In vitro studies for evaluation the antitumoral and immunomodulator effect of EGCG on Ehrlich Ascites

Claudia Gherman[1,2], Valentina Pileczki[3], Roxana Cojocneanu Petric[3], Cornelia Braicu[1], S. Rapuntean[2], Ioana Berindan Neagoe[1,4]

[1]Cancer Institute "Prof. Dr. Ion Chiricuta", Cluj-Napoca, Romania; [2]University of Agricultural Science and Veterinary Medicine, Cluj-Napoca, Romania; [3]Babeş-Bolyai University, Cluj-Napoca, Romania; [4]Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

SUMMARY

Research for natural compounds with beneficial biological activities or antineoplastic capacity is an important target in drug discovery. An example of this type of bioactive compound is epigallocatechin 3-gallate (EGCG), which is the major polyphenol in green tea. The treatment with 10 μM EGCG of Ehrlich ascites carcinoma cells collected from the ascitic fluid of BALB/c mice harbouring 8–10 days old ascitic tumor determined a reduction of cell viability versus control. The value for cell viability expressed as % of control being 86.6% at 24h, 62.6% at 48h, 72.3% at 72h, and 81% at 92h. The cell growth data sustains cell viability data; EGCG did not induce necrosis but was able to induce apoptosis in investigated Ehrlich ascites carcinoma cells. There were no significant differences observed in IL-6 and TNF-α production to any group throughout the study at 24h, meanwhile at 48h a decreased level for both cytokines evaluated was observed. However, IL-6 and TNF-α was slightly reduced in protein expression at 48h. Experimental data exhibits significant antitumor activity of EGCG, being time dependent, but still remains to be validated on in vitro models.

Keywords: EGCG, Ehrlich ascites, antitumor activity

INTRODUCTION

Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally occurring elements of the diet (Davalli et al., 2012). The cancer-preventive activities of tea and tea constituents have also been studied in different models (Tachibana, 2011).

Green tea polyphenols have emerged over the past two decades as important dietary factors for health promotion. Green tea is the main source of this class of phytochemicals. Other sources of polyphenols are chocolate,

grapes and grape seeds, apples and berries (D'Archivio et al., 2008; Braicu et al., 2009). Catechins are a leading example of natural compounds with antineoplastic potential (Ahmad et al., 2000) of witch EGCG seems to have chemoterapeutic and chemopreventive roles in many cancer cells at physiological concentrations (Tachibana, 2011). EGCG is able to modulate several key cell signaling pathways, including suppression of tumor growth, regulation of apoptotic processes or suppression of cytokines (Shimizu et al., 2008; Tachibana, 2009; Tachibana, 2011).

Experimental models have great significance for evaluating the effect of different natural or artificial chemical compounds in *in vitro* or *in vivo* conditions, and Ehrlich ascites carcinoma is one of the most common. It appeared, at first, as a spontaneous cancer tumor in a female mouse (Ozaslan et al., 2011), and then Ehrlich and Apolant (1905) used it as an experimental tumor by transplanting tumor tissues subcutaneously from mouse to mouse. Loewenthal and Jahn (1932) obtained the liquid form in the peritoneum of the mouse, which is known as "Ehrlich ascites carcinoma". Ehrlich ascitic cells grow in suspension in the peritoneal cavity of mice and they do not adhere to the synthetic surface *in vitro* (Ozaslan et al., 2011).

Ehrlich ascites carcinoma is a spontaneous carcinoma, adapted to ascites form and carried in outbred mice by serial intraperitoneal passages. Ehrlich ascites tumor cells have a rapid proliferation rate *in vitro* and *in vivo* systems (Bhattacharyyaet al., 2003). Since the characterization of Ehrlich ascites, scientists used it for chemotherapeutic studies. In one recent study, Bhattacharyya et al. (2003) investigated the apoptogenic effect of black tea against Ehrlich ascites with encouraging data. In addition to this, the literature also describes an inhibitory effect in cytokine production by certain polyphenolic compounds. There are other reports that present an association among cytokines and tumor growth inhibition (El-Mowafy et al., 2010). The principal cytokines implicated in this response are TNF-α, IL-1a, IL-2, IL-4, IL-6, IL- 10 and IL-13 that can act as inhibitory compounds or growth factors for tumor cells (da Silva, 2002).

Previous studies conducted in our laboratory showed that EGCG could induce apoptosis in several cancer cell lines. In this study we aim to evaluate the antitumor effects of the EGCG in Ehrlich ascites grown *in vitro* in vitro.

MATERIAL AND METHODS

Chemicals

(-) EGCG was purchased from Sigma–Aldrich, IL-6 and TNF-α Immunoassay kit from AbFRONTIER

Ehrlich Ascites Carcinoma

Ehrlich ascites carcinoma cells were obtained from the ascitic fluid of BALB/c mice harbouring 8–10 days old ascitic tumour and maintained in culture conditions for several passages before treatment. 1×10^7 cells were seeded in culture before EGCG treatment.

Cell culture

Ehrlich Ascites was grown in RPMI 1640 (Sigma–Aldrich) supplemented with 10% Fetal Bovine Serum (Sigma–Aldrich), 2mM glutamine, 100 UI/ml penicillin, 100 mg/ml streptomycin (Sigma–Aldrich, Bucharest, Romania). The ascitic cells were maintained by serial passage in 75 cm^2 flasks, incubated at 37°C, in a humidified incubator with a 5% CO_2 atmosphere. The CASY Cell Counter and Analyzer was used for basic quality control of the cell culture system, for evaluating cell numbers, cell viability, cell aggregation and cell debris.

Determination of Cell Concentration and Cell Viability

Cell viability was determined using the CASY Cell Counter and Analyzer (Roche Diagnostics), a dye-free system that allows the determination of cell number, the state of the cells (viable/death cells) which can be used to calculate the cell viability for treated, respectively untreated cells and the proliferation rate (ratio between total cell number at the end and beginning of the experiment), which is considered to express the actively dividing cells. Cell debris, dead cells and viable cells are determined in the same measurement. A cell-specific setup was used to define the explicit settings for the discriminate dead and viable cells. This was settled by quantifying a mixture of viable cells with a standardized specimen of dead cells, prepared using 70% ethanol. Cells were seeded at a density of 5 x 10^5 cells/mL and treated with 10 µM EGCG; non-treated cells served as controls. After treatment at 24 , 48, 72 hours, respectively, 50 µL of cellular suspension from each well was diluted in 10 mL CASY ton ready-to-use isotonic saline solution (Roche Diagnostics) for automatic multi-parameter cell counting.

Determination of cytokines production using. Immunoenzymatic assay

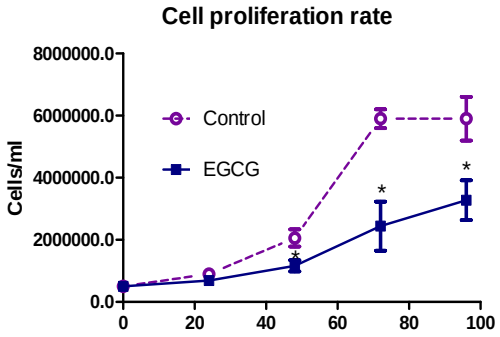
The cytokine profile was obtained through immunoenzymatic assay (enzyme-linked immunosorbent assay) using 100 µL supernatant aliquots of cell culture medium. The following cytokines were analyzed: TNF-α and IL-6. The Immunoenzymatic assay protocol was done according the manufacturer's recommendations (AbFRONTIER).

RESULTS

Effect of EGCG on tumor cell proliferation and viability

The effect of a xenobiotic compound, including for EGCG on cell proliferation rate and cell viability is shown in figure 1 (A, B). The reduction of cell proliferation rate and the decrease in cell viability induced by 10 µM EGCG was evaluated *in vitro* in Ehrlich Ascites. The effect of EGCG on tumor cell proliferation and viability was measured at different time points (24, 48, 72 and 96 hours). Figure 1A shows that 10 µM EGCG induce a reduction of the cell proliferation rate compared with the control set. In cell viability (Figure 1 B) the highest efficiency of EGCG treatment was observed at 42 hours, after that the efficiency decreasing steadily at 72 and 96 hours after treatment.

(A)



(B)

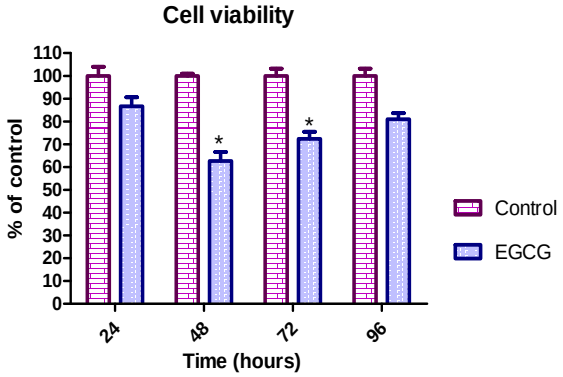


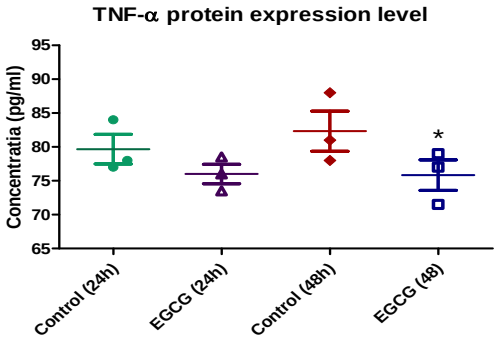
Figure 1. Effect of EGCG on tumor cell numbers and viability. (A) EGCG inhibits cell proliferation in a time-dependent manner and (B) reduces cell viability. Ehrlich Ascites cells were seeded at a density of 1 x 10^5 cells/mL, and tumor cell growth and viability was measured after 24, 48, 72 and 96 hours, respectively, after treatment, using the CASY Cell Counter and Analyzer. Data are presented as mean ± SD (n=6), (*p<0.05 compared to the control using t-test).

The treatment with 10 µM EGCG determined an increased level of cell viability reduction in the treated group compared to the control one. The value for cell viability expressed as % of control being 86.6% at 24h, 62.6% at 48h, 72.3% at 72h, and 81% at 92h. The cell growth data are in agreement with the cell viability data; EGCG did not induce necrosis but was able to induce specifically apoptotic mechanism.

Effect of EGCG on IL-6 and TNF-α release in the Ehrlich Ascites in vitro cell culture

IL-6 and TNF-α synthesis was measured in the presence of a single dose of 10 µM EGCG at 24h and 48 h post treatment. There were no significant differences observed in IL-6 and TNF-α production between groups throughout the study at 24h, meanwhile at 48h we observed a decrease in the protein levels of both cytokines evaluated. However, IL-6 and TNF-α was slightly reduced in protein expression at 48h (Figure 2, A and B).

(A)



(B)

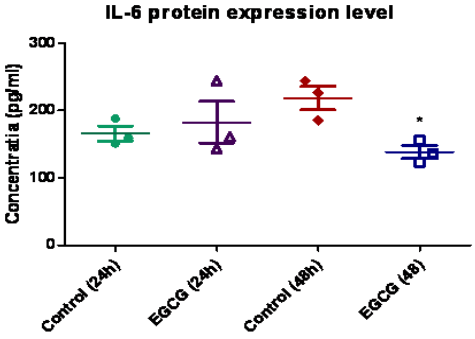


Figure 2 Quantification of IL-6 (A) and TNF-α (B) production from cellular supernatant, in the presence of EGCG, (*p<0.05 compared to the control using t-test), at 24 h, respectively 48h after the treatment.

DISCUSSION

Ehrlich ascites presents similarities with human tumors, more it is undifferentiated and that it has a rapid growth rate. Due to the similarity, investigators reported that some plant extracts were effective against Ehrlich ascites (Ozaslan et al., 2007, 2009a, 2009b). Also the investigation of cytostatic agents from natural sources has been conducted, worldwide (Cragg and Newmann, 1999).

Our studies confirmed that EGCG inhibits the growth of Ehrlich ascites in an effective manner. Hence antitumor effect against Ehrlich ascites could be attributed to its phenolic content and its antioxidant ability, as we can see by the reduction of the efficacy on cell growth rate at 72, respectively 92h. In a similar study using two honey samples and eugenol (one of the phenolic constituents of honey) the data reveals that they were able to significantly inhibit the growth of Ehrlich ascites. This study promotes the idea that polyphenols from honey or any other origin can be considered as promising candidates in cancer therapy (Kumar et al., 2010).

The reduction of tumor cell number and viability is associated with the modulation of cytokine profile mainly for TNF-α and IL-6. Our protein expression data being in agreement with da Silva et al. (2002), and it suggests an effective inter-relation among cytokines and the inhibition of Ehrlich ascites cell growth (da Silva et al. 2002). IL-6 was produced in significantly higher quantities than TNF-α, so we believe that IL-6 is the main cytokine associated with Ehrlich ascites growth, either as a growth factor or a host suppressor factor. IL-6 is considered to by a multifunctional cytokine that promotes tumor growth, metastasis and angiogenesis by modulating the VEGF expression (Zhu et al., 2011).

The pro-inflammatory and tumor sustaining effects of TNF-α are diminished by EGCG effect by blocking one of the major tumor pro-survival pathways. It was observed that EGCG lowers the binding activity of NF-κB gene expression factor (Hsu et al., 2011). In similar in vitro study was observed that TNF-α-induced cyclooxygenase 2 (COX-2) gene transcription and NF-κB cell signaling pathway activation was inhibited by other biological active compound (Plummer et al., 1999).

Inflammatory cell involvement and cytokine release was evoked as an important factor in tumor growth inhibition, and EGCG may decrease the inflammatory processes. Publications on neoplasias and cytokines are comprehensive, and its approach in the *in vitro* and *in vivo* models is highly difficult.

Future studies are needed on in vivo situation using flow cytometry, to discriminate which populations of mononuclear cells are involved in the inhibition response of the tumor growth, for a better knowledge of the

connections of tumor progress, the inflammatory medium, and the cytokine profile in mice Ehrlich ascites treated with EGCG. To increase the efficiency of EGCG many efforts have been made by chemical modification, to improve their bioavailability and stability. But by reduction of the OH groups was observed a reduction of the beneficial effects (Lu et al., 2003; Lam et al., 2004; Lambert et al., 2006).

CONCLUSIONS

Our studies confirmed that the EGCG inhibits the growth of Ehrlich ascites. This study presents valuable information concerning the beneficial effect of natural phytoterapeutic compounds like polyphenols, in variable concentrations. Experimental data exhibits significant antitumor activity of EGCG, being time dependent, but still remains to be validated on in vitro models.

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