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Original Research

Eugenol as a modulator of the immune dialogue between human mononuclears and colon cancer cells

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ABSTRACT

Objective: Cloves (*Syzygium aromaticum*), the dried flower buds of a tree belonging to the Myrtaceae family have achieved a top place in herbal medicine. To its main active compound eugenol have been attributed definite anti-anaphylactic, anti-inflammatory, and even anticancer properties. It was the object of the study to elucidate the immunomodulatory potential of eugenol and its ability to alter the crosstalk between human peripheral blood mononuclear cells (PBMC) and cells from two human colon carcinoma lines. **Materials and Methods:** Eugenol, at concentrations of 3.75, 7.5, and 15 μg/ml, was added for 24 h to PBMC alone or to cells co-incubated with either HT-29 or RKO colon carcinoma cells. Subsequently, the generation of the following cytokines was examined: Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IFN-γ, IL-1ra, and IL-10. **Results:** Eugenol did not affect the capacity of unstimulated PBMC or that of cancer cells to produce any of the cytokines examined. However, when eugenol was added to PBMC co-incubated with HT-29 cells, the production of all cytokines was inhibited, except for IL-6 and IFNγ. The capacity for cytokine generation by PBMC co-incubated with RKO cells was reduced concerning TNF-α and IL-1β. **Conclusions:** The results indicate that eugenol exerts an immunomodulatory effect on PBMC to produce inflammatory cytokines when stimulated by colon carcinoma cells from the two lines examined. This effect has been shown to be cancer cell type dependent. The capacity of eugenol to interfere with the crosstalk between immune and colon cancer cells may further explain the restraining effect of this compound on cancer development.

KEY WORDS: Cloves, colon carcinoma cells, cytokines, eugenol, mononuclear cells

INTRODUCTION

The health benefits of plants and spices for the treatment of various conditions have been soundly established. The Far East and Chinese medicine and pharmacopeias are prolific in experience and are highly regarded as principle leaders in exploration of spices' favorable properties. The health advantages of including herbs and spices in the culinary armamentarium have been extensively reviewed by Tapsell *et al.* [1] and Jessica Elizabeth *et al.* [2]. Cloves (*Syzygium aromaticum*), the dried flower buds of an evergreen tree belonging to the Myrtaceae family may be placed on the top list of spices with the highest antioxidant capacity [3,4]. Cortés-Rojas *et al.* [5] have characterized clove as "the most valuable spice that has been used for centuries." Eugenol, the major ingredient in clove buds and lives has been suggested as worthy remedy for a long list of illnesses and serves as a root canal filler and sealer in dentistry. The antibacterial, analgesic, antipyretic, anti-anaphylactic, antioxidant, anti-inflammatory and anticancer properties are only examples of the potential benefits that could be obtained by its usage [5-8]. The expressive anti-inflammatory and anticancer activities of eugenol are of interest. Its anti-inflammatory capacity is due to its both antioxidant properties and the capability to suppress cyclooxygenase-2 (COX-2) expression, an enzyme closely connected with inflammation and its progress. Moreover, since COX-2 is involved in the induction of carcinogenesis, it has been reported that inhibition of COX-2 by eugenol resulted in suppressed proliferation of HT-29 human colon cancer cells [9]. The linkage between chronic inflammation and increased risk for cancer is well confirmed. Development of small bowel adenocarcinoma and colorectal cancer following ulcerative colitis and Crohn's disease are examples of the role of prolonged inflammation in promotion of tumorigenesis [10-12]. Although studies have shown that the mechanisms involved in this process are rather copious and intricate, the role of inflammatory cytokines in incitement of cancer growth subsequent to chronic inflammation seems to be significant. Moreover, in a previous work, we have shown that the production of inflammatory cytokines by human peripheral blood mononuclear cells (PBMC) has been altered following contact with HT-29 or RKO human colon cancer cell lines [13]. It is conceivable that diverting the balance of cytokine production toward

increased release of anti-inflammatory ones will impede the inflammatory process with subsequent inhibition of cancer development. Based on the close relationship between chronic inflammation and carcinogenesis on the one hand, and the dual effect of eugenol on both processes on the other hand, this study has been conducted to evaluate the effect of eugenol on the immune dialogue between human PBMC and HT-29 and RKO human colon cancer cell lines.

MATERIALS AND METHODS

Cell Preparation

PBMC were separated from venous blood obtained from adult blood donors by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The cells were washed twice in phosphate buffered saline and suspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Biological Industries, Beith Haemek, Israel) containing 1% penicillin, streptomycin and nystatin, 10% foetal calf serum (FCS), and was designated as complete medium (CM).

Colon Cancer Cell Lines

HT-29 and RKO human colon cancer cell lines were obtained from the American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-Coy's 5A medium (Sigma, Israel) and modified eagle medium (Minimum Essential Medium - Biological Industries Co, Beth-Haemek, Israel), respectively, supplemented with 10% FCS, 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.

Eugenol Preparation

A stock solution of eugenol (2-methoxy-4-[2-propenyl] phenol, Sigma, Israel) was freshly prepared in 0.025% dimethyl sulfoxide (DMSO) in RPMI-1640 medium at 15 mg/ml and further dilutions were made in 0.025% DMSO in RPMI-1640 medium. The volume of eugenol solutions added to cell cultures was 10 μl/ml (1%) to reach final concentrations between 3.75 (22.5 μM) and 15 μg/ml (90 μM). To all control cultures, 10 μl/ml solution of 0.025% DMSO in RPMI-1640 medium was added.

Effect of Eugenol on Cytokine Production

About 0.5 ml of PBMC (4×10^6/ml of CM) was incubated with equal volume of CM (spontaneous) or with one type of the colon cancer cells (4×10^5/ml) suspended in appropriate CM. Eugenol was added at the onset of cultures at concentrations as described above. The cultures were maintained for 24 h 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 1500 rpm 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 the following cytokines: Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IFNγ, IL-10, and IL-1ra in the supernatants was tested using ELISA kits (Biosource International, Camarillo, CA), as detailed in the guideline provided by the manufacturer. The detection levels of these kits were: 15 pg/ml for IL-6 and 30 pg/ml for each one of the remaining ones.

Effect of Eugenol on Cell Proliferation and Viability

Since PBMC do not proliferate unless they are exogenously stimulated, the effect of eugenol on cell proliferation was determined on HT-29 and RKO cells only, using XTT proliferation assay kit (Biological Industries, Beth Haemek, Israel). Briefly: 0.1 ml aliquots of HT-29 or RKO cell suspension (2×10^5/ml of appropriate CM) were added to each one of 96 well plates and incubated for 24 h in the absence or presence of eugenol at concentrations as indicated. 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 h at 37°C in a humidified incubator containing 5% CO_2, and the absorbance was measured at 450 nm using ELISA reader.

The effect of eugenol on cell viability was determined by trypan blue exclusion test. PBMC, HT-29 or RKO cells were treated for 24 h with different concentrations of eugenol in 96 well plates and incubated at 37°C in a humidified incubator containing 5% CO_2. Cells that excluded trypan blue were counted with a hemocytometer and designated as viable cells. Vehicle treated cells (incubated without eugenol) were considered as control.

Statistics

A linear mixed model with repeated measures and assumption of compound symmetry was used to assess the effects of eugenol concentrations and the leverage of the cancer cell lines on cytokine levels. SAS versus 9.4 was used for this analysis. Paired *t*-test was used to compare between the level of cytokine produced with various concentrations of eugenol and that found in control cultures (incubated without eugenol). Probability values of $P < 0.05$ were considered as significant. The results are expressed as a mean ± standard error of mean.

RESULTS

Effect of Eugenol on Cell Proliferation and Viability

About 24 h of incubation of HT-29 or RKO colon cancer cells with eugenol concentrations between 3.75 and 15 μg/ml had no effect on cell proliferation as tested by XTT assay ($F_{3,30} = 1.37$, $P = 0.2849$; and $F_{3,30} = 1.75$, $P = 0.1936$, Table 1). The number of viable HT-29 or RKO cells as determined by trypan blue dye exclusion, and counted after 24 h of incubation was similar at all concentrations of eugenol and controls. The viability of PBMC tested by trypan blue dye exclusion was not significantly affected

Table 1: Effect of eugenol on cell proliferation and viability

Eugenol (μg/ml)	XTT proliferation test		Trypan blue dye exclusion		
	O.D. at 450 nm		% viable cells	Number of viable cells×10^6/ml	
	HT-29 (n=9)	RKO (n=9)	PBMC (n=7)	HT-29 (n=4)	RKO (n=4)
0	2223±70	1734±42	96.5±0.5	91±13	123±5
3.75	2200±114	1717±54	95.8±0.5	90±7	114±3
7.5	2284±105	1758±69	96.0±0.5	85±7	113±15
15	2138±70	1763±46	95.5±0.6	80±6	107±11

PBMC: Peripheral blood mononuclear cells. The effect of various concentrations of eugenol on cell proliferation examined by XTT test and on cell viability tested by trypan blue dye exclusion was assayed following 24 h of incubation, as described in materials and methods

following 24 h of incubation with eugenol at concentrations tested ($F_{3,17}$ = 1.37, P = 0.2849, Table 1).

Cytokine Production by PBMC and Malignant Cells

Supernatants from HT-29 or RKO cells incubated for 24 h in appropriate medium did not contain detectable concentrations of any of the cytokines tested in the study. Incubation of PBMC with HT-29 cells caused increased secretion of IFNγ (P < 0.001), IL-6 (P < 0.005), TNFα (P < 0.01), IL-1β (P < 0.005) IL-10 (P < 0.005), and IL-1ra (P < 0.05) and incubation with RKO cells induced higher production of IFNγ (P < 0.001), IL-6 (P < 0.01), TNFα (P < 0.05), IL-1β (P < 0.01), and IL-10 (P < 0.05) whereas that of IL-1ra was not affected.

Effect of Eugenol on TNF-α Production

The secretion of TNF-α by non-stimulated PBMC was not significantly affected following 24 h of incubation with the indicated concentrations of eugenol ($F_{3,15}$ = 0.53, P = 0.67). However, the secretion of TNF-α induced by HT-29 or RKO cells incubated with increased concentrations of eugenol was dependently reduced ($F_{3,30}$ = 4.46, P = 0.01; $F_{3,30}$ = 5.0, P = 0.0062). The production of TNF-α induced by HT-29 colon cancer cells was suppressed by 16% (P = 0.0025) and 21% (P = 0.0012) at eugenol concentrations of 7.5 and 15 μg/ml, respectively, and that induced by RKO was inhibited by 12% (P = 0.037) and 20% (P < 0.005) at the same concentrations of eugenol, respectively [Figure 1].

Effect of Eugenol on IL-1β Production

The generation of IL-1β by non-stimulated PBMC was not affected significantly following 24 h of incubation with the applied concentrations of eugenol ($F_{3,15}$ = 1.39, P = 0.285), whereas that induced by HT-29 or by RKO colon cancer cells was concentration-dependently inhibited ($F_{3,30}$ = 11.1, P < 0.0001 or $F_{3,30}$ = 3.5, P = 0.027, respectively). At eugenol concentrations of 3.75, 7.5, and 15 μg/ml the production of IL-1β by HT-29 stimulated PBMC was suppressed by 30% (P = 0.013), 40%, and 43%, respectively (P < 0.005), and that secreted by RKO-stimulated PBMC was lowered by 8% (NS) 12% (P < 0.012), and 13% (P = 0.008), respectively [Figure 1].

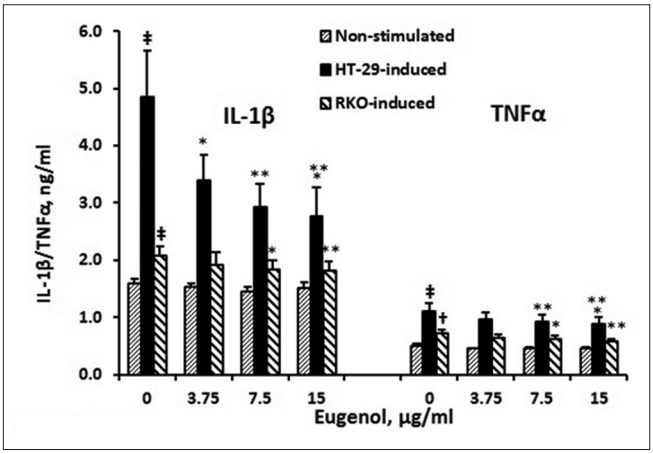

Figure 1: Effect of eugenol on tumor necrosis factor-α and IL-1β production. 2 × 10^6 peripheral blood mononuclear cells (PBMC) were incubated for 24 h in the absence or presence of 2 × 10^5 HT-29 or RKO colon cancer cells, without (0) or with eugenol at concentrations as indicated. The level of tumor necrosis factor -α and interleukin-1β in the supernatants was tested by enzyme-linked immunosorbent assay. Each column represents the mean of 11 experiments. Bars represent standard error of the mean. The statistically significant difference in cytokine production between PBMC cultured alone or those with colon cancer cells is presented by (†P < 0.01) or by (‡P < 0.005). Asterisks represent statistically significant difference from PBMC incubated without (0) or with eugenol (*P < 0.05; **P < 0.01; ***P < 0.001)

Effect of Eugenol on IL-6 Production

The generation of IL-6 by non-stimulated PBMC or that stimulated by HT-29 or RKO cells was not affected significantly by eugenol at the concentrations used ($F_{3,15}$ = 1.06, P = 0.395; $F_{3,30}$ = 2.54, P = 0.0754; $F_{3,30}$ = 0.78, P = 0.517, Table 2).

Effect of Eugenol on IFN-γ Production

The secretion of IFN-γ by non-stimulated PBMC or that stimulated by HT-29 or RKO cells was not affected significantly by eugenol at the concentrations used ($F_{3,15}$ = 0.88, P = 0.475; $F_{3,30}$ = 1.14, P = 0.348; $F_{3,30}$ = 2.83, P = 0.055, Table 2).

Effect of Eugenol on IL-10 Production

The generation of IL-10 by non-stimulated PBMC or by PBMC stimulated with RKO cells was not affected significantly by

Table 2: Effect of eugenol on IFNγ and IL-6 production

Eugenol concentration	0	3.75 μg/ml	7.5 μg/ml	15 μg/ml
TFNγ, ng/ml (n=11)				
Non-stimulated	0.81±0.03	0.82±0.02	0.85±0.03	0.78±0.04
HT-29-induced	2.98±0.25***	3.35±0.38	2.60±0.18	2.77±0.30
RKO-induced	3.08±0.31***	2.82±0.18	2.77±0.21	2.67±0.22
IL-6, ng/ml, (n=11)				
Non-stimulated	10.2±0.7	11.5±1.7	12.3±2.1	14.1±2.4
HT-29-induced	20.4±2.9**	19.8±3.0	18.1±2.9	18.7±3.0
RKO-induced	17.9±2.8**	17.1±3.0	18.2±2.9	19.0±3.0

TFN: Tumor necrosis factor, IL: Interleukin. 2×10^6 peripheral blood mononuclear cells (PBMC) were incubated for 24 h without or with eugenol at concentrations as indicated, in the absence (non-stimulated) or presence of 2×10^5 HT-29 or RKO colon cancer cells. The level of IFN-γ and IL-6 in the supernatants was tested by enzyme-linked immunosorbent assay. Each column represents the mean of 11 experiments. Bars represent standard error of the mean. Asterisks represent statistically significant difference from PBMC incubated without or with colon cancer cells (**P<0.01; ***P<0.001)

24 h of incubation with the above mentioned concentrations of eugenol ($F_{3,15}$ = 0.69, P = 0.572; $F_{3,30}$ = 1.07, P = 0.376). However, the secretion of IL-10 induced by HT-29 cells was dose-dependently reduced following incubation with increased concentrations of eugenol ($F_{3,30}$ = 6.16, P = 0.0022). At eugenol concentrations of 7.5 and 15 μg/ml the production of IL-10 induced by HT-29 cells was inhibited by 20 and 28%, respectively (P < 0.0001, Figure 2).

Effect of Eugenol on IL-1ra Production

IL-1ra production by non-stimulated or RKO-stimulated PBMC was not affected significantly by 24 h of incubation with eugenol at the concentrations tested ($F_{3,15}$ = 2.18, P = 0.133; $F_{3,30}$ = 2.57, P = 0.072). On the other hand, IL-1ra secretion by PBMC was significantly inhibited when induced by HT-29 cells ($F_{3,30}$ = 3.58, P = 0.025). At eugenol concentrations of 3.75, 7.5, and 15 μg/ml the production of IL-1ra was reduced by 10%, 19%, and 19%, respectively (P < 0.05, Figure 2).

DISCUSSION

The results of the study show that eugenol did not affect the secretion of the examined cytokines by both PBMC and cancer cells at all concentrations used in the course of the experiments. This observation is in accordance with other findings showing that eugenol was able to block the release of IL-1β, TNF-α and prostaglandin E2 by human macrophages stimulated by lipopolysaccharide (LPS) [14]. The possibility that eugenol affected cell viability and therefore became unresponsive can be excluded following by both our observations and those reported in other studies showing that macrophage viability was affected only by eugenol at doses of ≥100 μg/ml [15,16]. On the other hand, when cytokine secretion by PBMC was promoted by contact with malignant cells, 7.5 and 15 μg/ml of eugenol caused a pronounced inhibition of the pro-inflammatory cytokines TNFα and IL-1β secretion. Notably, this effect on IL-1β was less prominent when PBMC were immunologically triggered by RKO cells in comparison to

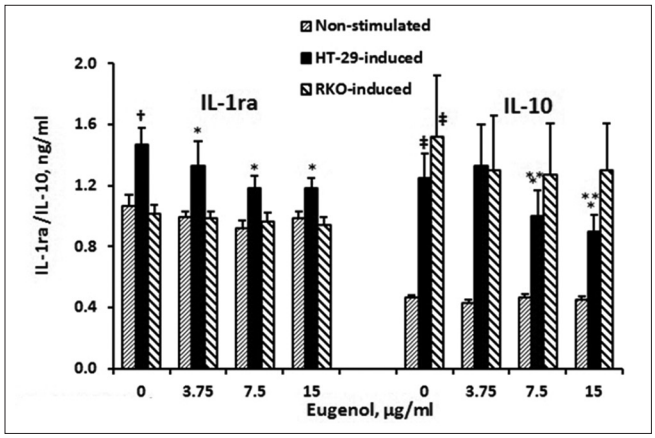

Figure 2: Effect of eugenol on IL-1ra and IL-10 production. 2 × 10^6 peripheral blood mononuclear cells (PBMC) were incubated for 24 h in the absence or presence of 2 × 10^5 HT-29 or RKO colon cancer cells, without (0) or with eugenol at concentrations as indicated. The level of interleukin (IL)-1ra and IL-10 in the supernatants was tested by enzyme-linked immunosorbent assay. Each column represents the mean of 11 experiments. Bars represent standard error of mean. The statistically significant difference in cytokine production between PBMC cultured alone or those with colon cancer cells is presented by (†P < 0.01) or by (‡P < 0.005). Asterisks represent statistically significant difference from PBMC incubated without (0) or with eugenol (*P < 0.05; **P < 0.01; ***P < 0.001)

HT-29 cells. It is of interest that the secretion of the anti-inflammatory cytokines IL-1ra and IL-10 by PBMC was reduced when they were activated by contact with HT-29 cells only. One possible explanation for this phenomenon may be linked to the fact that activation of the cytokine cascade triggers a close reliance in secretion of inflammatory cytokines, i.e., the lower pro-inflammatory cytokine production is, the lesser is that of the anti-inflammatory ones. Although eugenol is the major active component of clove's buds, it seems that it may act differently on the capacity of macrophages for cytokine production. Thus, Bachiega *et al.* [17] have found that clove inhibited IL-1β, IL-6, and IL-10 production independently on LPS stimulation. On the other hand, eugenol did not alter IL-1β release but inhibited IL-6 and IL-10 production. Considering the fact that the inhibition of these cytokines by eugenol prevented the LPS effect on macrophage activity, the authors suggested that the anti-inflammatory effect of cloves is exerted by inhibition of LPS activity. It is possible that macrophages from various species respond differently to eugenol. Thus, while in our hands unstimulated human mononuclears did not respond to eugenol as for cytokine production, Rodrigues *et al.* [18] have reported that eugenol inhibited the secretion of IL-1β and IL-6 by mouse macrophages. Apparently, Th1 and Th2 cells react adversely to eugenol - while Th1 cytokines TNF-α and IL-2 production by nicotine-induced murine macrophages were down-regulated by eugenol, the generation of Th-2 cytokines IL-10 and TGF-β was activated [19]. On the other hand, administration of clove to mice did not exert any effect on the Th1/Th2 balance [20]. The anti-inflammatory effect of eugenol has been demonstrated on rats with carrageenan-induced inflammation that was markedly alleviated by eugenol containing diet [21]. In mice with LPS induced lung injury, treatment with eugenol

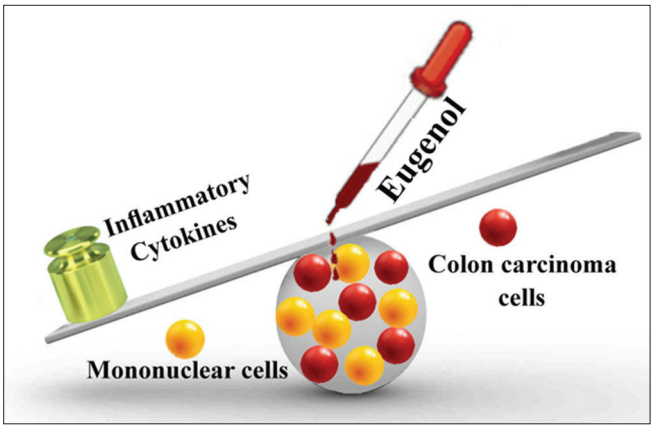

Figure 3: Schematic presentation of the effect of eugenol on the crosstalks between mononuclear and carcinoma cells from the two lines examined in the study. Addition of eugenol alters the dialogue between immune and cancer cells

significantly reduced lung inflammation and inhibited TNF-α level [22]. It is of interest that the anti-inflammatory effect of eugenol can be locally exerted. Thus, in a murine model of rheumatoid arthritis eugenol lowered the level of TNF-α, IFN-γ, and TGF-β within the affected joints [16]. Keeping in view the close association between chronic inflammation and cancer development [10,22] and particularly with colon carcinoma [11], the anti-inflammatory effect of eugenol may play a key role in prevention of carcinogenesis. Aggarwal *et al.* [23] have reviewed profoundly the potential of eugenol and the active components of other spices for prevention of cancer. Apparently, there are several ways by which eugenol exerts its protective effect against various types of carcinoma, including antioxidant activity, inhibition of apoptosis and malignant cell proliferation and restriction of angiogenesis [23-27]. Similarly to phytochemicals extracted from other foods and spices eugenol acts through a relatively long list of signaling pathways detailed by Khuda-Bukhsh *et al.* [28]. It is notable that the apoptotic effect of eugenol does not dependent on p53 presence [29]. Studies have shown that eugenol exerts a synergistic effect with anticancer drugs such as gemcitabine by increasing both anti-inflammatory and apoptotic events at the cancer milieu [30].

In short, as schematically detailed in Figure 3, the results of this study point to an additional way by which eugenol may attenuate carcinogenesis, i.e., by interfering with the immunological balance between PBMC and colon carcinoma cells, at least from the two lines examined. It is conceivable that a decrease in pro-inflammatory cytokine production triggered by the malignant cells will reduce the effect of tumor-associated inflammation on tumorigenesis.

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