Immunology: Current Research

On the Link Between Ellagic Acid and the Immune Balance Between Human Mononuclear and Colon Carcinoma Cells

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Abstract

Background: Pomegranate and particularly its phenolic compound ellagic acid (EA) have been well renowned as a therapeutic remedy for a long list of illnesses including inflammation and cancer. The beneficial activity of EA has been attributed to a number of factors comprising its immunomodulatory effect. Our aim was to examine the effect of EA on the immune relationship between human peripheral blood mononuclear cells (PBMC) and colon cancer cells from two human colon carcinoma cell lines.

Methods: Unstimulated and LPS stimulated PBMC were incubated with various concentrations of EA and the generation of the following cytokines was examined: TNFα, IL-1β, IL-6, IFNγ, IL-1ra and IL-10. In another set of experiments the production of the same cytokines was detected adding EA to PBMC co-cultured with HT-29 or RKO colon carcinoma cells.

Results: While EA at concentrations examined had no effect on inflammatory cytokine secretion by non-stimulated PBMC, it exerted a concentration dependent reduced production of IL-1β, IL-6, IL-10, IL-1ra and IFNγ by mitogen stimulated cells. Moreover, EA showed an inhibitory effect on the cross-talk between immune and colon cancer cells. Lower concentrations of EA decreased IL-1β, IL-6, IL-1ra and IL-10 production by PBMC incubated with HT-29 cells, whereas higher concentration inhibited the production of all cytokines examined except IFNγ. A concentration dependent inhibitory effect was observed when EA was added to PBMC co-cultured with RKO cells. However, in that setting, higher doses of EA did not affect IFNγ and IL-10 production.

Conclusions: The results imply that EA possesses the capacity to modulate the cross-talk between immune and cancer cells of the two lines examined, an activity that may serve as additional mechanism for understanding the way by which EA may inhibit carcinogenesis.

Introduction

Ellagic acid (EA) is a polyphenol existing in one of the most ancient fruits, i.e., pomegranate, also known by its botanical name Punica granatum. The tree has been cultivated since centuries, it has been depicted in the mosaics of Pompeii [1], and even recommended by Avicenna for treatment of uterine bleeding [2]. Moreover, the fruit has been considered as sacred by foremost religions, for reasons thoroughly described by Langley [3]. The fruit's juice has been highly appreciated following years of medical experience and research studies suggesting that its intake is linked with beneficial health properties such as anti-inflammation, antioxidant and anti-proliferative activities [4,5]. In addition, a long list of therapeutic applications has been attributed to pomegranate and its juice, such as cardiovascular conditions, reduction of elevated blood pressure, diabetes and dental problems [5,6]. The anti-inflammatory and anti-proliferative effect of EA warrants particular interest. A substantial number of studies have indicated that there is a close association between chronic inflammation and carcinogenesis (Figure 1). Development of colorectal cancer in patients suffering from ulcerative colitis or Crohn's disease is an appropriate example for this coexistence [7]. Although genetic predisposition cannot be excluded, clinical observations such as extended and prolonged duration of inflammation, coexistence of additional inflammatory conditions and the ability of anti-inflammatory drugs to slow-down cancer development support the role of the inflammatory process in cancerogenesis [8-10]. In this sense the function of mononuclear cells and particularly the macrophages as leaders in the battle against cancer development should be acknowledg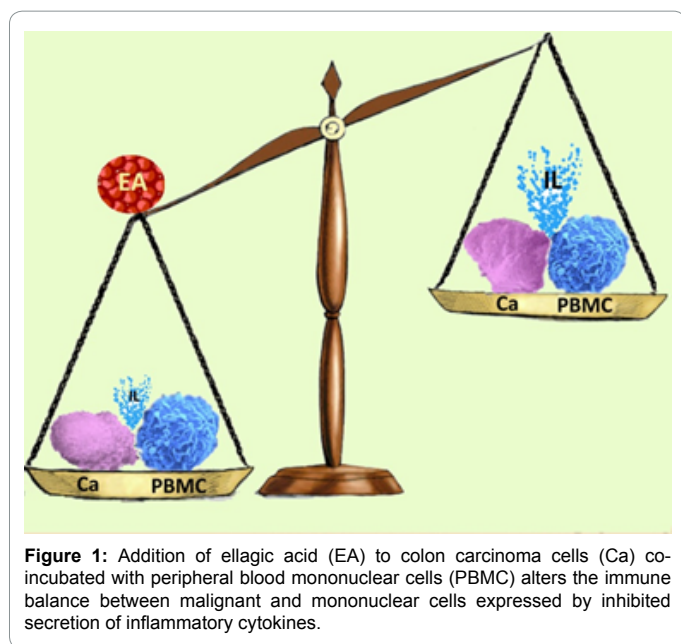ed. To be effective in that task, these cells possess an array of armaments, such as inhibition of cell growth, interference with cell migration and prevention of metastases, as well as ability to produce anti-inflammatory cytokines. It should be noted that peripheral blood and tumor associated macrophages differ in their effect on carcinogenesis as it has been reviewed by Caux et al. [11] Evidently, with suppression of inflammation the possibility of cancer development should be limited. Moreover, in a previous study we have shown that HT-29 and RKO human colon carcinoma cells may activate cytokine production by mononuclear cells and to alter the immune balance between immune and cancer cells [12]. Taking into consideration the anti-proliferative ability of EA it was the aim of the present work to detect if this principal polyphenol constituent of pomegranate will affect the immune interchange between human peripheral blood mononuclear cells (PBMC) and cells from two human colon carcinoma cell lines i.e., HT-29 and RKO. The results may enlighten the way by which pomegranate exerts anti-cancer activity.

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Figure 1: Addition of ellagic acid (EA) to colon carcinoma cells (Ca) co-incubated with peripheral blood mononuclear cells (PBMC) alters the immune balance between malignant and mononuclear cells expressed by inhibited secretion of inflammatory cytokines.

Materials and Methods

Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from venous blood of adult blood donors, using Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The study was approved by the Ethics committee of Rabin Medical Center. Blood bank donors gave written informed consent in which they expressed their approval that certain blood components, not required for patients' treatment, may be used for medical research. The cells were washed twice in phosphate buffered saline (PBS) and suspended in RPMI-1640 medium (Biological Industries, Beith Haemek, Israel) containing 1% penicillin, streptomycin and nystatin, 10% heat inactivated fetal bovine serum (FBS), and was designated as complete medium (CM).

Ellagic acid preparation

Ellagic acid (Sigma, Israel), was freshly dissolved in dimethyl sulfoxide (DMSO, Sigma, Israel) at a concentration of 2 mg/ml, and was further diluted in DMSO. EA was added to cultures at a final volume 10 µl/ml. DMSO was added to control cultures at 10 µl/ml.

Colon cancer cell lines

Two human colon cancer cell lines were used in the study - HT-29 and RKO obtained from American Type Cultural Collection, Rockville, MD. HT-29 cells were maintained in Mc-COY'S 5A medium and RKO cells in modified eagle medium (MEM- Biological Industries Co, Beth-Haemek, Israel) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beth-Haemek, Israel). The cells were grown in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2.

Effect of EA on cell proliferation

The effect of EA on cell proliferation was determined using XTT cell proliferation assay kit (Biological Industries, Beith Haemek, Israel). Briefly, 0.1 ml aliquots of HT-29 or RKO cell suspension (2×10^1/ml of appropriate CM) or PBMC (1×10^6/ml of CM) were added to each one of flat bottom 96 well plates and incubated for 24 hrs with either DMSO (10 µl/ml) or EA at concentrations of 5 µg/ml, 10 µg/ml and 20 µg/ml. At the end of the incubation period, the cells were stained for proliferation according to the manufacturer's instructions. The plates were incubated for 2-4 hrs at 37°C in a humidified atmosphere containing 5% CO_2 and the absorbance was measured at 450 nm using ELISA reader.

Effect of EA on cytokine production

0.4 ml of PBMC (4×10^6/ml of CM) was incubated with 0.4 ml of CM without and with LPS (100 ng/ml) or PMA/ionomycin (1 µg/0.5 µg/ml respectively). In another set of experiments, 0.4 ml of PBMC (4×10^6/ml of CM) were incubated with 0.4 ml cells of one of the colon cancer lines (4×10^1/ml) suspended in appropriate CM. EA was added at the onset of cultures at concentrations as indicated above. Control cultures contained DMSO at 10 µl/ml. The cultures were maintained for 24 hrs at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period the cells were removed by centrifugation at 450 g for 10 min, the supernatants were collected and kept at -70°C until assayed for cytokine content.

Cytokine content in the supernatants

The concentration of TNFα, IL-1β, IL-6, IFNγ, IL-2, IL-10, and IL-1ra in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA), as detailed in the guide-line provided by the manufacturer. The detection levels of these kits were: 15 pg/ml for IL-6 and 30 pg/ml for the remaining ones.

Statistics

A linear mixed model with repeated measures and assumption of compound symmetry (CS) was used to assess the effect of different concentrations of EA on spontaneous cytokine secretion by PBMC or that induced by mitogens or by colon cancer cells. SAS vs 9.4 were used for this analysis. Paired t-test was applied to compare between the level of cytokines produced with various concentrations of EA and that found in control cultures. Probability values of $p<0.05$ were considered as significant. The results are expressed as mean ± SEM.

Results

Effect of EA on cell viability

Incubation of PBMC for 24 hrs with 10 and 20 µg/ml of EA revealed reduced cell proliferation ($F2,10=8.22$, $p=0.0077$) being significantly lower at EA concentration of 20 µg/ml by 29% ($p=0.015$). 24 hrs of incubation of either HT-29 or RKO colon cancer cells with concentrations of EA between 5 µg/ml and 20 µg/ml had no effect on cell proliferation examined by XTT cell proliferation test ($F3,24=2.09$, $p=0.128$ and $F3,24=2.3$, $p=0.108$, respectively, Table 1).

Effect of EA on spontaneous secretion of cytokines

The spontaneous production of all cytokines tested in the current study was not modified by 24 hrs of incubation of PBMC with EA at concentrations as indicated ($p>0.1$, Tables 2 and 3).

Effect of EA on cytokine production induced by LPS or PMA

LPS induced TNFα generation was not affected by 24 hrs of incubation with EA at concentrations as indicated ($F3,15=1.84$, $p=0.183$). LPS- stimulated IL-1β, IL-6, IL-10 and IL-1ra production and PMA promoted IFNγ secretion were dose dependently reduced following 24 hrs of incubation with EA at 5 µg/ml-20 µg/ml ($F3,15=9.59$, $p<0.001$, $F3,15=4.71$, $p=0.016$, $F3,15=3.32$, $p=0.049$, $F3,15=5.3$,

EA	PBMC		HT-29		RKO	
	Absorbance at 450 nm	P*	Absorbance at 450 nm	P*	Absorbance at 450 nm	
0	1141 ± 119		2190 ± 172		1277 ± 250	
5 µg/ml	-	NS	2215 ± 84	NS	1202 ± 204	NS
10 µg/ml	1088 ± 76	NS	2345 ± 91	NS	1336 ± 210	NS
20 µg/ml	793 ± 58	0.015	2138 ± 164	NS	1214 ± 219	NS

Note: PBMC, HT-29 or RKO cells were incubated without (0) or with EA at concentrations as indicated. After 24 hrs, cell proliferation was tested using XTT test as described in Materials and Methods section. The results are expressed as Mean ± SEM of 9 experiments. P* value represents statistically significant difference from cells incubated without EA. NS - not statistically significa

Table 1: Effect of EA on cell proliferation (XTT test).

EA µg/ml	TNFα, ng/ml (n=6)		IL-1β, ng/ml (n=6)		IL-6, ng/ml (n=6)		IFNγ, ng/ml (n=6)	
	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*
	Spontaneous							
0	0.21 ± 0.04		1.91 ± 0.58		5.32 ± 1.61		0.62 ± 0.03	
5	0.19 ± 0.01	NS	1.29 ± 0.28	NS	7.56 ± 2.13	NS	0.61 ± 0.03	NS
10	0.21 ± 0.01	NS	1.50 ± 0.35	NS	9.90 ± 2.22	NS	0.64 ± 0.01	NS
20	0.19 ± 0.01	NS	1.67 ± 0.22	NS	8.60 ± 1.51	NS	0.60 ± 0.02	NS
	LPS-induced						PMA-induced	
0	0.50 ± 0.1		5.92 ± 0.26		17.04 ± 0.71		34.1 ± 6.09	
5	0.49 ± 0.09	NS	5.86 ± 0.12	NS	16.73 ± 0.41	NS	32.7 ± 5.20	NS
10	0.49 ± 0.08	NS	5.51 ± 0.20	0.063	16.10 ± 0.31	0.070	26.9 ± 3.90	0.054
20	0.41 ± 0.09	NS	4.36 ± 0.50	0.013	14.46 ± 0.66	0.045	27.8 ± 5.02	0.012

Note: PBMC were incubated for 24 hrs without (spontaneous) or with either LPS or PMA as described in materials and Methods, in the absence (0) or the presence of EA at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments. P* value represents statistically significant difference from cells incubated without EA. NS - not statistically significant

Table 2: Effect of EA on pro-inflammatory cytokine production.

EA µg/ml	IL-10 ng/ml (n=6)				IL-1ra ng/ml (n=6)			
	Spontaneous		LPS-induced		Spontaneous		LPS-induced	
	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*
0	0.66 ± 0.03		0.81 ± 0.16		0.79 ± 0.14		1.91 ± 0.20	
5	0.69 ± 0.03	NS	0.84 ± 0.06	NS	0.75 ± 0.08	NS	1.80 ± 0.15	NS
10	0.81 ± 0.06	NS	0.78 ± 0.03	NS	0.85 ± 0.07	NS	1.60 ± 0.16	NS
20	0.66 ± 0.03	NS	0.66 ± 0.03	0.026	0.73 ± 0.09	NS	1.47 ± 0.15	0.017

Note: PBMC were incubated for 24 hrs without (spontaneous) or with LPS as described in Materials and Methods in the absence (0) or the presence of EA at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments. P* value represents statistically significant difference from cells incubated without EA. NS - not statistically significant

Table 3: Effect of EA on anti-inflammatory cytokine production.

p=0.011, respectively, and $F_{3,15}=6.58$, p=0.0047 for IFNγ). The reduction of cytokine secretion by stimulated PBMC was statistically significant at EA concentration of 20µg/ml only and was lowered by 26% for IL-1β, 15% for IL-6, 18.5% for IL-10, 23% for IL-1ra and 18.5% for IFNγ. At lower concentrations of EA the degree of reduced cytokine secretion did not reach statistically significance (Tables 2 and 3).

Effect of EA on cytokine production induced by HT-29 and RKO cells

Pro-inflammatory cytokines: The secretion of TNFα, IL-1β and IL-6 by PBMC induced by cells of both colon cancer lines was significantly inhibited by 24 hrs of incubation with EA at concentrations between 5 and 20 µg/ml ($F_{3,15}=15.8$, p<0.001, $F_{3,15}=64.53$, p<0.001, and $F_{3,15}=44.49$ p<0.001, respectively for HT-29 induced cytokine secretion and $F_{3,15}=6.68$, p<0.001, $F_{3,15}=1816$, p<0.001, $F_{3,15}=24.34$, p<0.001, respectively for RKO-induced production). EA caused reduced IFNγ secretion induced by HT-29 cells only ($F_{3, 15}=4.46$, p=0.02), whereas that induced by RKO cells was not affected ($F_{3,}$

$_{15}=1.86$, p=0.18). Reduced IL-1β and IL-6 secretion induced by cancer cells was observed at EA concentration as low as 5 µg/ml, and reached 50% inhibition and more at EA concentration of 20 µg/ml (Table 4).

Anti-inflammatory cytokines: The generation of the anti-inflammatory cytokines IL-1ra and IL-10 by PBMC induced by HT-29 or RKO cells was significantly inhibited following incubation with 5-20 µg/ml of EA ($F_{3,15}=14.3$, p<0.001, $F_{3,15}=6.73$, p=0.0043, respectively for HT-29 induced cytokine secretion and $F_{3,15}=14.85$, p<0.001, $F_{3,15}=5.38$, p=0.01, respectively for RKO- induced production). IL-1ra production induced by both HT-29 and RKO cells was decreased by all three concentrations of EA used in the study, reaching 40% and 54% inhibition, respectively at 20 µg/ml of EA, whereas IL-10 secretion induced by HT-29 cells was only slightly reduced by EA (Table 5).

Discussion

The beneficial effect of pomegranate juice in general and EA in particular on the immune system is well recognized. In a double blind study Shema-Didi et al. [13] have administered 100 ml. of pomegranate

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EAµg/ml	TNFα, ng/ml (n=6)		IL-1β, ng/ml (n=6)		IL-6, ng/ml (n=6)		IFNγ, ng/ml (n=6)	
	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*
	HT-29-induced							
0	0.62 ± 0.06		7.40 ± 0.47		27.44 ± 3.32		2.26 ± 0.41	
5	0.56 ± 0.07	NS	6.05 ± 0.51	0.002	24.79 ± 3.36	0.02	2.00 ± 0.32	NS
10	0.57 ± 0.07	NS	5.75 ± 0.49	<0.001	23.61 ± 3.31	0.013	1.82 ± 0.23	NS
20	0.46 ± 0.07	<0.006	3.47 ± 0.29	<0.001	13.39 ± 2.31	<0.001	1.61 ± 0.19	0.049
	RKO-induced							
0	0.58 ± 0.09		5.54 ± 0.70		29.94 ± 3.16		3.69 ± 1.04	
5	0.67 ± 0.05	NS	5.57 ± 0.90	NS	26.09 ± 3.02	0.004	2.99 ± 0.40	NS
10	0.61 ± 0.06	NS	4.12 ± 0.44	0.018	23.40 ± 3.32	0.03	3.41 ± 0.43	NS
20	0.47 ± 0.08	0.047	2.50 ± 0.44	0.001	11.22 ± 1.61	<0.001	2.46 ± 0.37	NS

Note: PBMC were incubated for 24 hrs with HT-29 or RKO colon cancer cells in the absence (0) or the presence of EA at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 6 experiments. P* value represents statistically significant difference from cells incubated without EA. NS - not statistically significant

Table 4: Effect of EA on pro-inflammatory cytokine production.

EA µg/ml	IL-10 ng/ml (n=7)				IL-1ra ng/ml(n=7)			
	HT-29- induced		RKO-induced		HT-29-induced		RKO-induced	
	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*	Mean ± SEM	P*
0	0.79 ± 0.11		0.77 ± 0.14		1.50 ± 0.22		1.50 ± 0.28	
5	0.74 ± 0.12	0.031	0.90 ± 0.10	NS	1.35 ± 0.22	0.05	1.34 ± 0.19	NS
10	0.71 ± 0.12	0.022	0.76 ± 0.11	NS	1.20 ± 0.16	0.011	1.16 ± 0.16	0.045
20	0.71 ± 0.11	0.018	0.75 ± 0.12	NS	0.90 ± 0.15	0.006	0.69 ± 0.12	0.009

Note: PBMC were incubated for 24 hrs with HT-29 or RKO colon cancer cells in the absence (0) or the presence of EA at concentrations as indicated. The level of cytokines in the supernatants was tested by ELISA. The results are expressed as Mean ± SEM of 7 experiments. P* value represents statistically significant difference from cells incubated without EA. NS - not statistically significant

Table 5: Effect of EA on anti-inflammatory cytokine production.

juice three times a week to 101 patients being on chronic dialysis and concluded that the patients showed reduced oxidative stress and lower incidents of inflammation morbidity. Other studies revealed that EA was able to promote expression of innate immune mediators justifying its use in immune-mediated diseases [14-16]. It has been reported that EA caused a significant inhibition on the production of the pro-inflammatory cytokines TNFα and IL-6 by murine RAW macrophages [17]. As for its effect on human PBMC, Anderson and Teuber [18] have found that EA decreased the secretion of IL-13 and TNFα, but not that of IL-2 and IL-4. In our hands, while unstimulated cells did not show any immune response following incubation with EA, it inhibited the generation of almost all cytokines examined except that of TNFα by LPS-stimulated PBMC. The results became quite different when PBMC were induced for cytokine production by both HT-29 and RKO colon carcinoma cells. In both cases the cells were intensely stimulated and inhibited the production of all cytokines examined in the present work. These findings support the results observed in previous studies in our laboratory in which we have repeatedly demonstrated the existence of an immune dialogue between PBMC and colon cancer cells from the two lines [12,19-21]. Application of EA as an additional therapy for malignant diseases has been forwarded both *in vitro* and in experimental models *in vivo* [22]. MCF-7 breast cancer cells treated with EA showed inhibited proliferation due to interference with the cell cycle [23,24]. Cell proliferation assays in the present study showed that EA does not affect cancer cells of the both lines. These results are rather unanticipated since they are in certain discordance with reports in the literature. Observations demonstrating the inhibitory effect of EA on cell proliferation have been reported with colon and prostate cancer cells [25-27]. Administration of EA to rats with dimethylhydrazine induced colon carcinoma resulted in marked inhibition of a number of inflammatory mediators including IL-6 and TNFα [28]. Umesalma et al. [29] have shown that EA was able to decrease proliferation of HCT-15 colon adenocarcinoma cells via inhibition of alkaline phosphatase and lactate dehydrogenase activities. Similar findings have been reported with HCT-116 and CaCo-2 colon cancer cells lines [30-32]. Inhibited cell proliferation by EA has been reported in several types of malignant cells such as human glioblastoma cells [33] and human pancreatic carcinoma cells [34]. Human adenocarcinoma epithelial cells (A549) treated with EA showed repressed IL-8 and TNFα production [35]. Apparently, the anti-proliferative effect of EA is expressed by a several mechanisms reviewed by Chen et al. [34] and Zhang et al. [36]. The discrepancy between these reports and our findings might be related to the type of cells, EA concentration and the methods applied to assess cell proliferation. Thus, while pomegranate juice was shown to inhibit HT-29 colon cancer cells proliferation, EA itself, similarly to our findings was futile [37].

The main outcome from the current study was the activity that various EA concentrations exerted on the cross-talk between immune and cancer cells. While lower EA concentrations decreased IL-1β, IL-6, IL-1ra and IL-10 production by PBMC incubated with HT-29 cells, higher concentration inhibited the production of all cytokines examined, except IFNγ. A concentration dependent inhibitory effect was observed also when EA was added to PBMC co-cultured with RKO cells. However, in that setting, higher doses of EA did not affect IFNγ and IL-10 production. It is reasonable to assume that inhibited secretion of pro-inflammatory cytokines will reduce the process of inflammation and will cause a deceleration in cancer progress [38]. It is notable that in the living organism tissue associated macrophages are tightly related to tumor growth and progress in addition to PBMC, as it has been reviewed by Bingle et al. [39].

Conclusion

The observations in the present work suggest that the altered immune cross-talk between PBMC and colon carcinoma cells, at least from the two lines examined, is indicative for the existence of an additional mechanism by which cells specialized in immune protection may abolish cancer progression.

Acknowledgement

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