to compare the effect of EGCG at various time points to the untreated control. A p value of <0.05 was regarded as significant.

3. Results

3.1. HS-5 cells improves survival of isolated B-cells in vitro

Prior to evaluating the effect of EGCG on the in vitro survival of B-cells, the study required the maintenance of a B-cell population for at least 48 h. Initial culturing of isolated healthy and CLL patient derived B-cells in RPMI 1640 culture media observed rapid cell death with a loss of 90% of viable cells within 48 h (Fig. 1A). Replacement of RPMI 1640 culture media with HS-5 conditioned media (HS-5CM) improved survival over 48 h, with 45% of healthy controls B-cells (Fig. 1B) and 35% of CLL B-cells (Fig. 1C) surviving. Similarly survival of healthy B-cells was improved to 45% after culturing B-cells in the presence of HS-5 cells feeder layer separated by a Transwell membrane (Fig. 1D). However, direct co-culturing without Transwell membranes of healthy B-cells with the HS-5 cells feeder layer further improved survival, with 55% of B-cells viable after 48 h incubation with 0.5×10^6 HS-5 cells and 70% viable with 1.0×10^6 HS-5 cells (Fig. 1D).

3.2. EGCG induces apoptosis in isolated CLL B-cells and healthy control B-cells

Isolated B-cells, co-cultured with 0.5×10^6 HS-5 cells were exposed to various concentrations of EGCG (0, 25, 50, 75 or 100 μg/ml) for 60 h with apoptosis measured by Annexin V/PI staining. Approximately 40% of isolated healthy B-cells underwent apoptosis over 60 h when not exposed to EGCG. This increased in a dose dependent manner to 70% cell death at a concentration of 100 μg/ml EGCG (Fig. 2A). Approximately 25% of CLL B-cells underwent apoptosis over 60 h in the absence of EGCG and this increased in dose-dependent manner to 65% at 100 μg/ml EGCG (Fig. 2B).

3.3. Significant survival improvement of B-cells in the presence of both HS-5 feeder layer and whole white blood cell preparations

Although HS-5 feeder layer cells improved the survival of both CLL and healthy isolated B-cells, a significant proportion of cells died in culture over a 60 h period. We postulated that to maintain a higher rate of survival of B-cells *in vitro*, they require similar micro-environmental interactions as experienced *in vivo*. To investigate this, whole white blood cell preparations (whole blood following red blood cell lysis) were cultured *in vitro* for 60 h with or without HS-5 cells feeder layer. In the absence of an HS-5 feeder layer (RPMI 1640 only), healthy control B-cells and CLL B-cells showed a significant reduction in viability, whilst in the presence of an HS-