Immune Suppressive Effect of Cinnamaldehyde Due to Inhibition of Proliferation and Induction of Apoptosis in Immune Cells: Implications in Cancer

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Abstract

**Background:** Besides its anti-inflammatory effects, cinnamaldehyde has been reported to have anti-carcinogenic activity. Here, we investigated its impact on immune cells.

**Methods:** Activation of nuclear factor-kB by cinnamaldehyde (0–10 μg/ml) alone or in combination with lipopolysaccharide was assessed in THP1XBlue human monocytic cell line and in human peripheral blood mononuclear cells (PBMCs). Proliferation and secretion of cytokines (IL10 and TNFα) was determined in primary immune cells and the human cell lines (THP1, Jurkat E6-1 and Raji cell lines) stimulated with cinnamaldehyde alone or in conjunction with lipopolysaccharide. Nitric oxide was determined in mouse RAW264.7 cells. Moreover, different treated PBMCs were stained for CD3, CD20 and AnnexinV.

**Results:** Low concentrations (up to 1 μg/ml) of cinnamaldehyde resulted in a slight increase in nuclear factor-kB activation, whereas higher concentrations led to a dose-dependent decrease of nuclear factor-kB activation (up to 50%) in lipopolysaccharide-stimulated THP1 cells and PBMCs. Accordingly, nitric oxide, interleukin 10 secretion as well as cell proliferation were reduced in lipopolysaccharide-stimulated RAW264.7 cells, PBMCs and THP1, Raji and Jurkat-E6 immune cells in the presence of cinnamaldehyde in a concentration-dependent manner. Flow cytometric analysis of PBMCs revealed that CD3+ were more affected than CD20+ cells to apoptosis by cinnamaldehyde.

**Conclusion:** We attribute the anti-inflammatory properties of cinnamaldehyde to its ability to block nuclear factor-kB activation in immune cells. Treatment with cinnamaldehyde led to inhibition of cell viability, proliferation and induced apoptosis in a dose-dependent manner in primary and immortalized immune cells. Therefore, despite its described anti-carcinogenic property, treatment with cinnamaldehyde in cancer patients might be contraindicated due to its ability to inhibit immune cell activation.

Introduction

Cinnamon is widely used in the manufacturing industry as a spice and flavoring agent, but it is also an important compound in traditional herbal medicine. The essential oil of the cinnamon bark is constituted by >80% of cinnamaldehyde [1] and the aqueous extract of the cinnamon spice has been attributed with antioxidant properties [2,3]. Cinnamaldehyde (CA) is a bioactive compound that has been identified to have anti-bacterial [4,5], anti-inflammatory [6,7], hypoglycemic [8], anti-mutagenic [9,10] and anti-tumorigenic activity. Moreover, it was demonstrated to be anti-proliferative and pro-apoptotic on various cancer cell lines in...
The anti-carcinogenic property by CA is achieved by mitochondrial depolarization [16] leading to elevated reactive oxygen species, activation of the pro-apoptotic Bcl-2 family proteins, caspase-3 and mitogen-activated protein kinases [16,17] as well as inhibition of NF-κB and AP-1 activity [15,10].

Compounds possessing both anti-cancer as well as anti-inflammatory properties like CA may therefore provide an attractive therapeutic tool for cancer therapy. It is well known, that chronic inflammation is a trigger for cancer promotion. However, in an established tumor an immune-suppressive environment already exists and further immune-suppression leads to tumor promotion [19–23]. In this respect, death or accumulation of dysfunctional dendritic cells [24,25], downregulation of regulatory T cells [28] within tumors have gained attention. Immunosuppression in the tumor environment leads to reduced proliferation of peripheral T cells in vitro, which correlates with a more negative outcome in cancer patients [29]. Consequently, successful stimulation of the immune system as a result of cancer therapy has been shown to reduce disease relapses and to improve survival [30].

Hence, various strategies in cancer immunotherapy have emerged in recent years to combat immunosuppressive factors and to create an anti-tumor environment. Approaches in active anti-cancer immunotherapy include 1) the introduction of tumor-associated antigens or derivatives thereof as vaccines in an immunogenic context to break tumor tolerance; [31] 2) isolation of immune cells from cancer patients, followed by antigen pulsing and/or stimulation ex vivo before re-infusion into the patient [32] as well as 3) blocking immunosuppressive molecules like cytotoxic T lymphocyte-associated antigen 4 (CTLA4), and programmed cell death protein 1 (PD1) with monoclonal antibodies [33].

The anti-tumorigenic properties, which have so far been attributed to cinnamaldehyde, were deduced from models concentrating on cancer cells. However, considering the importance of tumor-infiltrating immune cells, we aimed in this study to critically assess its effects on primary and immortalized immune cells.

Materials and Methods

Ethic statement

The study was approved by the institutional ethics committee of the Medical University of Vienna (EK-Nr. 949/2011) and informed written consent was obtained from all subjects before their participation in the study. Healthy volunteers with no reported allergy to cinnamon donated 15 ml blood.

PBMC isolation and cell lines

Peripheral blood mononuclear cells (PBMCs) of 6 healthy volunteers with no reported allergy to cinnamon were isolated from whole blood using Ficoll-paque density gradient centrifugation as previously described [34,35]. The THP1-XBlue human monocytic cell line, obtained from InvivoGen (San Diego, CA, USA) as well as THP1, Jurkat E6-1, Raji (all from ATCC, Rockville, MD, USA) cell lines and peripheral blood human mononuclear cells (PBMCs) were maintained in suspension in RPMI-1640 (Gibco Invitrogen, Darmstadt, Germany) containing heat inactivated 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% L-glutamine. According to the manufacturer’s protocol, 200 μg/ml Zeocin were added upon propagation to THP1-XBlue cells. Murine RAW264.7 macrophages, purchased from ATCC were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Invitrogen, Darmstadt, Germany) supplemented with 10% FCS plus 1% penicillin/streptomycin.

Cell stimulation

All cells were stimulated with CA (SAFC chemicals supply/ Sigma Aldrich, Steinheim, Germany) in a concentration range from 0 up to 10 μg/ml with or without 5 μg/ml (15 000 EU/ml) of LPS from E. coli 055:B5 (Sigma, St. Louis, MO, USA).

Nuclear extraction

PBMCs (1×10⁸ cells/well) were seeded into a 48-well plate and stimulated with CA (0 to 10 μg/ml) alone or in combination with LPS (5 μg/ml, 15 000 EU) for 24 h. Subsequently, nuclear extracts were obtained using a commercial available nuclear extraction reagents and according to manufacturer’s protocol (Thermo Scientific, Pierce, Rockford, IL). In brief, cells were washed in PBS and lysed in cytoplasmic extraction reagent I and II. After removing cytoplasmic fraction, nuclear proteins were extracted using nuclear extract reagent and stored at −80°C.

phospho-NFkB p65 assay

Phospho-NFkB p65 were determined of nuclear fractions using a phospho-NFkB p65 ELISA, (InstantOne ELISA, eBioscience, San Diego, CA) according to the manufacture’s protocol. Briefly, to nuclear extracts, an antibody cocktail mix were added for one hour before washing plate and adding detection reagent for 30 minutes, stopping the reaction and measuring optical density at 450 nm.

NF-κB activation assay

NF-κB activation assays were performed using THP1-XBlue reporter cells, stably expressing an NF-κB/AP-1-inducible secreted alkaline phosphatase reporter (SEAP), according to the company’s protocol. In brief, 1×10⁶ cells/well were seeded into a 96-well plate and stimulated with CA (0 to 10 μg/ml) alone or in combination with LPS (5 μg/ml, 15 000 EU/ml) for 24 h. NF-κB activity was determined adding QUANTI-Blue as a substrate of secreted alkaline phosphatase in the supernatants and further incubation for 8 h at 37°C and 5% CO₂. Subsequently, optical density (OD) was measured at 625 nm using a spectrophotometer Tecan InfiniteM200 PRO (Tecan Group Ltd, Männedorf, Switzerland).

Nitric oxide determination

Nitric oxide (NO) concentrations of supernatants were determined using Griess reagent (Sigma Chemical Co. St. Louis, MO, USA). RAW264.7 cells (1×10⁶ cells/ml) were incubated with CA (0 to 10 μg/ml) in the presence or absence of LPS (5 μg/ml) for 24 h. Subsequently, equal volume of Griess reagent was added to supernatants. After 10-minute incubation, OD was measured at 550 nm. Zero to 100 μM of sodium nitrite (NaNO₂; Sigma) was used as standard to calculate NO-levels.

Cytokine analysis

RAW264.7 cells and human PBMCs (1×10⁶ and 2×10⁵ cells/ml, respectively) were incubated with CA (0–10 μg/ml) in the absence or presence of LPS (5 μg/ml, 15 000 EU/ml). Cell-free supernatants were recovered and stored at −80°C until use. The concentrations of IL-10 and TNF-α in the supernatants were determined by ELISA according to manufacturer’s protocol (eBioscience, San Diego, CA).
Cinnamaldehyde negatively affects viability and proliferation of immune cells

The data so far showed that regardless of cytokines (pro- or anti-inflammatory, TNF-α or IL10), of signaling molecules (NO) as well as pathways (NF-κB/AP-1) investigated, a decrease in activation and production was observed when using higher concentration of CA than 1 μg/ml (≥ 8 μM). Therefore, we next tested, whether the observed results where due to reduced viability in CA-treated immune cells.
As depicted in Fig. 3, low concentration of CA (<1 μg/ml) led to a significant increase in proliferation of primary human PBMCs, similar to the concentration-dependent NF-κB-profile of CA (Fig. 1). A similar tendency was also observed when using immortalized cells like the human monocyte-like cells THP1, the B cell line Raji and the T cell line Jurkat E6-1, even though this did not reach statistical significance. However, all immune cells tested (THP1, Raji B cells, Jurkat E6-1 and human PBMCs) had in common that higher concentration of CA (>1 μg/ml) significantly reduced cell viability and proliferation. Hence, our data point out that the immune suppressive capacity described by CA is due to its ability to reduce the viability of immune cells at higher concentrations than 1 μg/ml.

Cinnamaldehyde rather affects T cells than B cells

In a next step, we investigated the susceptibility of B and T cells in PBMC-preparations to CA via flow cytometry. PBMCs were stained for CD3 (T cell receptor) as a marker for T cells, CD20 (B lymphocyte antigen) as a marker for B cells and for the early apoptosis marker Annexin V. As depicted in Fig. 4, concentration above 1 μg/ml of CA led to apoptosis of both T cells (Fig. 4A) and B cells (Fig. 4B) in a dose-dependent manner, whereas loading with lower concentrations failed to change cell numbers. Treatment with high concentrations of CA (5 and 10 μg/ml) increased the number of apoptotic T cells up to 8-fold whereas increase of apoptotic B cells was 3.5-fold compared to untreated controls.

Discussion

CA has been attributed many pharmacological properties, such as being anti-inflammatory, anti-ulcerogenic, antipyretic, antimicrobial, anti-diabetic, but also having anti-tumor activity [39]. We intended to investigate these proclaimed prodigious beneficial properties in greater detail, particularly with the focus to understand its impact on immune cells and thereby evaluating its possible indirect contribution to cancer progression. Cancer is defined as the unregulated growth of malignant cells, which is caused in 90-95% by environmental factors such as pollutants, diet, sun exposure, infections, physical inactivity and obesity, and only in 5–10% can be linked to genetic defects [41]. The probability to develop cancer increases with age and has been associated with a decreased immunosurveillance in the elderly [42]. Indeed, the cancer microenvironment is very immunosuppressive and hence reactivation and modulation of the immune system is the primary goal of cancer vaccines [43]. Immuno-modulatory regimens offer an attractive approach as they often have fewer side effects than the existing chemotherapeutic drugs. In this respect, blocking of the inhibitory CTLA4 antigen on T cells with anti-CTLA4 antibodies leads to activation of T cells [44,45] and has been proven to lead to significant survival benefits in two randomized phase III trials in patients with advanced melanoma, emphasizing the importance of immune activation in cancer [46,47]. Hence, we investigated the immune-modulatory capacity of CA on immortalized and primary immune cells. In our first approach, we investigated its impact on NF-κB activation, since NF-κB is constitutively activated in a number of hematologic and solid tumors and is one of the major transcription factors associated with cancer progression [48], inhibition of apoptosis, tissue invasion and metastasis [49]. Thus, inhibition of NF-κB in malignant cells is regarded beneficial. We were able to reproduce data from literature, that NF-κB activation of LPS-stimulated immortalized monocytes is inhibited by the addition of CA in concentrations above 8 μM [36–40], but interestingly we observed a significant increase in NF-κB activation when lower concentrations of CA were used. We thus hypothesize, that an immunosuppressive tumor micro-environment could be shaped when CA could be applied in high enough concentrations. On the other hand low concentration of CA, which is achieved by moderate consumption of cinnamon, further activates the immune system, although not sufficient for combating fast-growing tumors. Hence moderate consumption of CA-containing food seems rather to be a prophylactic, rather than a therapeutical mean. Importantly, our data indicate that high doses might even be contraindicated.

Concerning its bioavailability cinnamaldehyde and its alcohol as well as acid derivate are rapidly absorbed from the gut, metabolized and excreted primarily in the urine, which seems to be independent of dose (up to 250 mg/kg), species and sex. Blood concentrations of cinnamaldehyde following intravenous administration of 5,15, or 25 mg/kg in male and female F344 rats decreased in a biphasic manner [50,51]. The initial phase correlates with the rapid appearance of cinnamic acid in blood, in which estimated 37–60% of the cinnamaldehyde is oxidized to cinnamic acid within 30 min. The second phase with a half-life of 1.7 h is hypothesized as a release of cinnamaldehyde from protein adducts formed during the initial phase [52]. In a recent study oral administration of CA resulted in a bioavailability of 20% [53].
Moreover in a similar report after oral administration, CA concentration in blood were found to be 1 mg/ml [52] and up to 10 mg/ml for cinnamic acid [54] and maintained for 24 h despite its relatively short biological half-life of 1.7 h.

Hence, high local concentration of cinnamaldehyde and its derivates are achievable in vivo upon ingestion and could exert a sustained immune-suppressive effect.

In our next approach, we further focused on the immune-suppressive qualities of cinnamaldehyde by investigating its impact on cytokine secretion in primary PBMCs activated with LPS. In line with suppression of the NF-κB pathway, we observed a concentration-dependent downregulation of the inflammatory and regulatory mediators TNF-α, IL10 and NO being implicated in development and progression of various cancers [55,56]. However, the prominent decline in mediator secretion of primary immune cells upon incubation with CA suggested that this might be due to changes in the metabolic rate. Indeed, our data clearly demonstrate that CA leads to a pronounced reduction in cell viability of immortalized as well as primary immune cells. Further analysis of the immune cell population in human subjects revealed that especially T cells were more prominently susceptible to CA-induced apoptosis than B cells. The obtained results are highly relevant, since cytotoxic T lymphocytes remain potent mediators of anti-tumor immunity and tumor infiltration by T cells have been shown to be a good prognostic factor in ovarian, colon, breast, renal, prostate and cervical cancers [43]. Furthermore, there are two in vivo studies, using cinnamon extract as a cancer therapeutic agent. They report reduced tumor growth of mouse melanoma, when feeding cinnamon extract in large doses (400 µg/g body weight) over 20 to 30 days [37,57] to mice. However, they also showed a severe reduction of secondary immune organs like the spleen and lymph nodes [28]. In these studies very high doses of CA are used, considering that CA is known to have a low toxicity with a lethal dose low (LDlow) by parental application of 200 µg/g body weight [58]. The observed reduction of fast growing cells like immune and cancer cells might hence be the general characteristic of the described low toxicity of CA.

In contrast, cancerogenic concerns were risen by Mereto et al [59], who proposed cinnamaldehyde as a weak promoter of liver carcinogenesis due to its potential to be clastogenic and its ability to induce micronuclei in rat liver in vivo. There exist also one human case report, which associated oral carcinoma formation after the consumption of up to five packs of cinnamon chewing gum a day in a 24-year-old non-smoker [60].

Still, in a 2-year NTP study conducted in mice and rats, in which up to 200 mg/kg body weight of CA, were fed, and which correspond to the maximal concentration of no-observed-adverse-effect-level, NOAEL for long-term effects, no signs of neoplasia were observed [61]. The study highlights the fact, that the consumption of CA may not be cancerogenic when the amount ingested is moderate.

On a molecular level CA as an aldehyde possessing α, β-unsaturated olefinic substituents is known to form adducts with cellular thiol-groups most notably with nonprotein sulfhydryls such as cysteine and glutathione via nucleophilic addition to the β-carbon [62]. Thus, by depriving cells of the antioxidant glutathione, cells are limited in their ability to neutralize free radicals.
Impact of Cinnamaldehyde on Immune Cells

Figure 4. Cinnamaldehyde induced apoptosis in human primary immune cells. Human PBMCs were incubated with CA and stained for CD3, CD20 and AnnexinV and analysed by flow cytometry. Data of three independent experiments are presented as mean ± SD of AnnexinV positive A. CD3+ cells and B. CD20+ cells.

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Author Contributions

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